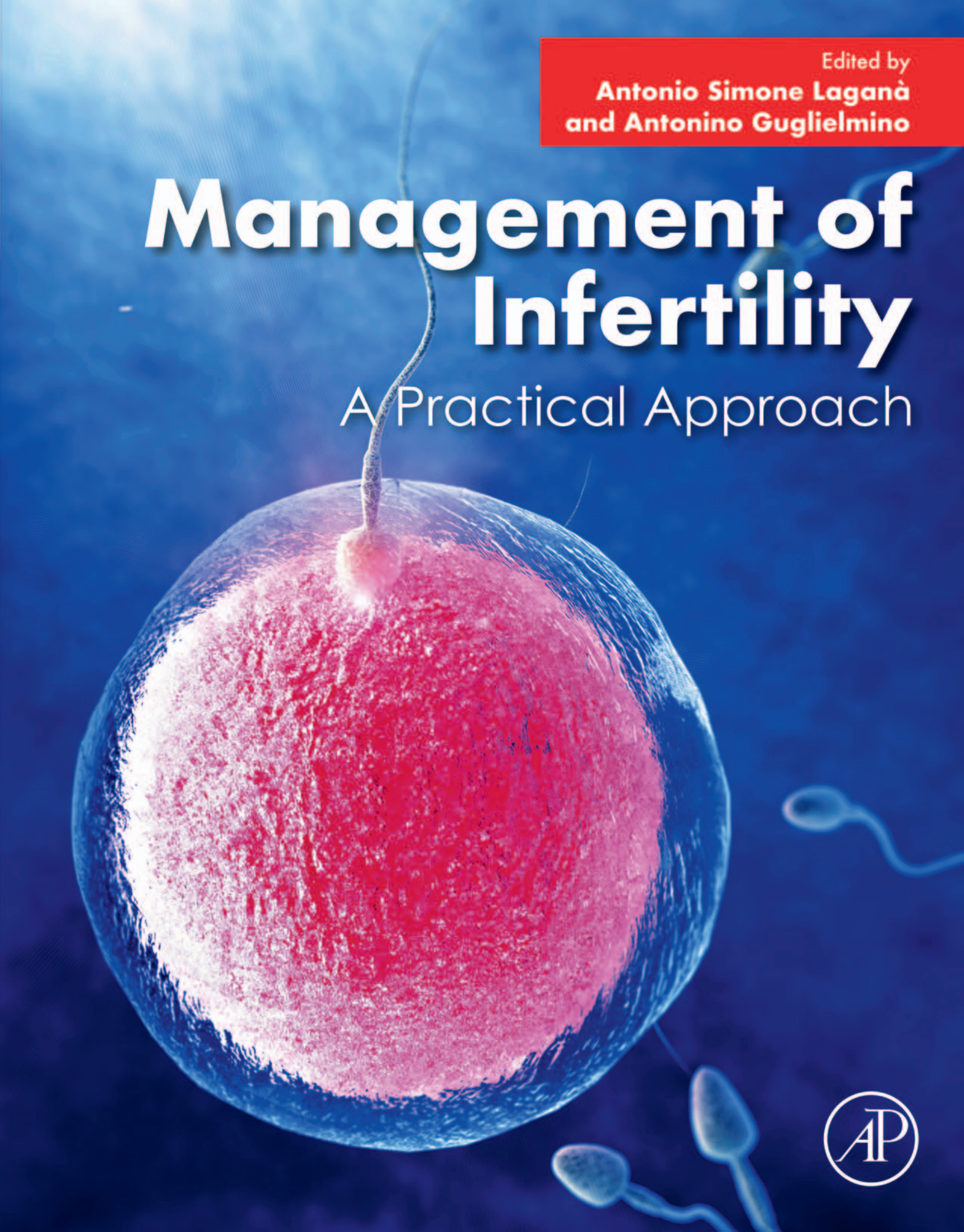
Edited by
Antonio Simone Laganà
and Antonino Guglielmino

Management of Infertility

A Practical Approach



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A PRACTICAL APPROACH

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Preface

Management of infertility is rapidly evolving, due to the worldwide increased rate of this condition in the general population. In the current scenario, the aim of this book is to offer a proper, accurate manual for the management of infertility and a robust step-by-step guide for assisted reproduction technologies (ARTs), including how to plan, design, and organize the clinical setting and laboratory.

This book is precisely designed to help gynecologists, biologists, general practitioners, nurses, midwives, healthcare managers, and patients to gain a complete knowledge about both basic and advanced methods for the diagnosis and management of infertility, in males and females. In addition, considering the high-quality and completeness of the contents, the textbook would be appropriate also for physicians and biologists who already have experience in the field of ART and would like to master one particular technique.

The practical approach to male and female infertility, with detailed and step-by-step descriptions about how to perform all the different types of ARTs, makes this book a unique guide for a robust and generalizable decision-making approach, even in low-resource settings and considering the limitations due to the ongoing COVID-19 pandemic.

Considering all these elements, we are very glad to offer this book to readers, aiming to implement an evidence-based and practical guide for the management of infertility.

Antonio Simone Laganà
Antonino Guglielmino

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CHAPTER

1

History and epidemiology of human fertility

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The history of fertility is the history of mankind. Since the dawn of humanity, fertility has played a major role in human thought, culture, and activities, and the mystery of reproduction was one of the earliest dilemmas facing the human race. In fact, for a good part of their early history, humans did not understand how a woman became pregnant, and the discovery of the relation between sexual intercourse and pregnancy must have been one of the earliest concepts achieved by the human brain.

Fertility in the ancient world

In the ancient world, humans related fertility to superpowers and many fertility deities were worshiped in various parts of the world to seek their help in understanding the mystery of fertility. Most of these deities were female goddesses, as the fertility myth was perceived to reside mainly in the females who bring the offspring to this world. In ancient Egypt, Isis was the goddess of fertility, while Hathor was the goddess that protected women in labor (Fig. 1.1). In ancient Greece, Aphrodite was the goddess of fertility. She was also the mother of Eros, the god of love, while in Roman mythology, Venus was the goddess of love, sex, beauty, and fertility. In African culture, the goddess was Ashanti, and in the Inca culture, she was Mama Oclio. In China, she was Jiutian Xuanwu, while in India, she was Banka-Mundi. In Sumerian and Babylonian cultures, she was Ishtar, and in Ireland, she was Brigit. Each goddess had powers that were also helped by certain rituals and flowers that attracted fertility, mainly the rose, the lotus, and the orchid [1].

On occasions, attempts were made to develop more mundane solutions for infertility, but these were not successful due to the absence of the basic tools and the scientific method. For example, ancient Egyptians developed a primitive pregnancy test: women were asked to urinate on barley or wheat seeds and sprouting

FIGURE 1.1 Headless sculpture of Isis, goddess of fertility in ancient Egypt made from basalt showing the characteristic knot on her chest from the Graeco-Roman Period (332 BCE–395 CE) found in Alexandria (Bibliotheca Alexandrina collection).

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seeds indicated pregnancy. While this may sound like pseudoscience, Ghalioungui et al. reported that it correctly identified 70%–85% of pregnancies [2].

In ancient Greece, attempts at explaining fertility and infertility were made but offer little to help in our current understanding of the fertility process. The Hippocratic Corpus contains three texts related to fertility, "Diseases of Women" (*Gynaikeia*) 1 and 2 and "On Infertile Women" (*Peri Aphorôn*) with various empirical treatments and recipes. Even Aristotle (384–322 BCE), the most enlightened of the Greek philosophers, believed that only male semen was incorporated into the fetus and that the female played no role in the generative material. However, Soranus of Ephesus, one of the leading scientists of the old Alexandria Medical School, and who was the first to describe the human uterus, contradicted Aristotle, and wrote in his book "Gynecology" that both the male and female produce "seeds" necessary for conception [3]. He also noted that masculine-appearing females and those exercising excessively failed to menstruate and commented on contraception, noting that blockade of the cervical os was an effective means of preventing conception [4].

Galen (129–200 AD) was a leading Roman physician who also trained in Alexandria before traveling to Rome to become the personal physician of the Emperor Marcus Aurelius and his son Commodus. He described the "female testes," which he thought corresponded to the male testes, and thought that menstruation was a form of auto-phlebotomy and represented a means to eliminate unfavorable circulating humors, a concept that remained alive well into the Middle Ages [5]. However, few advances were made during the Middle Ages, and even during the Arab/Islamic golden age in Andalucia, no notable discoveries were made in the field apart from the primitive obstetrics forceps described by Abulcassis of Cordoba [6].

Fertility in the post-Renaissance era

It is only after the Renaissance and subsequent age of enlightenment that various discoveries started to shed light on our current understanding of the processes of human reproduction. In 1506, Leonardo da Vinci (1452–1519) began his anatomical drawings in Milan and later collaborated with the physician-anatomist Marcantonio della Torre in Pavia and made an accurate sketch of the fetus in utero [7]. Subsequently, Gabriele Falloppio (1523–62) professor of anatomy in Padua described the Fallopian tube, which bears his name to this day. However, the real breakthrough came with the invention of the microscope when Antonie van Leeuwenhoek (1632–1723) a Dutch scientist and businessman living in Delft was the first to observe and describe the spermatozoa using his primitive instrument and called them "animalicules" [8].

It was also the Dutch physician and anatomist Regnier de Graaf (1641–73) also working in Delft who summarized the work of his predecessors and made key discoveries in reproductive biology. He described the testicular tubules, the efferent ducts, and corpora lutea and was probably the first to understand the reproductive function of the Fallopian tube, but his most important discovery is probably the description of the ovarian follicles (later called after him: Graafian follicles), which he thought were the oocytes [9]. Subsequently, the Italian priest and physiologist Lazzaro Spallanzani (1729–99) working in Pavia was the first to show that fertilization requires physical contact between the sperm and the ovum and used this information to perform successful artificial insemination in dogs in 1770 [10]. Ten years later, the Scottish surgeon John Hunter (1728–93) working in London performed the first successful artificial insemination in humans [10]. However, it was the Baltic-German scientist Karl Ernst von Baer (1792–1876) who eventually discovered the human oocyte in 1827 while working at Königsberg University in Kaliningrad and showed that it resided inside the follicle [9]. Finally, it was Oscar Hertwig (1849–1922) working in Berlin who, by studying sea urchins, proved in 1870 that fertilization occurs due to the fusion of a sperm and an egg cell [11].

At the same time, the concept of hormones was introduced by Arnold Berthold (1803–1861) in 1846 while working in the University of Göttingen by finding that castrated cock chickens lost their aggressive male behavior and characteristics, but it was Ernest Starling and William Bayliss of University College London who introduced the term "hormone" in 1905 [12] (Fig. 1.2).

Fertility in modern times

With the dawning of the 20th century and the understanding of the basic principles of fertility, major discoveries were made in a remarkably short time. These included the understanding of the hypothalamic-pituitary-ovarian axis, the discovery of gonadotrophins and the isolation of gonadal steroids, the understanding of the hormonal changes involved in the control of the menstrual cycle, culminating in the success of in-vitro fertilization and its allied techniques.

The hypothalamic-pituitary-ovarian axis

In 1910, Samuel Crowe, working at Johns Hopkins, showed that partial pituitary ablation resulted in

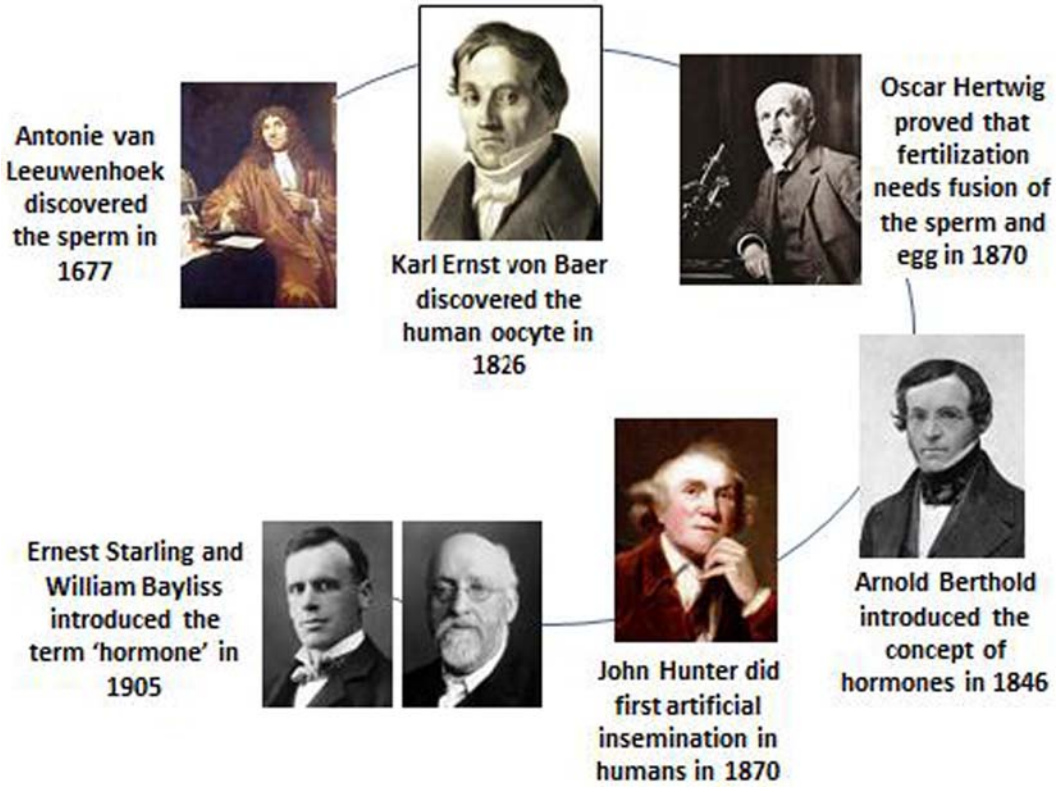

FIGURE 1.2 The fathers of human reproduction in the post-Renaissance era. *Adapted from Lunenfeld B. Gonadotropin stimulation: past, present and future. Reprod Med Biol. 2012;11(1):11–25.*

atrophy of the genital organs in adult dogs [13], and in 1912, the Austrian physician Bernhard Aschner (1883–1960) working in Vienna observed that men and women with diseases, tumors, or injuries of the hypophysis and pituitary stalk suffered the same fate [14]. Subsequently, in 1926 Philip Smith (1884–1970) working in Berkeley and later in Columbia showed that daily implants of fresh anterior pituitary gland tissue into immature male and female mice and rats induced precocious sexual maturity [15].

At the same time (in 1926), Bernhard Zondek (1891–1966) working in the Charité Hospital in Berlin implanted anterior pituitary glands from adult cows, bulls, and humans into immature animals and showed that this led to rapid development of sexual puberty [16]. It was also Zondek who proposed in 1929 the idea that the pituitary secretes two hormones that stimulate the gonads—Prolan A and Prolan B—and in 1930, he showed that the blood and urine of postmenopausal women contained gonadotropins. He proposed that Prolan A stimulated follicular growth and the secretion of "foliculin" (estradiol) and that Prolan B induced ovulation, formation of the corpus luteum, and secretion of "lutein" (progesterone) [16]. He also suggested in 1930 that the synchronization of Prolan A and Prolan B secretion by the anterior pituitary was responsible for the rhythmic activity of the ovary and the cyclic preparation of the endometrium [17]. However, it was in 1931 that Fevold working in Wisconsin actually extracted the two hormones from the pituitary and called them follicle-stimulating (FSH) and luteinizing (LH) hormones [18].

Simultaneously, in 1927, Selmar Ascheim (1878–1965), working again with Bernhard Zondek at the Charité Hospital in Berlin, showed that the blood and urine of pregnant women contained a substance that stimulated the gonads. They also showed that injecting this substance into intact immature female mice produced follicular maturation and luteinization, which was to become the Ascheim Zondek pregnancy test [19]. However, Ascheim and Zondek believed that this substance was produced by the anterior pituitary, and it was in 1943 that Georgeanna Seegar-Jones (1912–2005) working at Johns Hopkins showed that this gonadotropin was produced by the placenta and not the pituitary gland and called it human chorionic gonadotrophin (HCG) [20].

The gonadotrophins

With the understanding of the role of gonadotrophins, attempts at using them for treating infertile women started. Pregnant mare serum gonadotrophins (PMSG) were the first to be used, and in 1945, Hamblen et al. of Duke University in North Carolina introduced the two-step protocol for women with hypofunctioning ovaries: administration of PMSG during the follicular phase followed by HCG 12–18 days later [21]. In parallel, and in the same year of 1945, HMG was purified and isolated from urine of menopausal women and the first pregnancy was reported by Lunenfeld et al. in 1962 [22].

On the other hand, in 1958, Carl Gemzell, working in Uppsala, Sweden, extracted gonadotropins from human pituitary glands and used them to treat anovulation. However, in 1990, four cases of Creutzfeldt–Jakob disease (CJD or mad-cow disease) were discovered in Australia, France, and the United Kingdom, and the production of these human pituitary gonadotrophins was stopped [16]. HMG therefore became the drug of choice, with each ampoule containing 75 IU of FSH and 75 IU of LH.

With the use of HMG, it became clear that the patients' response to stimulation varied. Patients with polycystic ovarian syndrome who already had a high LH/FSH ratio were particularly liable to ovarian hyperstimulation syndrome. Work on the purification of HMG using polyclonal antibodies to remove LH by immunechromatography started, and in 1982, purified HMG (urofollitropin) was available on the market, with each ampoule containing 75 IU of FSH and 25 IU of LH. Highly purified HMG (urofolllitropin-HP) was introduced next using monoclonal antibodies with each ampoule containing 75 IU of FSH and less than 1 IU of LH [16]. With increased demand and the proliferation of IVF units, recombinant FSH was introduced by incorporating the FSH gene into the nuclear DNA of Chinese hamster ovary cells. Follitropin-α was produced in 1988 and Follitropin-β in 1996 (Table 1.1).

TABLE 1.1 Comparison of HMG and FSH preparations.

Product	Purity (%)	Specific activity
HMG (menotropins)	5	Variable
Purified FSH (urifollitropin)	5	100–150 IU/mg
Highly purified FSH (urifollitropin-HP)	95	10.000 IU/mg
Recombinant FSH (follitropin α and β)	>99	Mass/μg

Gonadal steroids

As in the case of gonadotrophins, the discovery of estrogens went through various stages. In the 1880s, Robert Battey (1928–1895) working in Atlanta, Georgia, performed oophorectomy as a treatment for dysmenorrhea and bleeding from fibroids. After removal of the ovaries, he observed that patients developed amenorrhoea, hot flashes, and vaginal atrophy. This meant that the ovaries were secreting a substance responsible for menstruation. In 1896, Emil Knauer (1867–1935) working with Josef Halban (1870–1937) and Ludwig Fraenkel (1870–1951) in Vienna removed the ovaries from rabbits and observed uterine atrophy, which he could prevent by transplanting the ovary at a distant site, confirming the theory of internal secretion by the ovaries. Finally, in 1897, Hubert Fosbery successfully used ovarian extracts to treat a patient with severe hot flashes [23].

Thus with the beginning of the 20th century, work started in earnest to isolate this substance secreted from the ovary called "estrogen." In 1929, the German biochemist Adolf Butenandt (1903–95), who received the Nobel Prize in 1939, and the American biochemist Edward Adelbert Doisy (1893–1986), who also received the Nobel Prize in 1943, independently isolated and purified estrone, the first estrogen to be discovered. Subsequently, estriol and estradiol were discovered in 1930 and 1933, respectively [23].

On the other hand, the discovery of progesterone followed a different path. In 1929, Georges Corner (1889–1981) and William Allen (1904–93) working in the United States extracted a substance from the corpus luteum of a pregnant rabbit. They injected the extract into another rabbit that was castrated just after mating and found that the pregnancy continued. They called the substance "progestin" [24]. However, it was again Adolf Butenandt who isolated the same substance in 1934 and discovered that it contained a ketone group and called it progesterone [25].

The discovery of the aromatase system responsible for the conversion of androgens to estrogens involved the collaboration of many scientists from the Worcester Foundation for Experimental Biology, established in 1944 in Shrewsbury, Massachusetts, and from Harvard. They included Ralph Dorfman (1911–85) and the enzymologist Mika Hayano (1920–1964) who used radiolabeled tracer steroids in their experiments [26]. But it was Kenneth Ryan and Lewis Engel at Harvard who utilized human placental microsomal preparations to convert androgens to estrogens in high yields [27]. Subsequently, Armstrong and Dorrington working in Ontario, Canada, suggested the 2 cell 2 gonadotrophin theory to explain the interplay between the gonadotrophin and ovarian hormones in the ovary [28].

Immuno-assays and the female hormonal interplay

Rosalyn Yalow (1921–2011) and Solomon Berson (1918–72) working in New York cooperated in their discovery of immunoassays, and Yalow received the Nobel Prize in 1977. This meant that it was then possible to measure compounds present in biological fluids (blood or urine) in nmol and even pmol concentrations [29]. This immediately opened the door for the discovery of the intricate relations between FSH, LH, estrogens, and progesterone. It was also possible to measure estradiol, estriol, and estrone separately. Thus the temporal relationships between the pituitary hormones and the gonadal hormones became clearer, and the classical diagram showing these relationships and which we now take for granted was published simultaneously in 1970 by two groups: the Columbia University group headed by Raymond Vande Wiele (1922–83) [30] and the California group headed by Robert Jaffe (1933–2020) [31].

Other milestones in the history of fertility

Some other important discoveries supplemented our current understanding of human fertility. In 1971, Roger Guillemin (Baylor College of Medicine) and Andrew Schally (Tulane University) discovered the gonadotrophin-releasing hormone (GnRH) and jointly received the Nobel Prize in 1977. This development helped our further understanding of the fertility process and opened the door for the manufacturing of GnRH agonists and antagonists that proved of great value in assisted reproduction in later years [32,33]. On another front, Peter Medawar (1915–87), while working at the National Institute for Medical Research in the United Kingdom, received the Nobel Prize of 1960 for his discovery of the mechanisms involved in acquired immunological tolerance, which was instrumental in our understanding of the embryo implantation process [34].

The IVF revolution

The birth of Louise Brown on Tuesday July 25, 1978, was an extraordinary milestone in the field of human fertility and was the culmination of numerous years of hard work for all involved. In the early 1960s, Patrick Steptoe (1913–88), a consultant gynecologist in Oldham near Manchester, had paid a visit to Professor Raoul Palmer (1904–85) in Paris who had pioneered the then new technique of laparoscopy. Upon his return to England, Steptoe gave a talk on laparoscopy at the Royal Society of Medicine in London in 1968, and although his fellow gynecologists were not impressed by this new technique, he was approached by Robert Edwards who was a young scientist working in Cambridge University [35]. Edwards had been working on fertilizing mammalian oocytes since 1955 and had started working with human oocytes in 1965 [36]. Following this encounter, one of the most important collaborations in the field of human reproduction started with Edwards regularly traveling from Cambridge to Oldham and vice-versa to fertilize oocytes collected by Steptoe through laparoscopy.

After 4 years of basic research, Steptoe and Edwards started their first human transfers in 1972, but none of their first 40 patients became pregnant [35]. In 1976, they achieved their first IVF pregnancy after a blastocyst transfer, which unfortunately turned out to be an ectopic pregnancy. Two years later and after 102 failed attempts, Leslie Brown became pregnant following the transfer of an 8-cell embryo in a nonstimulated cycle and gave birth to a full-term, normal, fit, and healthy baby "Louise" by caesarean section as reported in the Lancet the following week [37]. On January 4, 1979, they achieved the birth of their second baby, Alastair Macdonald, who was the world's first boy conceived by IVF.

Steptoe and Edwards had originally suggested that IVF should be done in nonstimulated cycles to avoid any negative effect of the stimulation drugs on the endometrium. However, the team of Carl Wood and Alan Trounson in Monash succeeded in achieving the first successful IVF in Australia in June 1980 in a clomiphene-stimulated cycle, and the birth of the fourth baby in the world [38]. And shortly afterward, Howard and Georgeanna Jones working at the Jones institute of Eastern Virginia School of Medicine achieved the birth of the first IVF baby in the United States in an HMG-stimulated cycle on December 28, 1981 [39]. Both Steptoe and Edwards received many honors in recognition of their pioneering work including a CBE from the British Queen and Edwards received the Nobel Prize in 2010, although he could not receive it in person due to his illness [35].

Further developments in assisted reproduction

It is important to note that until 1981, monitoring folliculogenesis was effected mainly by the daily measurement of plasma estradiol, and the time of oocyte retrieval was decided on the basis of serial measurement of LH in blood or urine, as follicles could not be seen by the linear ultrasound machines available then. And although Alfred Kratochwil working in Vienna had reported the visualization of ovarian follicles with static B-mode ultrasound in 1972 [40], follicular scanning became more realistic with the introduction of abdominal sector scanners in the early 1980s, and the first series of monitoring gonadotrophin therapy with ultrasound,

without hormonal assays, was reported by Schmidt and von Holst in 1981 [41] and Sallam et al. in 1982 working at King's College Hospital in London [42]. The first successful attempt at oocyte retrieval by transabdominal transvesical ultrasound was reported by Lens et al. working at the Rigshospitalet in Copenhagen in 1981 [43]. However, by 1985, vaginal ultrasound machines were introduced, and transvaginal ultrasound-directed oocyte retrieval was first reported by Dellenbach et al. in Strasbourg [44], and it rapidly became the universal method of oocyte retrieval.

Simultaneously, other developments were taking place on the laboratory front. Advances in cryopreservation allowed the freezing of embryos for transfer in subsequent cycles. The first ever pregnancy derived from a frozen human embryo was reported by Alan Trounson and Linda Mohr in 1983 but ended in spontaneous abortion at 10 weeks of gestation [45]. The first babies (twins) derived from frozen embryos were born December 26, 1983, in the Netherlands [46]. At the same time, the world's first successful preimplantation genetic diagnosis was performed by Handyside et al. at the Hammersmith Hospital in London. Female embryos were selectively transferred in five couples at risk of X-linked disease, resulting in two twins and one singleton pregnancy [47].

The story of ICSI

Toward the end of the 1980s, micromanipulation of the human oocytes was introduced in an attempt to treat couples with unexplained and male factor infertility. As direct injection of sperm in the cytoplasm of the oocyte had not been tried in animals before, various groups experimented with milder forms of micromanipulation such as subzonal insemination (SUZI). The first successful case of SUZI, a twin pregnancy, was reported in 1990 by Simon Fishel working in Nottingham [48]. Subsequently, in an apparently lucky event for humanity, Gianpierrro Palermo working under the chairmanship of André van Steirteghem at the Free University of Brussels accidently injected a spermatozoon in the cytoplasm of an oocyte, and found that fertilization and cleavage occurred. The embryo was replaced and pregnancy resulted in the birth of a healthy baby [49]. Intracytoplasmic sperm injection (ICSI) was born, starting another revolution in the treatment of male infertility.

Embryo selection, fertility preservation, and the future

In an attempt to improve the clinical results of IVF and ICSI, various methods for embryo selection were introduced including the use of time-lapse systems and the analysis of various components in the spent medium of cultured embryos (genomes, metabolomes, and proteomes). However, so far, none of these methods has proven its superiority [50,51]. Preimplantation genetic testing for aneuploidy (PGT-A) is now being advanced as the method of choice. However, it is still under scrutiny [52].

On another front, fertility preservation is now a real option for men and women who survive cancer treatment or opt for delaying their fertility for social reasons [53]. Advances were made in cryopreserving oocytes, ovarian tissue, and even a whole ovary for future transplantation [54,55]. Indeed, the story of human fertility is a never ending story and each day brings new developments in this exciting field.

Epidemiology of human fertility

No treatise on the history of human fertility is complete without a thorough discussion of its epidemiology. We will now discuss normal fertility trends, the prevalence and causes of infertility, the burden of infertility, and finally the need for fertility services and whether they are adequately met both in developed and developing countries.

Normal fertility patterns and the definition of infertility

In a study of 340 couples practicing natural family planning methods to conceive, Gnoth et al. found that 310 couples achieved a pregnancy within 1 year. The cumulative probabilities of conception based on Kaplan–Meier survival analysis were 38%, 68%, 81%, and 92% at 1, 3, 6, and 12 months of regular sexual intercourse, and although pregnancy could happen afterward, the probability of conception diminished significantly with time [56]. This work confirmed earlier observations by Collis et al., Gleicher et al., and also of Hull et al. [57–59]. Consequently, and based on these findings, WHO defines infertility as the failure to achieve a clinical pregnancy after 12 months of regular, unprotected sexual intercourse [60].

Prevalence of infertility

In a study by Boivin et al., based on surveys involving 172,413 women (52,253 from developed countries and 120,160 from developing countries), the prevalence of infertility ranged from 3.5% to 16.7% with a median figure

of 9% in women aged 20 to 44 in married and consensual unions. This median estimate of 9% was nearly the same in developed as well as in developing countries with a sensible range of 5%–15% in both groups [61]. These data contradict previous reports showing a higher incidence of infertility among developing countries (particularly in Africa) compared to developed countries, where infertility was mainly blamed on genital and sexually transmitted infections [62].

At the same time, the total worldwide population of infertile people is very difficult to estimate due to the heterogeneity of the definitions used, the populations studied, and whether infertility is defined as being located in women, couples, people, or individuals. Nevertheless, various studies put the figures in the many millions [63]. For example, a WHO-supported study of 47 Demographic and Health Surveys had found that more than 186 million women in all of the developing countries surveyed (except China) were infertile, more than one-quarter of ever-married women of reproductive age in these countries [64]. However, the more realistic estimate based on the aforementioned study by Boivin et al. of 172,413 women from 25 populations (from developed and developing countries) estimated that there were 72.4 million infertile women in 2007 [61]. More recently, the 2010 Global Burden of Disease Study supported by WHO and the Gates Foundation analyzed 277 reproductive and health surveys from 190 countries and territories and estimated the number of infertile women at 48.5 million. However, this study defined infertility as the inability to achieve a live birth after a 5-year exposure period [65]. According to WHO, reducing the time frame from 5 to 2 years would increase the total number of infertile couples to 121 million [63].

Seeking infertility treatment

Despite these large numbers of infertile couples, only about half of them seek medical services, and even a smaller percentage succeed in receiving them, both in developed and developing countries. In their same study, Boivin et al. found that the proportion of infertile couples seeking medical care was, on average, 56.1% (range 42%–76.3%) in more developed countries and 51.2% (range 27%–74.1%) in less developed countries. They also found that the proportion of people actually receiving care was substantially less at 22.4% in both groups [61]. Based on these estimates, they calculated that about 40.5 million couples were seeking infertility medical care then (2007) [62].

Factors affecting the success of infertility treatment

Whether pregnancy occurs with or without treatment depends on various factors, which can be summarized as follows [66]:

1. Knowledge of the maximum fertile period. Many couples assume wrongly that the day of ovulation is the best day for conception. In their analysis of 225,596 menstrual cycles from 98,903 women, Faust et al. confirmed previous studies and found that the probability of conception was highest when intercourse took place 1 day before ovulation (42%) followed by 2 days before ovulation (33%), 3 days before ovulation (27%) and 20% when it occurred on the day of ovulation [67] (Fig. 1.3).
2. Time of unwanted nonconception. The chances of a couple in achieving a pregnancy diminish the longer the time they have been trying to conceive. As mentioned before, Gnoth et al. found that 81% of the pregnancies occur in the first six cycles with regular intercourse in the fertile period. One out of two couples of the remaining 19% will conceive spontaneously in the next six cycles. After 12 unsuccessful cycles, 8% of the couples will remain infertile, and after 48 months, 5% of the couples are definitively infertile with a nearly zero chance of achieving a spontaneous pregnancy [57].
3. Age of the woman. Female fertility starts to decline around 25–30 years of age. In their seminal paper, Eijkemans et al. showed that the age-related loss of

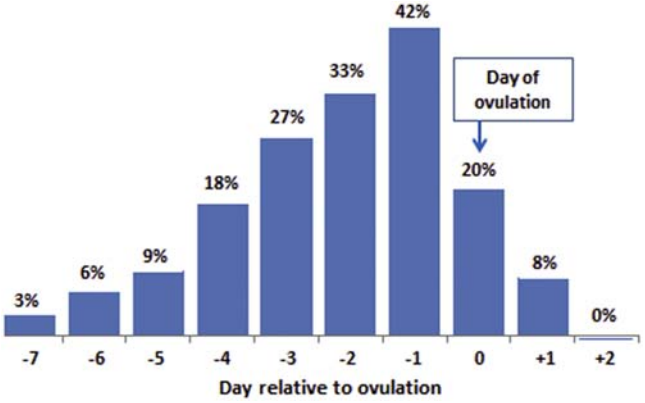

FIGURE 1.3 Chance of conception per day of cycle. *Adapted from Faust L, Bradley D, Landau E, Noddin K, Farland LV, Baron A, Wolfberg A. Findings from a mobile application-based cohort are consistent with established knowledge of the menstrual cycle, fertile window, and conception. Fertil Steril. 2019;112(3):450–457.e3.*

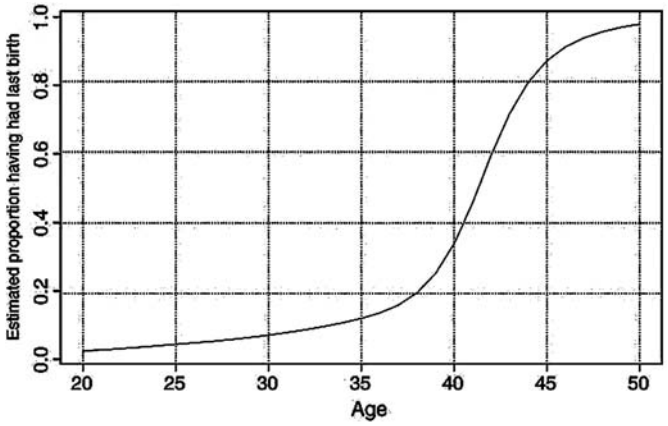

FIGURE 1.4 Cumulative age at last birth (ALB) curves showing declining fertility with age. *From Eijkemans et al. with the kind permission of the Editor of Human Reproduction Eijkemans MJC, van Poppel F, Habbema DF, Smith KR, Leridon H, te Velde ER. Too old to have children? Lessons from natural fertility populations. Hum Reprod. 2014;29(6): 1304–1312. https://doi.org/10.1093/humrep/deu056*

fertility slowly increases from 4.5% at age 25 years to 7% at age 30 years, 12% at age 35 years, and 20% at age 38 years. It increases rapidly afterward to about 50% at age 41, almost 90% at age 45 years, and approaching 100% at age 50 years [68]. This decline in fertility is related both to the continuous depletion of oocytes stored in the ovaries as well as a decline in oocyte quality (Fig. 1.4). Unfortunately, studies show that most women are not aware of the fact that delaying childbearing increases the risk of infertility, and moreover, many women believe that modern treatment modalities such as IVF can treat the fertility decline associated with advancing age [66].

4. Cause of infertility. The success of infertility treatment depends also on the cause of infertility. In their classical study of a population of 1,850,000 in three French regions, Thonneau et al. found that women alone were responsible for infertility in 33% of the cases, while the man alone was responsible in 20% of the cases. The cause resided in both partners in 39% of cases, while in 8%, infertility was unexplained [69]. Most causes of infertility are nowadays amenable to treatment, and even intractable cases such as absence of the uterus, ovarian failure, or absolute testicular failure can be helped by gamete and embryo donation, uterine transplantation, and surrogacy, whenever the law of the land permits.

Burden of infertility

Infertility exerts a burden both on the infertile couples as well as on the national health systems. On a personal level, infertility is known to cause significant psychological and social effects, particularly in low and middle income communities, such as fear, anxiety, depression, self-blame, marital stress, emotional abuse, intimate partner violence, and divorce. Other negative consequences include social isolation, economic deprivation, loss of social status, and in some regions of Africa and Asia, violence-induced suicide and even loss of dignity in death [70]. Unfortunately, in many of these societies, the infertility burden falls disproportionately on women, who are often marginalized, socially excluded, and stigmatized [71].

At the same time, infertility exerts an economic burden on the national systems, and unfortunately, in many parts of the world, authorities still claim that infertility is not a health problem, is not a serious health problem, or that contraception is a more pressing need. As "reproductive rights" are now an integral part of human rights, all governments that are signatories of the Universal Declaration of Human Rights cannot advance these arguments anymore and are obliged to include infertility services in their family health programs [72].

Access to infertility services

Infertility services offered by specialists and institutions can be stratified at three different levels: (a) a basic level offering laboratory investigations, ovulation induction with or without artificial insemination, (b) an intermediate level offering IVF with diagnostic endoscopic services with or without cryopreservation services, or (c) an advanced level capable of offering ICSI with or without preimplantation genetic testing (PGT) as well as operative endoscopic surgeries and other advanced services [71].

At the top of these services, assisted reproduction is considered a state-of-the-art technique capable of solving most infertility problems. However, in many parts of the world, this service is not accessible to those who need it most. In 2001, the European Society for Human Reproduction and Embryology (ESHRE) had suggested that 1500 couples per million population required ART treatment annually [73]. However, with the exceptions of Australia, Israel, and the Scandinavian countries, few developed nations have met this ESHRE benchmark, and even in North America and the United Kingdom, only 25% and 40% of the optimal number of ART cycles were being carried out, respectively, as of 2009 [74]. Unfortunately, in less developed countries, these services are only available to very few people (e.g., only 1.5% of the needs are met in sub-Saharan Africa) [75]. It is hoped that with time, infertility services will be available to more couples in developed as well as developing countries [70].

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CHAPTER

2

Setting up an ART unit: planning, design, and organization

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Planning

Location

The location of a medically assisted procreation (PMA) center is strategic for obtaining good results [1,2]. Before thinking about building an assisted reproductive technology (ART) center, it is a good idea to evaluate the number of inhabitants, and consequently the number of infertile couples that will need it. Areas that are able to satisfy most needs must be privileged: maximum proximity to the catchment area, environmental quality, and any availability of subsequent extensions, as well as a convenient position to transports.

Luo et al. found that electromagnetic fields could cause DNA damage in embryos *in vitro*; however, the electromagnetic field in this study was applied directly next to the culture dishes inside an incubator [3]. It is important to underline that a magnetic field's power is inversely proportional to the square of the distance. Electrical equipment, especially those accredited for use in operating rooms, must meet regulatory standards for electromagnetic fields to avoid interference with other electronic equipment. However, it may be advisable to space out this type of equipment away from incubators. The health of laboratory personnel should also be considered from this perspective, as there is growing evidence that some individuals may be sensitive to electromagnetic radiation [4].

The area must be far from humid soils or land subject to infiltration or stagnation, must not be in areas with potential for landslide, and must not be exposed to strong winds or be located downwind of areas from which fumes or noxious fumes may originate or be unpleasant [5].

A determining factor contributing to this choice is the quality of the air [2,6]. Nowadays, however, for economic and population needs, the PMA centers are located in the city center to serve a large portion of the population. The designer should first assess whether there are areas around the structure potentially subject to demolition or renovation that could subsequently compromise the air quality [7]. Activities taking place near the center could have an unfavorable impact on the center; in particular the wind direction, industrial emissions, pollen and dust and ozone quantity present in the area should be determined. In fact, one of the most important polluting components is the presence of VOC (volatile organic compounds) particles that come from construction, renovation, or demolition of buildings [8].

It should be considered that within this structure, outdoor spaces play a prominent role: car parks, roads, and in some cases, green areas. The area must include a large independent car park externally and areas reserved for handicapped people, both external and internal ones. Pathways for the handicapped must also be studied.

Reachability

It seems trivial, but the easy accessibility of a structure and the ease of parking is a primary thing; only those who have had an adverse experience can report it. In some cases this problem can be so serious as to orient the patient's choice differently. The structure should be located in an area that can be easily reached by public transport and near parking lots. Having parking spaces or being in an area full of parking spaces is indispensable.

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Communication

Another important factor to evaluate is the type of accessibility, especially in regard to public and private transports and the communication network. In addition to road communication, telephone and data communication must be guaranteed. Even now the most widespread communication standard should be a free WIFI area, located in the waiting room or in nearby areas. However, it would be advisable that this area does not extend beyond the waiting room, so the WIFI signal does not invade the technical areas.

Design and building

Required surface

It is difficult to assess the amount of square meters needed to build an ART center Table 2.1, but there is an assessment according to American standards that can help [9].

Design

The figure that a professional wants to convey is shown through many components: among these is the environment in which the patient is received.

Waiting room

It would almost always be wise to welcome patients in the most relaxing way possible. Let us remember that they come to do something they do not like to do.

So let us welcome them in a place that is as relaxing as possible, with comfortable seats, soft colors, background music, recent newspapers. We create, if space permits, a sideline area for children, set up with comics or cartoon videos, with the double result of not bothering other waiting adults and to entertain them without doing damage. The number of seats must also be adequate Table 2.2.

Examination room

The examination room should allow you to dialogue appropriately with the patient or the couple but also to carry out the normal investigative tasks. To do this, a space is needed in which the patient's privacy is protected: an area where the patient can undress without being seen and therefore not be uncomfortable and where the path from that place to the examination table is as short as possible.

Semen pick area

The room or rather the bathroom where the sperm is taken should be particularly comfortable with a video

TABLE 2.1 Analysis of spaces in a medically assisted procreation center (according to American standards). The estimate is made on a center that carries out between 300 and 600 oocyte retrievals per year and has three gynecologists.

			Total
Administration			229 sqm
Business area (payment area, workroom, data)	46 sqm		46 sqm
Waiting room	36 sqm		36 sqm
Reception	18 sqm		18 sqm
Meeting room	26 sqm		26 sqm
Staff lounge	14 sqm		14 sqm
Toilet staff	5 sqm	n. 2	10 sqm
Storage area	7 sqm		7 sqm
Director	12 sqm		12 sqm
Lab director	8 sqm		8 sqm
Psychologist	12 sqm		12 sqm
Resource/educational patient area	12 sqm		12 sqm
Conference room	28 sqm		28 sqm
Clinical area			
Examination room	9 sqm	N. 6	54 sqm
Infirmary	9 sqm	N. 2	18 sqm
Blood sampling	4 sqm		4 sqm
Sperm sampling	9 sqm		9 sqm
Toilettes	7 sqm	N. 3	21 sqm
Consultation room	13 sqm	N. 3	39 sqm
Andrology lab			12 sqm
Embryology lab			60 sqm
Micromanipulation area			
Cryoroom			
Storage			
Surgical area			132 sqm
Surgery room	36 sqm		36 sqm
Minor procedures room	20 sqm		20 sqm
Recovery/preoperative room	12 sqm		12 sqm
Hospitalization area	15 sqm	N. 2	30 sqm
Dirty storage	6 sqm		6 sqm
Clean storage	6 sqm		6 sqm
Operators' washing area	4 sqm		4 sqm
Locker room	12 sqm		12 sqm
Various	6 sqm		6 sqm
Subtotal			578 sqm
20% circulation			116 sqm
Total			694 sqm

TABLE 2.2 Formula for determining the seats.

$2P \times D - E = S$	
P =	Average of patients/hour/doctor
D =	Number of doctors
E =	Number of doctors' offices
S =	Seats

device, on which it is possible to choose to watch films that can help to carry out the act itself. In fact, it is not unlikely that some patients will not be able to produce the sample because they are strongly affected by the psychological situation.

The only way we have to help these patients is to provide them a comfortable and hygienic environment with the right precautions to take this sample. Another precaution that may seem trivial but is considered particularly useful is direct communication between the sampling area and the seminal laboratory. This prevents the patient, after collection, with the sample in hand asking where to deposit it.

Semen laboratory

This environment must in any case be contiguous with the *in vitro* fertilization laboratory (not necessarily communicating) since some operators are often divided between the two areas; and in any case it is good that the sperm is treated and prepared in another area.

IVF laboratory

The laboratory must be in a low-traffic and secure area with limited access. In the embryology laboratory, the workflows must be carefully evaluated: from egg retrieval (with a window or door for communication between the laboratory and the surgical room where the pickup takes place), oocyte processing under a laminar flow hood, incubators, eventual sperm injection with an inverted microscope and micromanipulator, again incubators, and microscopic preparation of the catheter for the transfer. It will be appropriate for the air quality [1,2,9] and for quality certifications that access to the laboratory [10] is controlled (e.g., by badge) and allowed after washing and wearing suitable clothing (the same as the sterile one in the operating room).

Storage areas

The operating room and IVF laboratory use many different consumables; these require storage space and should not be stored in the laboratory or operating room. First, cardboard packaging is a source of dust, bacterial contamination, and most cardboard is saturated with VOCs. Consumables should be removed from the cardboard packaging outside and transferred to plastic tubs for storage near the laboratory. Plastic packaging that surrounds consumables (e.g., plastic items) can also be a source of VOCs. It is preferable to store consumables in a warehouse area outside the laboratory and transfer to the laboratory only a small amount of what is needed for use.

Controlled accesses

There are areas such as the IVF laboratory or the surgical area and the cryoroom where access should be controlled, to prevent unauthorized personnel from entering, so there is a trace of the last person who entered and left.

Emergency access

In the planning phase, emergency routes should be taken into account, for services such as ambulances and firefighters. Paramedics can request access to the operating room in the event that a patient suffers a complication that cannot be treated with the medical equipment available in the clinic. Passages and doors should be wide enough to allow for easy passage of a stretcher to remove patients. Similar consideration should be given to the access that may be required for firefighters to enter the building in the event of a fire. It would also be advisable to provide, in case of danger, an emergency access and an evacuation plan for the gametes and embryos stored in the cryoroom.

Materials and implants

The prevention and control of workplace contamination is one of the main problems. It is appropriate to provide that the conditions of the environments are such as to guarantee the following:

- the optimal conditions for patients
- the health of the operators
- the protection of the external environment

To these objectives, we must add one, the most important, that is that the materials used can affect cell cultures. It must be considered that, although cell culture is carried out in the IVF laboratory and, in particular in incubators, there are also passages that occur outside of them and which are highly sensitive to the external environment. Having used materials that are sources of toxic substances for cells would compromise much of our work [2]. To achieve the above, "controlled

contamination" environments are required; these are identified in premises characterized by particular constructive and operational measures aimed at minimizing the risk of contamination of crops, patients, and exposure of operators [5,11].

Materials

Floors

The floor must be nonslip, connected to the walls, smooth and uniform, resistant to chemical and physical agents; the walls must be connected to the ceiling, also smooth and uniform, disinfectable at full height and fireproof; the ceiling, on the other hand, must be continuous and smooth.

Some materials for walls, floors, and ceilings may contain chemicals (for example formaldehyde and VOC) and, once installed, emit these pollutants with a negative contribution to indoor air quality. Furthermore, these pollutants are highly harmful to cell cultures. Some of these materials are porous and absorbent and can trap both odors and chemical products derived from other activities and construction materials, to be then re-emitted and pollute the air [8,10,12].

Resilient coatings

They include a series of products composed mainly of PVC, linoleum, or rubber. PVC is an easily disposable and nonpolluting material; it does not contain potentially allergic or toxic substances and is a naturally stable polymer.

Materials for thermal and acoustic insulation

There are some problems related to indoor air quality that can be associated with the materials used for thermal insulation of buildings: problems related to the emission of chemical substances (in particular VOC and formaldehyde) but also related to humidity [12–14]. For insulation and waterproofing, synthetic materials such as polystyrene panels or urea-formaldehyde foams should be avoided as much as possible; these release potentially dangerous substances, and being particularly impermeable, they compromise the breathability of walls.

False ceiling

It happens more and more frequently, even in healthcare environments, that the ceiling is a false ceiling. This allows the fixtures to run into the false ceiling. In this way it is easy to intervene in case of breakdowns. The false ceiling, however, precisely because of its structure can be a source of dust stagnation and a source of infections spread through the air.

Therefore, the false ceilings applied in healthcare facilities must have particular characteristics and certifications that prevent such inconveniences.

Paints

Paints are among the most important sources of emission of VOCs; it would therefore be advisable to choose to adopt a plan to reduce the formation of VOCs. This can be done using application cycles and/or paint products with lower emission of solvents [15]. The paints with the highest VOC content are the nonwater-based ones; therefore, when setting up a PMA center, only and exclusively water-based paints should be chosen.

Plants

In the 1980s, in the hope of creating an ecological system to purify the air in spacecraft to be sent into space, NASA carried out a series of experiments on plants. Much of this information has been taken from the text "Friendly plants" by B.C. Wolverton, one of the NASA researchers who participated in this project. He exposed that certain houseplants (Fig. 2.1) remove 50% of toxic substances from a closed environment, such as benzene or formaldehyde, which would otherwise be free in the air. These particles are absorbed by leaves and conveyed from stem to roots where the microorganisms metabolize and eliminate them [16]. To ensure that our daily environment, including the one of our centers or

FIGURE 2.1 *Nephrolepsis exaltata* able to purify the environment of VOCs.

departments, is full of clean and fresh air, it will be important to surround ourselves with some "friendly" plants. More precisely, three types of common plants are specialized in converting harmful elements of the air such as formaldehyde and transforming CO_2 into oxygen. The University of Georgia published in October 2009 in *Hort Science"* journal a list of plant species that can prove to be valid allies to clean the air from harmful VOCs, such as benzene or other toxic hydrocarbons that come from adhesives, clothes, solvents, building materials, paints, and even tap water.

Installations

Light

Hospitals and sterile environments have very specific lighting needs that must be solved with luminaires with peculiar construction and lighting characteristics. In the laboratory, however, some things must be considered. Embryos show and possess a wide capacity to adapt to different culture conditions. However, suboptimal situations of the environment can disturb not only gene expression, but also the occurrence of important repercussions on postnatal development as well as on growth and offspring.

Over the years, particular attention has been paid to constituents of culture medium, temperature [17], and pH, while less to the potential role of light and its effects. It is commonly believed that light has no effect on the physiology of early oocytes, zygotes, and embryos. Over the years, different effects of light have been observed on oocytes, sperm, and embryos in different animal species, and it has been possible to conclude that the presence of light was not always harmful [18,19].

To date, there are still no important issues regarding the assessment of a possible impact on gametes and human embryos *in vitro* of the type of light, duration of exposure, or exposure to different wavelengths. Most of the time, the available results derive from studies on animal models. In mammals, the natural incubator, the uterus, is equipped with homeostasis conditions that allow for minimal environmental changes, unlike the external environment that, on the other hand, is quite variable. PMA laboratories are equipped with modern incubators nearly capable of reproducing this internal environment.

The greatest interest in controls on incubators therefore focuses, in particular, on temperature and pH management. This is because, unlike what happens under normal conditions, in IVF laboratories, these parameters are subject to wider and faster excursions. During ART procedures, embryos, sperm, and oocytes are exposed to different light sources. It is hypothesized that oocytes and embryos do not have a system of protection or repair against the potential damage of light during the various steps of *in vitro* fertilization and therefore irreparable damage can be generated [18]. There are several ways that light can damage a cell. Subsequent studies have shown that there can be a direct effect when light stresses the cell often, directly damaging DNA through ionization [20–22]. Light can also indirectly damage mammalian cells through photooxidation, which is a chemical reaction between light and components of the culture medium and oil [23–25]. It has been shown that photooxidation can lead to the production of toxic hydrogen peroxide in the components of a culture medium. The same mechanism described for the elements of the culture medium can similarly involve sperm and membranes, producing changes that can potentially inhibit [25].

There are, in fact, numerous examples of how light itself can damage gametes or embryos. Sensitivity to light has been reported for hamster embryos. In fact, the first intracytoplasmic sperm injection success with hamster oocytes was obtained by filtering the light of the microscope with red light in a dark room [19,26]. As proof of this, it has been reported that just 1 hour of exposure of hamster oocytes to cold fluorescent light determines an inhibition of the normal meiosis process, and only 30 min of exposure to light (380–760 nm) blocks the development of the embryo at the 2-cell stage. Embryonic development is even more compromised when at the stage of two to eight cells there is an exposure of even just 5 min to light [27]. It has also been widely demonstrated that reactive oxygen species levels in hamster and mouse zygotes after exposure to cold fluorescent light or warm fluorescent light for 15 min at 37° C especially increased after exposure to cold fluorescent light, and most of it is produced in hamster zygotes more than in mouse zygotes. These results lead to the conclusion that warm fluorescent light and incandescent light appear to be less stressful to oocytes and embryos when compared to cold light.

The most frequent and common effect resulting from exposure to light can be translated into a failure of normal chromosomal development after the metaphase and in formation of numerous small pronuclei. In many cases there is no expulsion of the second polar globule.

In humankind, the same repair and cell cycle blocking mechanisms are present. However, it is not known whether these systems are in operation and capable of acting in the event of damage resulting from exposure to light during IVF techniques. Therefore, the exposure of oocytes, zygotes, and embryos to visible light and in particular to light at low wavelengths (close to UV) should be minimized or avoided to ensure *in vitro* development as similar as possible to *in vivo* [27]. For this

purpose, safety and convenience, the use of warm white fluorescent light appears, containing light with shorter wavelengths. The incandescent light, coming from common microscopes, should not produce any serious problem unless it is used excessively [27].

Power and UPS

All the critical functions of an IVF laboratory depend on electricity. Not only is the lack of electricity supply a major trouble, but the quality of electricity supplied to the clinic is also important. Power fluctuations can cause problems for electronic equipment. Backup in the event of a power outage is essential and options can include generators and uninterruptible power systems (UPS). Many backup systems also provide power filtering to remove problems associated with spikes and surges. Large UPS groups can cope with the absence of current for a fair period and do not produce VOCs because they use the conversion of direct to alternating current.

Generators, on the other hand, are usually located outside the building in a secure room, which is easily accessible for maintenance, often near the parking lot. Generators usually run on diesel or petrol. Fuel is a potential source of VOCs, so it should be positioned as far as possible from the air conditioner vent.

Gas station

The IVF laboratory requires a special gas mixture of N_2 and CO_2 for its incubators, and for the operating room, it will require anesthetic gases. Gas cylinders are heavy and dirty and difficult to move. The ideal location for cylinders is next to the parking lot or in a place where exhausted cylinders can be collected and stored, possibly in a place easily accessible by the delivery service.

It is advisable to assign a small room or an area with a cage for storing cylinders and regulators, but even better is that this area is possibly in a parking lot and that it is protected from the sun; in fact, gases are sensitive to thermal excursions and temperature variations often cause gas leaks from their fittings.

Keep gas cylinders out of the culture laboratory whenever possible.

Adequate shelving for securing cylinders will reduce the risk of personnel falling. Any gases supplied to the laboratory or operating room must be connected via an automatic switch system, where in case of emptying of one cylinder, the other continues to supply gas. The quality of the regulators used in the exchange units is important, as poor ones may not work. Some may contain neoprene diaphragms, which have the ability to release VOCs into the gas stream. During the installation of the gas supply system, the plumbing for the gas supply in the laboratory will be installed. This can be copper or stainless steel. Another option is to use polytetrafluoroethylene (PTFE) pipes. PTFE is an inert, nonembryotoxic material, ideal for special gas mixtures because it is not permeable to CO_2. Silicone is another inert plastic but is permeable to CO_2, so it is not suitable for premixed gas as the CO_2 concentration will decrease proportionally to the length of the tube, leading to incorrect gas concentrations entering the incubator.

Liquid nitrogen and cryoroom

Liquid nitrogen is an important consumable used in cryopreservation and storage of gametes and embryos. It is also dangerous and requires care in handling. If cryopreservation will be performed in the laboratory, a regular supply of LN2 will be required. Small units and standard dewars containing 6 to 10 containers are usually used; but there are also much larger units that can hold many samples. Large cryobanks are served by having a large storage container on site, preferably positioned directly outside the laboratory and in a room easily accessible by delivery personnel. Also in this case, great attention must be paid to positioning of the liquid nitrogen storage container, which must be outside the structure. In fact it is advisable to place it in a safe place and where sun does not arrive. The pipes connected by this to the cryoroom can then supply LN2 where it can be used to top up dewars, or feed directly to the storage tanks via an automated top-up system. Depending on the volumes used, LN2 is delivered in the form of smaller tanks, or for high-volume utilities the external LN2 tank is filled by a delivery truck. In both cases, external access by delivery vehicles and a transport route must be planned.

The cryolab or room where LN2 will be dispensed and used requires special attention to design. Some leakage of LN2 from dewars is inevitable, so floor coverings need to be able to withstand the sudden change in temperature. Forced ventilation is critical to ensure that LN2 vapor is quickly removed and replaced with fresh air and an oxygen meter installed to alert personnel if nitrogen gas has reduced oxygen in the room. In fact, this can lead to asphyxiation.

Furthermore, it is essential to remotely control alarms linked to liquid nitrogen level in the various dewars. Unfortunately even the most sensitive probes currently experience a reduction only when the loss is already abundant enough. Infrared cameras have recently been proposed that can immediately notice the slightest leak.

	Limits of maximum concentration (particles / m^3 air)					
	0,1 µm	0,2 µm	0,3 µm	0,5 µm	1 µm	5 µm
ISO Class 1	10	2				
ISO Class 2	100	24	10	4		
ISO Class 3	1000	237	102	35	8	
ISO Class 4	10000	2370	1020	352	83	
ISO Class 5	100000	23700	10200	3520	832	29
ISO Class 6	1000000	237000	102000	35200	8320	293
ISO Class 7				352000	83200	2930
ISO Class 8				3520000	832000	29300
ISO Class 9				35200000	8320000	293000

FIGURE 2.2 ISO class.

Air quality

Kukadia and Palmer have shown that the quality of the outdoor air has a proportional impact on the quality of the indoor air itself. The most frequent contaminants include external sources of civil and industrial pollution present in the air (which flow inside through ventilation ducts or openings) and volatile particles (Fig. 2.2) deriving from building materials such as wood, paints, resins, carpets, sealants, and fiberglass, produced within the environment itself [28]. In fact, according to studies by the American Environmental Protection Agency, indoors, where many people spend more than 90% of their time, some harmful substances can reach levels 2–5 times higher than to the external environment, also due to the presence of internal sources of pollution.

Although there is a limited amount of data on the study of air quality inside PMA centers, it has nevertheless been shown that indoor air quality in healthcare facilities is slightly lower than in other public and private environments (for example homes, firms, schools). This happens not only because of products used for sterilization (ethylene oxide) and cleaning containing pesticides with teratogenic action, or because of the airborne particles dispersed by workers in the laboratory, but also for the plastic materials components of medical equipment, solvents, fixatives, perfumes, chlorhexidine itself (toxicity for sperm), or even anesthetic gases that can dissolve in culture media and alter embryonic metabolism [12,29]. It is extremely important to avoid core materials such as chipboard, wood panels, dry stone walls, adhesives, carpets, and paints that are sources of substances such as VOCs, aldehydes, or compressed gases that act as contaminants not only of the internal air but, at the same time, also of the quality air of *in vitro* embryos with sometimes harmful effects in terms of decreases in *in vitro* development of embryos and reduced pregnancy rates [13].

Most of studies involving the toxicologic effects of VOCs on embryos have been conducted *in vivo* on animal and human embryos. Once an embryo has been implanted, it is partially protected from environmental contaminants by the maternal defense system. Furthermore, *in vitro* embryos have not developed an immune system and lack barrier systems, such as an epithelium, excretion mechanisms, or respiratory function to counter contamination phenomena [11].

Therefore, although the risk of air contamination is common in PMA centers, there are currently no toxicologic data about air contamination or its effects during and after reproduction techniques. Similarly, there are no standards on the limits for the content of the air or the emission of gas.

Hence, there is a need not only to monitor PMA centers for contaminants but also to reduce the amount of VOCs in the air supplied in the IVF laboratories themselves and to have an additional system to purify air that can help eliminate the particles generated in the laboratory [5]. Therefore, the center must guarantee measurement, monitoring, and maintenance not only of levels of contaminating particles through microbiological control, but also, in relation to the air pressure, the exchange of the same, and the verification of the systems used for ventilation and filtration.

For an ART center to work well, a suitable temperature with relative humidity around 40% must be guaranteed. In addition to this in the operating room and in the ART laboratory, there must be a positive air pressure to push the air outside.

LAN network

It is now essential that a LAN network must be present in every building. In the context of an ART center this is even more important because all scientific equipment has a network port for remote management of the same. It would be advisable to wire the entire structure as the WIFI mode must be limited to some areas, possibly excluding sensitive areas such as the operating room and laboratories.

PMA and home automation

The new approach to medicine is to take advantage of increasingly advanced technologies. The aim is to minimize human error. The application of computer and telecommunication systems to medical sciences has made concrete, unimaginable prospects until recently. Technologic innovation can provide a significant contribution to increasing the effectiveness, efficiency, and equity of access to healthcare services: think, for example, of the collection of clinical data from multiple separate diagnostic systems, of the remote monitoring of clinical parameters, of the widespread distribution of medical information.

Telemedicine is today probably the most important application as a connection between medical science and communication sciences in a broad sense. Methodologically and technologically, it offers new opportunities for connection according to "geographical axes"; from an organizational point of view, it offers a valid and effective tool for linking different levels of care. Above all, it allows the doctor to immediately have all the data of a patient available and to carry out his indispensable support even from a distance, in those cases where direct intervention could be problematic for various reasons. However, it is necessary to envisage a reorganization and rationalization phase of the healthcare structures that intend to fully exploit the opportunities offered by these technologies, to set the issue in an innovative way that is oriented toward the solution of the problem ("problem solving").

The first step to take, to creating a PMA center that is partially or totally home automated, is certainly the analysis of the client's needs, starting from the obvious ones up to trying to understand the latent and unexpressed ones. Home automation, in addition to automating some building control systems, such as antitheft and fire alarms, allows a tangible savings of electricity, estimated on the order of 20%–30%, and safer operation of the loads, avoiding blackouts and overloads of current. The different solutions offered are characterized by high flexibility, in fact they easily adapt to both very large and more reserved environments, while retaining all the characteristics of modularity, efficiency and effectiveness. The healthcare environment itself presupposes the existence of particular safety precautions: access protection, local protection, gates and bars, control via TVCC system, fire and flood detection, etc.

The challenge of designing a PMA center with home automation integration is linked to the ability to make all this opportunity possible. The services that home automation offers are so many that they can make the management of such an extremely engineered process completely automatic.

To get an idea of what are some of the potentials of this system, which being an open system lends itself to any future evolution, we will review some of the most common uses:

- Management of video distribution in the various premises of the center
- Audio management
- Management of cameras aimed at equipment
- Intrusion management and security cameras
- Microclimate and diversified humidity in the various areas
- Management of health alarms (oximeter, etc.)
- Telemedicine with real-time reporting
- Interrupt management of UPS and generators in the laboratory and operating room
- Verification of the air sanitation conditions in the laboratory and operating room thanks to interfacing with specific VOC-type sensors and particle counters
- Management of accesses in restricted areas and their tracking
- Management of incubator alarms
- Communication system between the various areas, in particular between the IVF laboratory and the seminological laboratory and areas such as the surgical area, the transfer area, and IUI, through computer systems
- Alarm management of medical gases and laboratory gases
- Remote management of equipment with the possibility of remote control (e.g., time lapse systems)
- Management of O_2 concentration and alarms in cryorooms

Ergonomics and flows

Workflows

When we relate to others, initially our concept of the interlocutor is dictated by the signals that come from him, and in particular those that in the first instance can capture our attention. Therefore, a good-looking figure, kind ways, and helpfulness are evaluated as positive signs.

When we find ourselves interfacing with a health facility, regardless of its size, in addition to the characteristics mentioned above, we add at least two priorities

to the evaluation: hygiene and cleanliness of place and organization. The latter seems to be the most important impression that users make and becomes a way of qualifying the quality of the service, since the patient at first contact has no other criteria than this. Therefore, even the mere perception of disorganization places the patient and his family in a position of distrust because it conveys the idea that the center is unable to take care of itself.

To understand in depth what the problems related to disorganization may be [30–32], it is necessary at least to know the actors of the organization:

if everyone knows their every task
if they implement what they know
if there are conflicts between the different competences
if the conflicts belong to the category of doing or not doing
the capital goods that an organization has to carry out its work
human resources

When we talk about workflow (Fig. 2.3), we are imagining a series of repetitive actions that lead to a generally

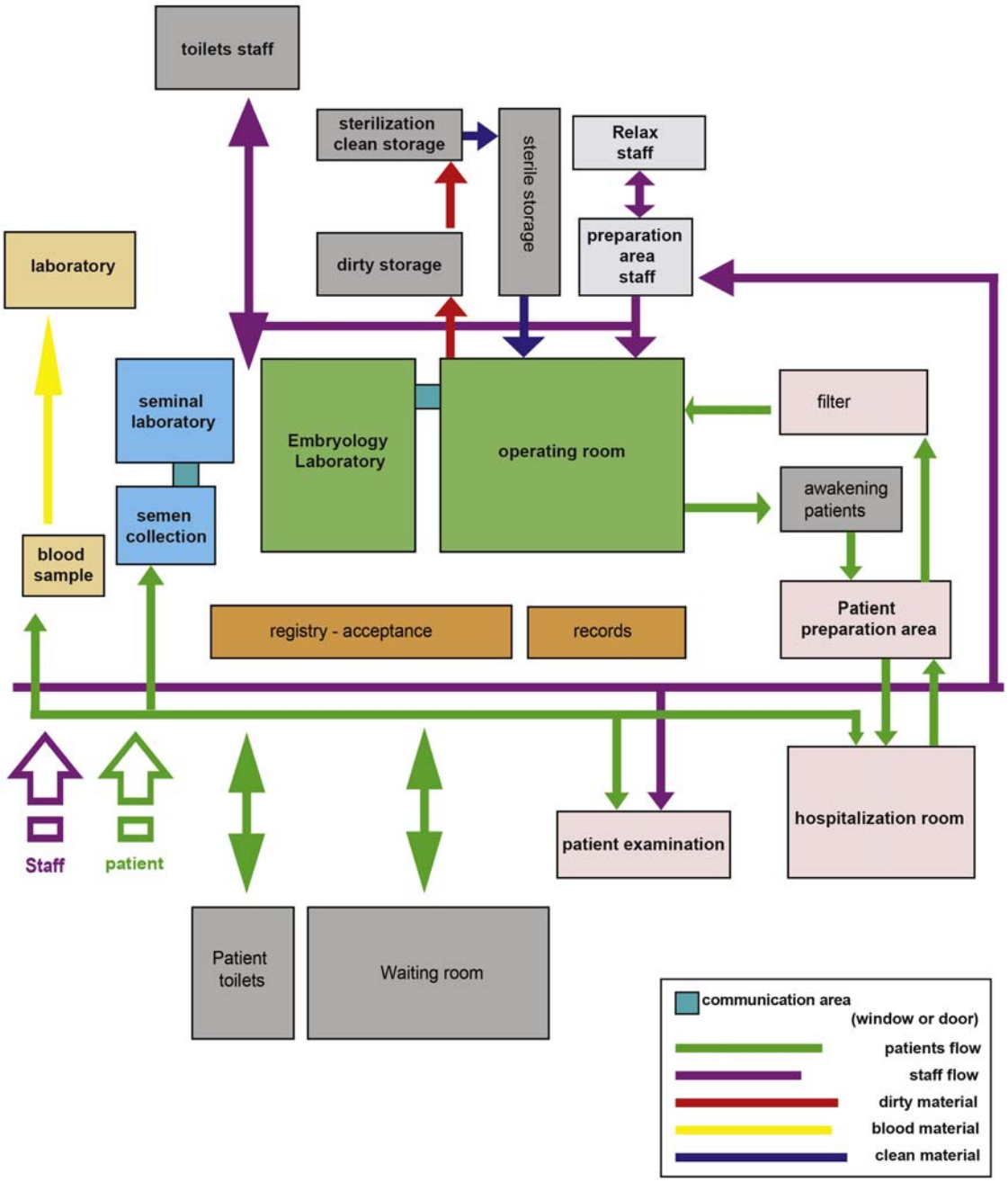

FIGURE 2.3 Demonstration of flows in an IVF center.

identical result, which are often repeated throughout our working day.

The flows must be studied in detail to avoid unnecessary crossings and waiting: for this reason the functions are well divided, with dedicated areas.

By leading logistics, we mean the nerve center and most critical points of the healthcare company, that is, where the flow of patients begins, or where there are important organizational hubs. Concentrating a lot of energy in evaluating these key points is an excellent choice because in the event of rapid success, an effect that can also be amplified in other structures that will be obtained, the good way to proceed and solve problems will physiologically expand. For each focal point it takes no less than 3–4 months of sharing problems to start a method that proceeds without further hesitation.

To apply these assessments in pathways, we practice designing value flow maps for individual disease or surgery or trauma management profiles. The frequency of review of the processes must be high enough (every 2–3 months) to allow learning the most effective system for applying the principles set out above. It is known that the timing that regulates the flow of patients is certainly not one of the most common qualities in a hospital environment, if it is true that the greatest evidence of waste is precisely in the number of people waiting: not only patients, but also doctors, nurses, technicians, and auxiliaries [33–35]. In summary:

choose a sector where it is possible to rigorously put activities in flow
simplify the flow
standardize the activities of the group
redefine priorities
introduce improvement indicators

Ergonomics

Ergonomics has as its object human activity, analyzed in relation to the environmental, instrumental, and organizational conditions in which it takes place. The purpose of ergonomics is the adaptation of these conditions to the needs of man, in relation with his characteristics and his activities. The environmental, instrumental, and organizational conditions are deemed to comply with ergonomic principles when their whole is consistent with the characteristics of those who work in the system and with the objectives of their activity [36]. Compliance is assessed in relation to the safety, productivity, and satisfaction of those who work in the system and/or those who refer to the system as a user. The ergonomist designs, manufactures, and evaluates the performance of environments, tools, products, services, and procedures to make them compatible with the characteristics of operators and users [37,38].

His intervention will therefore be aimed at the realization of the following:

physical and cognitive interfaces of environments tools
products and services, consistent with the anthropometric, physiological, psychological, and socio-cultural characteristics of operators and users
procedures and life and work activities that favor the development of skills and the improvement of the overall quality of the system

Having to identify the objectives of the ergonomic intervention, these can be identified (according to the classification of A. Chapanis) as follows:

1. basic operational objectives: reduce errors, increase safety, increase performance
2. objectives relating to reliability, durability, and utility
3. objectives relating to users and operators, improvement of the working environment, comfort, ease of use
4. other objectives to reduce waste

Examples about ergonomics in an embryology laboratory

The laboratory must be in a low-traffic area in a secure area with limited access. In the embryology laboratory, the workflows (Fig. 2.4) must be carefully evaluated: from egg retrieval (with a window or door for communication between the laboratory and the surgical room for the pickup), oocyte cleaning under a laminar flow hood, incubators, eventual sperm injection with an inverted microscope and micromanipulator, again incubator, to microscopic preparation of the transfer catheter. It will be appropriate, for air quality and quality certifications, that access to the laboratory is controlled (e.g., by badge) and allowed after washing and wearing suitable clothing (the same as the sterile one in the operating room).

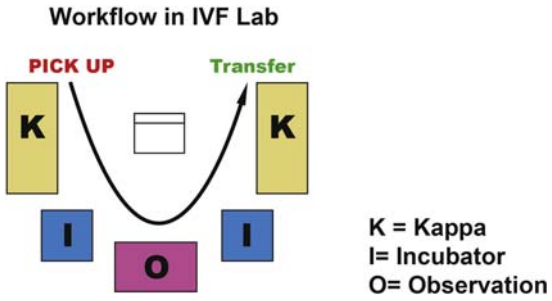

FIGURE 2.4 Example of ergonomics in an IVF laboratory.

Responsibility and organization

Reproductive medicine is certainly a teamwork where a series of scientific professionals make their contribution.

A concept is increasingly affirming that reproductive medicine is an independent branch of obstetrics and gynecology. The very high specialization of assisted reproduction centers requires specialized and dedicated staff, whether it is an integrated center in an obstetrics and gynecology department, or whether it is autonomous.

Staff and experience

Almost all the operators of an ART program educated through training do not exist or there are few institutions such as universities that prepare for this work.

Good results depend on a cautious and rational assessment of individual skills, so laboratory staff, directors, and embryologists must consider their experience in the context of what will be required of them. Some regulatory bodies such as the College of American Pathologists in the United States and the Human Fertilization and Embryology Authority in the United Kingdom provide guidelines and licenses for embryologists. However, the license does not necessarily guarantee skill (or success), and the licenses are not valid across borders from one country to another.

Stafff requirements

Practical experience in all aspects of clinical embryology is an absolute requirement when starting a new program. Even many experienced embryologists and scientists should be supervised directly by experienced clinical staff.

While a "traditional" IVF cycle took about nine staff hours a few years ago, a contemporary cycle can take up to 20 h. This leads to an increase in the number of embryologists required for the safe and efficient operation of the laboratory. However, it is important that the workload is not such as to neglect time for quality control and continuing education and training to maintain the high standards required for success.

If there are already doubts about skills and certifications of embryologists, the evaluation and certification for medical personnel is even more complex. To date, there are no shared data on which organization must certify the requirements to start working; only the European Society of Human Reproduction and Embryology (ESHRE) and the American Society for Reproductive Medicine (ASRM) have made an effort to write guidelines on this topic.

However, ESHRE and ASRM guidelines that regulate the organization of a PMA laboratory show some substantial differences. While the European model pays particular attention to the laboratory and everything that is necessary to guarantee an improvement in quality in terms of control, management, assistance, and results, the American model (ASRM) focuses attention on the professional figures who work in an ART center as well as on the organization of the same.

Organization chart

The organization chart is a fundamental thing when we talk about quality of treatment since all the actors of the treatments in the various stages of the procedure must be clearly identifiable, so the person responsible for a problem is easily identifiable and what initiatives we can take to avoid it. An organized structure requires a very specific organization chart where everyone performs specific tasks.

European model [39]

Staff and management

Personnel are crucial in the IVF laboratory. The number of embryologists should be related to the number of cycles performed. Generally, for up to 150 egg retrieval and/or cryopreservation cycles per year, it would be advisable to have at least two qualified clinical embryologists. This initial value will grow in relation to the complexity of the methods carried out and the number of cycles. Furthermore, activities such as administration, training, education, quality management, and communication inevitably fall on biologists. Equally important is having adequate staff to provide support to embryologists.

Laboratory director

The laboratory should be headed by a person with officially recognized qualifications and skills in clinical embryology and biological/medical sciences. In accordance with ESHRE survey's results about education and professional status of clinical embryologists, this would include a higher academic degree (MD, MSc, Ph.D.) with a minimum of 6 years of documented experience in human embryology and preferably ESHRE certification as a senior clinical embryologist or similar.

The laboratory director is responsible for managing many aspects, ranging from evaluation of materials to be used, equipment, quality management system, risk prevention, evaluation of appropriate skills, personnel management, up to evaluation of results.

Laboratory supervisors

Some laboratories include the figure of the supervisor. This requires specific qualifications, e.g., at least a

bachelor's degree in biomedical sciences, 3 years of documented experience in human embryology, and preferably the achievement of the certification of clinical embryologist by ESHRE or similar. The supervisor has the task of organizing work phases, establishing a valid communication system, staff training, and continuous improvement.

Clinical embryologists

Clinical embryologists are those who physically carry out daily clinical practice. New staff should take a structured training program under the supervision of experienced clinical embryologists. The activities of the clinical embryologist include execution of standard operating procedures, expressing their opinion on the decisions of the laboratory, communication with the various subjects, and training of new embryologists.

There is no mention about positions of the medical staff.

American model (ASRM) [40]

Personal

The staff must be sufficient to carry out all critical operations without interruption in case of someone's absence. A single individual can meet the competence requirement in one or more areas. An ART program must include the following personnel.

Doctors

A doctor with certification for obstetrics and gynecology or gynecological endocrinology is required, as well as a physician experienced in male reproduction. If there is no urologist, a urologist consultant should be available.

Nurses

A nurse is required with training and/or experience in reproductive medicine and coordination of clinical care ART.

Laboratory

The laboratory staff must include an expert in andrological procedures, someone with specific training in the techniques of cryopreservation of the gametes of embryos and gonadal tissues, someone capable of performing micromanipulation techniques, and appropriate personnel to perform hormone tests.

Auxiliary personnel

A gynecological ultrasound expert provides follicular development monitoring. This role can be filled by a doctor, nurse, or ultrasound technician. A mental health professional with experience in ever-present fertility counseling or at a least consultant is needed, as well as a genetics expert.

Training and specialized experience

Study director

The director is the one who takes care of communications with ASRM and with the registers; this person is not a doctor.

Medical director

Starting from January 1, 2000, the medical director of a PMA cycle must be certified by the REI board by American Board of Obstetrics and Gynaecology (ABOG) or be an active candidate for it. The medical director is responsible for verifying the data communicated to Society for Assisted Reproductive Technology (SART).

Doctor performing egg retrieval and embryo transfers

Both the doctors who perform the egg retrieval and those who perform the transfer must have adequate training and must have performed an adequate number of these procedures under a supervisor. Successful completion of this training should be documented by the medical director. To continue to be qualified for these procedures, every doctor must perform a minimum number of them every year. Physicians responsible for ultrasound follicular monitoring must be familiar with basic ultrasound principles and equipment.

Nurses

ART-licensed nurses are involved in education, counseling, support, and nursing care to patients seeking care for pregnancy.

Director of the embryology laboratory

The director of an embryology laboratory must have a PhD from an accredited institution in a chemical, physical, or biological science as a major subject, or a medical degree (MD or DO) from an accredited institution or have qualified as a laboratory director before July 20, 1999; they must have at least 2 years of industry experience, must be proficient in biochemistry, cell biology, and reproductive physiology with experience in experimental design, statistics and problem solving, quality management skills, and at least 60 supervised ART procedures. Furthermore, they must have at least 24 h of continuous training every 2 years in ART. Starting from January 1, 2006, all new laboratory directors need a certification as a High Complexity Clinical Laboratory Director or Embryology Laboratory Director (ELD) or its equivalent from the American Board of Bioanalysis.

The director of the embryology laboratory must write the protocols and report to the medical director anything

that may affect the laboratory aspects and to the other doctors the laboratory assessments about the specific treatment. Furthermore this person has to assess conditions and maintain sterile conditions in the laboratory, provide the staff manuals of standard operating procedures, organize a quality management program and continuous training of laboratory operators, and organize the work guaranteeing always a sufficient staff for the activities. Preparing a contingency plan is also needed.

Embryology laboratory technician

They must have a bachelor's or master's degree in chemical, physical, biological, or medical technology or clinical or reproductive laboratory science from an accredited institution. They must have performed at least 30 ART procedures as a minimum and be certified by an ELD.

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CHAPTER

3

Building the assisted reproduction laboratory

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Introduction

In 1978, in England, the first child conceived through an in vitro fertilization (IVF) procedure was born, a pioneering work of Drs. Edwards and Steptoe. Since then, a large improvement of IVF techniques has occurred in laboratories. The laboratory used for in vitro embryo culture is a key ingredient in the structure designed to replace the maternal womb for the first days of the prospective child's life [1]. To achieve successful embryo development and a positive clinical outcome, the embryos must be maintained in a stable environment [2].

Over more than 40 years, advances in the field of assisted reproductive technology (ART) have been made by gynecologists, embryologists, and geneticists to increase success rates of the procedure and the availability for the patients. As a result, over 200,000 babies are born worldwide each year by ART [3]. Up to now, approximately five million infants have been born through ART globally [4]. The awareness of the importance of quality assurance for laboratory systems, including disposables, culture media, and instruments specifically designed for assisted reproduction, has increased. A key component of a successful IVF program is a reliable laboratory, and what is truly important in an IVF laboratory: everything! (as highlighted at Cairo Consensus Guidelines on IVF Culture Conditions) [5].

Purposes of the lab

The conception of the project for ART procedures requires a clear vision of what services will be performed by the clinic and who will be the target customers. The clear definition of these aspects will be decisive for the allocation of the necessary space and conduction of effective planning [6].

When defining the clinic objectives, it is necessary to establish which services will be contemplated in the wide range of possibilities: whether it will be complete services, from elementary diagnostic tests to the use of the most advanced technologies, whether it will have a small team to have personalized treatments, or whether it will be a clinic with the capacity to meet a great demand from patients [6].

The project must take into account the expected service volume, as well as the number of procedures to be performed. It is necessary to define which subspecialties will be carried out in the same place. It is also important to estimate the size of the laboratory based on growth and expansion forecasts for the next decade, or period defined in the strategic plan. Therefore, it is essential that the project has a flexible design to allow further changes in the configuration of the rooms when expansion is necessary [6].

Location

One of the first aspects to consider when developing the basic design of a new laboratory is its location. Some clinics were built in places further away from large centers, as environmental factors such as stress can negatively affect the outcomes of reproductive treatment. However, not all factors can be isolated, so the convenience of access can be more decisive for some potential customers. Thus, laboratories today have favored more central locations in cities or metropolitan areas. Thus, more people will be able to be served, and it will make access to the place easier [6].

https://doi.org/10.1016/B978-0-323-89907-9.00023-5

On the other hand, some construction sites can be intrinsically harmful to cell culture. The level of pollution and the concentrations of volatile organic compounds (VOCs) inside and outside the laboratory premises must be considered. Although the embryo is not completely protected from pollution in the maternal tract, the mother's lungs, liver, and kidneys do provide considerable filtration and detoxification of VOCs, thus reducing the exposure of the embryo. In vitro the embryo has no such protective mechanisms, so steps must be taken to actively reduce toxic substances, in the general laboratory air and within the incubator in particular [7,8]. Air quality can be controlled in laboratories to a certain extent, but this protection against the external environment may not be complete, or its installation and operating costs may not be feasible for the dimensions of the proposed laboratory [8,9].

Laboratory

The laboratory of a human assisted reproduction center plays a decisive role in a significant part of the outcomes of IVF [6]. Therefore, the investment in the design of this type of facility must include particularities and specificities that will have a direct and indirect impact for patients and, therefore, for clinicians. Not only for the process efficiency, but due to the necessity of a very close interaction with the clinical staff, the configuration of the laboratory requires a clear definition of the workflows within the clinic.

The configuration of a new laboratory or the refurbishment of an existing one requires logistical and structural decisions that allow the flow and transit of personnel, supplies, and samples for clinical procedures. Thus, the project must prioritize a structure that values an adequate and restricted workflow, aiming at the safety of the samples and means of guaranteeing good laboratory practices.

The layout of the different areas of the laboratory must be based on the routine workflow, from the entry and reception of the samples, to their delivery after the procedures. The cryopreservation rooms, seminal processing, incubators, and culture media preparation area must be separate, allocated in areas adjacent to the gamete manipulation laboratory. This, in turn, must have air flow control systems to maintain a constant pressurizing level [1].

Inside the laboratories, incubators, gamete handling areas, and other micromanipulation stations should be positioned to minimize the distance between embryologists. Ideally, the embryologist should be able to complete a procedure without moving more than 10 feet in any direction. In this way, work becomes more efficient and safer, promoting less exposition of gametes and embryos to nonideal conditions [9].

Finally, it is recommended that the layout should not be defined by managers, engineers, or architects without the participation of the laboratory team and the clinical team [10]. The IVF laboratory is a complex environment, and at least 200 confounders affect IVF success [11]. Most of these are concerned with monitoring, staffing, equipment, and procedures in the embryology laboratory. The goal of this lab is to provide conditions that will lead to the production of embryos that have the same developmental potential as the embryos that develop in vivo [8].

Andrology laboratory

The seminal sample collection is part of the infertility treatment, but it can be embarrassing for some men. Although there is the possibility of carrying out the collection at home, clinics generally have at least one room dedicated to this purpose. The collection room must be positioned in a reserved area, so that patients can access it without any embarrassment. Soundproofing facilities should also be considered to improve patient comfort and tranquility [6].

Diagnostic andrology laboratories are likely to have a wide variety of potentially toxic chemicals, as well as pathogens, such as human immunodeficiency virus, hepatitis C virus, or hepatitis B virus. ART laboratories routinely safeguard against risk of sample mix up, equipment failure, and contamination by other organisms [12]. These areas should be physically separated from other laboratories and have exclusive air conditioning systems to ensure that no crossing toxins may affect the system among them [6].

Liquid nitrogen containers storage room

Liquid nitrogen is an input widely used in the cryopreservation and storage of gametes and embryos [6] that requires careful handling. The rooms in which the liquid nitrogen containers are stored must be located outside the laboratory, as close as possible to avoid evaporation and wastage during transport [1]. Smaller centers generally use tanks for supply, while larger ones may be able to maintain larger local reservoirs. In such a case, pipelines can be an option to supply the area of the nitrogen tanks. It is important to prioritize shorter, insulated pipes to avoid losses along the path to the final destination [6].

In addition, a reimplantation test (PGT) is currently performed on a large scale in many centers worldwide.

This development has been supported by significant improvements in cryopreservation methods. The genetic analysis can be time-consuming and as a consequence requires cryopreservation and storage of all tested embryos until results are obtained, and even after depending on the results [13].

Gas cylinders central and management

The gas storage and supply room must be installed outside the IVF laboratory, in facilities that allow easy access to authorized personnel, outdoors, or in fireproof shelters and protected from electric power transmission lines. The floor covering material must be a noncombustible composite resistant to liquid nitrous oxide and liquid oxygen. The gas supply pipes (CO_2 and N_2) must be made of stainless steel, which will conduct these gases from the central area to the usage points. The cylinders must be connected to pressure-regulating valves capable of maintaining the maximum continuous flow of the system. Those must be protected from heat sources so they do not reach temperatures above 54°C (129°F) [14].

General material storage

A wide variety of materials are used in the laboratory and require storage space. Cardboard packaging and other package materials are sources of bacterial contamination, dust, and dirt accumulation in addition to containing a high concentration of VOCs. Consumables must be removed from the cardboard packaging outside the laboratory complex and transferred to plastic containers for storage in small quotas for daily use only [6]. The storage facility should be large enough to accommodate bulky items, as well as movable shelves for containers. In addition, when possible, the storage location should be close to the laboratory to optimize logistics and an avoid excessive number of consumables in the laboratory [9].

The building structure

The adequate choice of construction material is essential as building materials are main sources of VOCs in the laboratory, which can negatively impact the results of cell cultivation. All materials from flooring, paint compounds, and furniture must be suitable for clean room standards, minimizing toxicity to gametes and embryos [14]. The laboratory floor requires a nonslip surface, impermeable to fluids, easy to clean, and that does not release harmful gases. Surgical or monolithic vinyl floors (polyurethane or epoxy) are the most used and should be tiled with heat-welded sections, extending to the walls with no angled corners. The purpose is to eliminate cracks that can accumulate dirt, bacteria, or fungi [1,6,14]. Finally, the area where the management processes of the laboratory are carried out must be separate from the laboratory itself, with a different air treatment system from the main laboratories [9].

Ceilings must be sealed to prevent particles from entering and constructed with materials that can be thoroughly cleaned. Coated steel or plasterboard are the main options. Likewise, the walls must be sealed, with nonpermeable material that can be easily cleaned or completely decontaminated if necessary. It can be made of aluminum, coated steel, plaster brick, or plasterboard. Regarding the paints, there are specific products with zero VOC, which would be the ideal option if the acquisition is within the reach of the clinic [6]. In spite of the illuminating system, two types of lighting must be installed in the laboratory: total, for maintenance and cleaning when the gametes and embryos are not exposed, outside the incubators; and for the laboratory routine, with yellow incandescent lamps, adjustable for intensity grading. Some light spectra can be harmful to cell development because they are associated with the formation of reactive oxygen species. Light wavelengths <400 nm can be potentially harmful to gametes and embryos, and the extent of the damage is related to the exposure time and intensity. It is recommended to reduce the light intensity during the evaluation of the embryos and to use filters to reduce the radiation energy in this range [1,6,14].

Air flow

Air quality is fundamental to the success of IVF programs. Atmospheric contaminants such as smoke, dust, chemicals, and inorganic gases are potentially toxic to embryos, negatively affecting their development and impacting implantation rates [14]. Modern laboratories are considered biologically clean rooms, which in addition to the air treatment system, microbiological control, and emphasis on VOC control (mainly of aldehydes), must associate other variables such as air conditioning control, adequate architecture, material flow, and professional vesting [1,14]. Relevant consideration must be made in relation to the position of the air conditioner. Cold air currents can cool the incubators, so the air vents must be positioned away from the benches to avoid this cooling [6]. The control of temperature and relative humidity, obtained by cooling and heating the air, allows maintaining favorable conditions for the embryo [14].

For laboratories adjacent to the operating room, it is essential to minimize the air entry into the laboratory, since the anesthetic, sterilizing gases, cleaning products, and disinfection solutions used between the procedures are potentially toxic compounds to embryos and should be excluded from the laboratory environment [6].

The laboratory must be subjected to particle counting and air flow verification by a certified agency each 6 months. If necessary, the ceiling filters should be replaced each 3 months [15]. A filtering system with filter batteries guarantees the contaminant retention. The filters known as HEPA (high efficiency particulate air), thin (F8 and F9) and thick (G3 and G4), are used for particle retention, and activated carbon filters guarantee the retention of VOCs. An inflation flow system in the rooms will provide the air volume changes per hour or m^3/h x m^2, and will certify the cleaning class and, consequently, the particulate material filtrated. The external air flow guarantees the pressurization of the environments [14].

Thus, with sterile air in the proper conditions of temperature and humidity being blown into the environments, and with the balance of air through the diffusers and grilles, it will result in a pressure escalation from the cleanest areas to the least clean areas of the laboratory obtaining a positive pressure. This pressure will protect clean rooms from entering contaminants from adjacent, less clean or rooms without cleanness control [14].

Furniture

A very common material used for the production of cabinets and countertops is MDF (medium-density fiberboard) coated with laminated plastic. This material is made essentially of wood particles joined with a variety of resins, which can release volatile gases such as formaldehyde, classified by the World Health Organization's International Cancer Research Agency as a known carcinogen, potentially toxic to the embryo. Plywood is another material commonly used with the same problem as MDF. If any of these are present in the laboratory, it is suggested to paint the crude surface to inhibit the release of VOC's. Materials that do not release gas, such as stainless steel and stones both natural or not, can therefore be a better option for use in the production of countertops [6].

Mobile chairs are commonly used in laboratories; however they can pose a danger as they move, causing accidents. The most recommended would be to adjust the height of the benches and microscopes so that professionals can work standing up. In this way, there would be a benefit in the ergonomics for the team, there would be no release of VOCs from the plastic materials used in the manufacture of these chairs, and it would make more space available, making a more organized working environment [6].

Equipment

The inclusion of new technologies in the assisted human reproduction laboratories, such as incubators with a time-lapse system or even bench tops, allow the laboratories to become smaller, which is relevant in cities with high prices for a square meter. Architects must be informed about the specifications of all equipment, as well as their ideal location for the optimal functionality [9]. The equipment specifications must be detailed to meet the needs and requirements. It is also important to consider the inclusion of spare equipment and tools in the event of an unexpected malfunction that may put procedures at risk [9]. Two or more incubators should not be seen as excessive, as well as one more micromanipulator. Eventually replacement parts, equipment maintenance, and sterilization will be necessary. For cryopreservation laboratories, extra nitrogen tanks should be in place to temporarily relocate samples when necessary [16].

In general, all devices have to be daily monitored regarding their performance, and the necessary maintenance must be programmed according to the guidelines of each manufacturer [9].

Safety

It is necessary to have an emergency electric power system in both the embryology and andrology laboratories, guaranteeing the supply for the necessary time. All equipment must be connected to an uninterrupted power supply system, preventing them from being disconnected during procedures [14]. Attention should be paid to operator comfort to provide a safe working environment that minimizes the risk of distraction, fatigue, and the consequent occurrence of mistakes. Taking into account occupational safety, it is necessary to pay attention to some points: bench height, adjustable chairs, adequate workspace for each person, the height at which microscopes and magnifiers are in relation to the operator, efficient use of space and surfaces, and adequate lighting [17].

Ensuring the safety of the laboratory working team is essential. So, once the layout has been decided, an appropriate place closer to the workstations should be considered for disposal of infectious waste and sharps. After the collection of this waste, it must be stored in a safe external area until a specialized company makes the appropriate collection [6]. Safety extends to areas

for hand washing, eye washing, and safety showers that need to be located in close proximity to the IVF and diagnostic laboratories [6].

Personal experience

The success of the IVF treatments is almost entirely dependent on the level of experience and skill of the medical and laboratory staff. Good clinical results require careful assessment of individual skills [9]. The embryologist's duty is to manage and cultivate gametes and embryos. In addition, it must maintain quality control standards, carrying out routine checks and tests, recording in detail possible complications, changes, and corrective measures [10]. It is also important to develop the ability to communicate with patients, basic knowledge of genetics, carrying out maintenance, administrative issues, and purchasing/receiving inputs [9].

Based on individual experience and activities performed by an embryologist, there is the possibility of occupying seven positions: director, supervisor, senior embryologist, embryologist, intern, assistant, and technician. However, these positions may vary according to each clinic [10]. Even experienced embryologists should be evaluated on their skills and time to perform specific tasks. The audits and accreditation of laboratories play a positive role in improving results by inducing standardization and quality management [9].

According to estimates, a single traditional IVF treatment requires about 9 hours of work by a professional, whereas contemporary cycles may require up to 20 hours to be complete. For this reason, the number of embryologists needed to perform laboratory processes safely and efficiently has also increased over time [10]. Considering a comprehensive analysis of the tasks that are carried in a laboratory and its complexity, an interactive calculator was created with the objective of helping directors and administrators to determine the ideal number of employees to organize their work schedule. In general, it is safe to say that the proportion of embryologists and the number of procedures must be equivalent, since these professionals perform not only technical tasks, but also management and continuing education and training, aiming to maintain the high standards necessary for succeed [10].

In addition, the increasing utilization of PGT has drastically increased the need for specialized genetic counselors [13]. Essential genetic counseling skills, which have remained largely unchanged over time, include the ability to explain genetic concepts and technologies at an appropriate level of complexity, communicate uncertainties, and interpret information to convey clinical implications and usefulness (Accreditation Council for Genetic Counseling, 2015). Therefore, genetic counselors draw on their skills in translating complex genetic information into practical and decision-making information [18,19].

Concluding remarks

Many variables can interfere in the processes performed at an assisted human reproduction clinic. The ideal scenario is to design a physical structure considering the workflow, although it is not uncommon to find clinics that have been renovated and then adapted to a preexisting space. In the process of defining the layout of the laboratory, it is essential to pay attention to the layout of the rooms, the materials and equipment used, the air flow system, as well as the qualification, training, and number of professionals needed to work in the sector. Proper laboratory planning can impact not only the clinical and laboratory results, but also influence the work environment, which can generate a more comfortable and rewarding place for employees.

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CHAPTER

4

Workup of female infertility

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Introduction

Female infertility is defined as the failure to achieve pregnancy within 12 months of unprotected intercourse in women younger than 35 years or within 6 months in women older than 35 years [1]. Infertility is a condition affecting up to 15% of couples trying for a child [2], and various pathologies can be responsible for that, although in 30% of cases it is not possible to identify the condition. As for other pathology approaches, for infertility a technical and efficient diagnostic approach is strongly recommended to maximize the probability of finding an underlying condition. For this reason, a medical interview with the gynecologist must be administered with a clear focus and high attention in detecting possible clues, both in the woman and the partner. A technical approach should include clear questions, exams, and test prescriptions and, above all, combine that with great empathy [3,4]. In this chapter we will clarify the path for the infertility diagnosis in women, giving attention to the medical interview practical issues such as the exams required for infertility assessment. For the specific male infertility diagnosis, we refer to the appropriate chapter of this book.

Classification of infertility interview

An infertility interview depends on the timing of when it is assessed and the aim it is focused on. The first access for the couple to an IVF center is called the "first infertility interview," and its focus is the anamnestic collection and exams prescription. The second access is called the "decision interview or secondary interview," and its focus is the treatment proposal. A third infertility interview would be necessary in cases of failure of previous treatment, and it is called the "follow-up interview."

Approaching the interview

In our experience it is highly recommended to conduct the medical interview as follows:

1) One gynecologist must be present, well-trained in infertility diagnosis and treatment. Other medical figures, such as residents or fellows, should be present only under tutoring and be as few as possible to minimize the "white coat stress" for the couple entering the interview room.
2) A nurse or midwife should be present only if necessary, especially when a gynecological visit or echography is requested.
3) The main focus should be reducing stress and other psychological factors as much as possible, which could impair women's willingness to answer questions. We must remember that an infertility interview probes the deepest aspects of both partner's intimacy.
4) The interview room must be welcoming but, at the same time, professional. A gynecological table and an ultrasound device should be present beyond a curtain. Nonessential objects should be out of sight. Eye-to-eye contact between the gynecologist and the woman should be easy and free from obstacles (i.e., computer, printer, etc.).
5) Eventually, informed consent, sheets, stamps, and other useful stationery items should be at hand. However, the desk should be as clean as possible.

Psychological care is mandatory in infertility clinics, since the World Health Organization defines health as a "state of complete physical, mental and social well-being and not merely the absence of disease or infirmity" [5]. For this reason, the European Society of Human Reproduction and Embriology (ESHRE) guidelines [3] suggest that fertility staff should pay attention to the

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emotional impact of infertility. In particular, infertile couples suffer a typically lengthy diagnostic and treatment period and the uncertainty of achieving a pregnancy [6]. Both partners should be equally involved in decision-making and in the treatment process. Great efforts should be made by the healthcare staff to reduce waiting times, offering infertility counseling or psychotherapy before, during, and after IVF treatment. Information should be as clear as possible. For example, informed consent and treatment-relevant information should be written and concise. Although a personal interview should preferably be administered with eye-to-eye contact, the recent SARS CoV-2 pandemic has forced the usage of telephone calls and video consultations [7,8]. Actually, they are both useful and appreciated by patients and should be equally proposed to couples.

First interview

The focus of the first interview is the anamnestic record and exam prescription. Every exam prescribed should answer a question regarding infertility, and its result should confirm a diagnosis or modify a therapeutical approach.

Anamnestic interview

An ideal anamnestic record should be divided in the following sections:

1) General data: in particular age and marital status are included.
2) Gynecological anamnesis: attention should be given to the menstrual cycle (regularity, timing, length, presence of pain, and intensity, etc.) and to previous pregnancies or miscarriages. Previous assisted reproductive technology (ART) treatment should be carefully evaluated, and hormonal treatments should be specifically reported. The fertility specialist should also check and report coital frequency and timing, eventual sexual dysfunction, the sexual history, the presence of pelvic inflammatory disease, endometriosis, leiomyomas, or sexually transmitted infections.
3) Familiar anamnesis: the main focus should be cardiovascular pathologies, oncologic disease, psychiatric disorders, and genetic diseases. At the same time, data about female relatives' attempts at pregnancy and success should be retrieved. Possible cardiovascular disease should be taken into consideration for thromboembolic disease related to genetic mutation [9]. Oncologic disease can be both related and unrelated to genetic mutations such as BRCA1. In particular, women who have pathogenic breast cancer gene (BRCA) mutations show an easier loss of ovarian reserve after chemotherapy treatment. Recent findings suggest that DNA repair deficiency is a mechanism involving aging, infertility, and cancer [10]. For this reason, they should be carefully evaluated before administering estrogenic therapies. Genetic diseases can be transmitted to offspring and should be carefully assessed before starting an ART treatment. Psychiatric or mental disorders should be considered for the possible correlation to genetic disease, such as the fragile X mental retardation 1 (FMR1) mutation. In this case, FMR1 triplets expansion could be related to primary ovarian insufficiency (POI) and, if transmitted, could be responsible both for female and male mental retardation.
4) Personal anamnesis: records should be retrieved about personal chronic pathologies, previous hospitalizations, serious illnesses or injuries, previous surgery (in particular if focused on abdomen and pelvis), actual therapies, possible allergies, and physiologic lifestyle. Allergies should be always considered, especially to potential hormonal therapies. Usage of nicotine or alcohol should be carefully considered and investigated. Also, the person's occupation should be considered for the eventual presence of an environmental hazard risk.

Physical examination

The physical examination, including the gynecologic inspection, can be performed during the first interview. However, in some cases it could be postponed to the ovarian reserve assessment, especially when considering the gynecologic examination. The fertility specialist should determine the perfect timing regarding the IVF center facilities. However, some female parameters are mandatory in the first interview such as weight, height, body mass index, blood pressure, and heart rate. Moreover, attention should be given to any sign of androgen excess such as hirsutism and acne. Careful attention should be given to weight. Indeed, obesity is a recognized negative effector for maternal and fetal health, and additionally, it also exerts a negative effect on female fertility [11]. For this reason, an increase in female weight should be recognized and adequately treated from the first approach of the couple to an IVF center. By contrast, an anorexic habitus could subtend a hypogonadotropic hypogonadism. Anorexia nervosa involves a reduction in caloric intake, loss of weight, and amenorrhea, either primary or secondary. Patients with anorexia show an alteration in the hypothalamic-pituitary-gonadal axis, which is responsible for menstrual disorders [12,13]. On the other hand, regarding the gynecologic inspection, attention should be given to dyspareunia and vulvodynia, which could impair both female sexual well-being and be caused by other pathologies (e.g., endometriosis) [14]. Moreover, the

infertility specialist should also check for uterine size, shape, position, and mobility, adnexal masses or tenderness and cul-de-sac masses, tenderness, or nodularity. Vaginal or cervical anomalies, secretions, or discharge should also be considered and adequately treated [4].

Exams required

We can divide the exams requested into two categories:

1) depending on methods: blood exams and imaging exams;
2) depending on question focus: first-line fertility assessment and second-line fertility assessment.

Ovarian reserve test (ORT)

The ovarian reserve is the most important exam for female fertility assessment [15]. The aim of this test is to determine an estimate of ovarian oocytes before follicle development. A diminished ovarian reserve predicts the response to controlled ovarian stimulation for ART. This test is composed of two tests: a serum hormone assessment and a transvaginal ultrasound echography.

Serum hormone assessment

The serum hormone assessment must be determined between the second and the fifth day from the beginning of menstruation, and it consists of the following:

a. Anti-Müllerian hormone (AMH)

AMH is the most important serum dosage to assess ovarian follicle reserve. AMH is normally produced by the granulosa cells of antral follicles, and its serum value is quite stable throughout the menstrual cycle. For this reason, it can be easily dosed in any day [16]. In particular, it correlates with age and progressively decreases during a woman's life. Its measure unit could be pmol/L or ng/mL, where the conversion factor is 7.14. To convert pmol/L to ng/mL, the value must be divided by 7.14, and to convert ng/mL to pmol/L, the value must be multiplied by 7.14. We suggest that one AMH value should have been recorded in the last 12 months for women younger than 35 years or the last 6 months for women older than 35 years. The AMH value assessment should be executed by a professional laboratory, since it is of great importance in deciding a possible infertility therapy.

b. follicle-stimulating hormone (FSH)

c. estradiol (E2)

FSH is the second most important hormone serum dosage. Before AMH introduction, it was the preferred hormone for assessing ovarian follicle reserve. However, its accuracy in the prediction of poor ovarian response is adequate only at very high threshold levels, and for this reason, its role is actually only related to a screening test for counseling purposes [17]. In particular, it is strictly linked to E2 due to a negative feedback. For this reason, basal E2 levels should be lower than 60−80 pg/mL, or the FSH value could be falsely decreased because of hypophysis inhibition. To find this E2 value the hormonal assessment must be administered between the second and fifth day after menstruation [2].

d. luteinizing hormone (LH)

LH is similarly assessed between the first and fifth day after menstruation. Its value could be helpful in two cases:

- To help the polycystic ovarian syndrome (PCOS) diagnosis: in this case, we can notice an inversion FSH/LH ratio with a value lower than 1 (Poretsky [18]). However, this finding is not sufficient to diagnose PCOS. PCOS is notably known to affect infertility and it should be adequately treated [19,20].
- To find an LH deficiency: if an LH deficiency is detected, associated with an FSH deficiency, a hypophysis assessment is needed. In particular, a GnRH test is required. If the test is negative, a hypogonadotropic-hypopituitarism is diagnosed.

Second-line hormonal assessment should be requested in case a pathology is suspected, which lies under the infertility cover. For this reason, they are not first-line-assessment suggested in an infertility interview.

e. thyroid-stimulating hormone (TSH).

Thyroid disease and hyperprolactinemia can be responsible for ovulatory dysfunction. This dysovulation can range from a luteal support deficiency to oligo-ovulation to amenorrhea. For this reason, serum thyrotropin (TSH) should be measured in women with ovulatory dysfunction, infertile women, or those with signs of thyroid disease to detect dysthyroidism [21]. A correct value should be under 2.5 mU/L [22]. In case of higher values, a levothyroxine supplement should be administered.

f. prolactin (PRL).

On the other hand, PRL should be measured in infertile women with irregular menses, galactorrhea, pituitary tumor, or other signs and symptoms of hyperprolactinemia. PRL is normally assessed to find possible hypopituitarism problems. In particular, a single punctual value higher than 25 ng/mL should be investigated. Primarily, a three-point PRL dosage should be administered. In this case, three samples are obtained at time 0, 20′, and 40′ with a single cannula insertion. This test helps avoid stress impairment in PRL values. If a three-point PRL dosage confirms a PRL value over the threshold, a cerebral magnetic resonance should be assessed to find possible adenoma affecting the hypophysis [23,24].

Ultrasound assessment

To complete the ovarian follicle reserve, an ultrasound assessment is needed. It is best administered from the first to the fifth day of menstruation, although it can also be performed at any point during the menstrual cycle. For this reason, a transvaginal ultrasound in often performed with bleeding and an appropriate chair assessment is needed, to make the woman as comfortable as possible. Disposable and waterproof drapes are preferrable. The ultrasound assessment targets the following issues:

a. The uterus: special attention should be paid to the dimensions (length, width, depth) and the endometrial thickness. A greater dimension could be caused by myomas, and in this case, they must be checked and measured. If a myoma protrudes into the endometrial cavity a secondary line ultrasound will be needed, a 3D ultrasound. Attention should be given to the presence of polyps, both in the uterus or in the cervix. A normal uterus should have a homogeneous pattern. Differences can suggest presence of adenomyosis.
b. The ovaries: dimensions must be measured (length, width, depth) and position must be stated (normal, retrouterine, above the uterus). Moreover, and this is the most important issue, ovarian reserve must be determined. Preantral follicles with a <7 mm mean diameter dimension should be counted [25,26]. They should be anechogenic and attention should be given also to their position. A centrifugal position could suggest a polycystic ovarian syndrome and must be correlated by the clinic. Moreover, endometriosic cysts must be detected and measured since they could affect both oocytes quantity and quality.
c. The salpinx should not be visible. However, a tubal enlargement (sactosalpinx) should be taken into consideration. Indeed, it could affect embryo implantation, and in IVF/ICSI cycles, its removal is necessitated.

The greatest importance of the ORT is in assessing the ovarian predictive response to controlled ovarian stimulation. Women can be classified depending on ovarian reserve into a large spectrum from high to poor follicular count. However, since different parameters are used to detect the ovarian reserve, from serum hormones, to age, to ultrasound antral follicular count, a consensus is necessary to define poor ovarian response (POR) women. Actually, the Bologna criteria are a milestone in the POR definition [27]. In particular, two of the following three features must be present:

I. advanced maternal age (≥40 years) or any other risk factor for POR;
II. a previous POR (≤3 oocytes with a conventional stimulation protocol);
III. an abnormal ORT (i.e., AFC five to seven follicles or AMH 0.5−1.1 ng/mL).

In addition, two episodes of POR after maximal controlled ovarian stimulation are sufficient for POR diagnosis even without advanced maternal age or abnormal ORT.

However, the Bologna criteria include a broad spectrum of clinical conditions in the POR category. For example, young women with a low ORT associated with a previous episode of POR and older women with a normal ORT and a previous episode of POR would be included in the same category even though the clinical management is strategically different.

For this reason, a new classification was proposed in 2016 by the Poseidon Group (Patient-Oriented Strategies Encompassing Individualized Oocyte Number). Four groups have been proposed as follows:

I. group 1: patients <35 years with good ORT (AFC >5, AMH >1.2 ng/mL) and with an unexpected poor or suboptimal ovarian response. This group could be further divided into the following:
  a. subgroup 1a, composed of patients with fewer than four oocytes retrieved
  b. subgroup 1b, composed of patients with four to nine oocytes retrieved after standard controlled ovarian stimulation (COS)
II. group 2: patients >35 years with good ORT (AFC >5, AMH >1.2 ng/mL) and with an unexpected poor or suboptimal ovarian response. This group could be further divided into the following:
  a. subgroup 2a, composed of patients with fewer than four oocytes retrieved
  b. subgroup 2b, composed of patients with four to nine oocytes retrieved after standard ovarian stimulation
III. group 3: patients <35 years with poor ORT (AFC <5, AMH <1.2 ng/mL)
IV. group 4: patients >35 years with poor ORT (AFC <5, AMH <1.2 ng/mL)

This new classification adds two new terminologies to the ORT concept:

a) "suboptimal response," defined as the retrieval of four to nine oocytes
b) "hyporesponse," defined as the need for a higher dose of gonadotropins and more prolonged stimulation to obtain an adequate number of oocytes retrieved (>3)

The aim of this new classification is mainly related to the individualization of COS [28]. All these classifications are mandatory for an infertility specialist to classify patients according to the COS proposal. It is probable that experience can help the summing together

of both POR classification and other patient features, to maximize the ART success. If POI is suspected, when a woman shows unexplained ovarian insufficiency or failure, or an elevated FSH level before age 40 years, fragile X carrier screening is recommended to determine whether a FMR1 premutation is present or not [29].

Ovulation and luteal phase support

Infertility can be caused also by anovulation. Amenorrhea can suggest this condition, but serum exams and assessments must be performed [30]. In particular, to detect ovulation, we use LH serum assessment and midluteal progesterone (P4) assessment. Biphasic basal body temperatures and/or cervical mucus changes are not reliable method.

P4 is assessed in the luteal phase, to detect ovulation and if the corpus luteum progesterone supplement is sufficient to maintain pregnancy. A progesterone value greater than 3 ng/mL is evidence of ovulation [31]. However, in women with menstrual cycles longer than 28 days the P4 assessment could be postponed and repeated every week until the serum peak is detected. Luteal phase deficiency (LPD) is a condition of insufficient progesterone production to maintain a normal secretory endometrium and allow embryo implantation [32]. This condition can be suspected if a shortened luteal phase is detected (lasting less than 9 days from ovulation). Moreover, LPD can be considered if spotting appears many days before menstruation [33]. However, currently there is no consensus in LPD diagnosis. Progesterone is known to have a great serum fluctuation during the midluteal phase, and it can preclude sufficient precision [34]. Contrastingly, endometrium biopsies to detect histologic changes of the secretive endometrium are imprecise [32]. Currently, genetic tests seem to be more precise and adequate [35]. By contrast, LH assessment can help to detect the pre- or postovulation timing. Although the LH surge can be difficult to achieve, due to the reduction in LH half-life, the ascending or descending phase of its curve can be detected. This datum can be added to P4 assessment to detect not only ovulation but also the timing of it [36].

Coagulation profile assessment

Coagulation is extremely important when assessing fertility for a woman. In particular, it can help decide if an estro-progestinic therapy can be safely given; it determines the need of anticoagulation during a COS therapy. In particular, the basal exams required are the following:

a) blood count, with attention to platelets (PLTs)
b) prothrombin time (PT) or INR
c) activated partial thromboplastin time (aPTT)
d) antithrombin III (AT III)

However, in case a coagulation problem is suspected, a second-line assessment is recommended. In particular, serum dosages of the following should be executed:

e) C-protein (PC)
f) S-protein (PS)
g) activated protein C resistance
h) serum homocysteine
i) lupus anticoagulant (LAC)
j) anti-cardiolipin antibodies (aCL), both IgG and IgM
k) anti-beta-2 glicoprotein-1 antibodies (B2GP1 Ab), both IgG and IgM
l) prothrombin (factor II) gene assessment
m) V factor Leiden gene assessment
n) methylene tetrahydrofolate reductase (MTHFR) gene assessment

When a coagulation profile is assessed, it must be adequately interpreted. Firstly, we should divide prothrombotic conditions from prohemorrhagic conditions. A prothrombotic condition can present the following conditions: a reduced PT/INR, aPTT, ATIII, PC, PS and an increased homocysteine, LAC, aCL, B2GP1, mutation of prothrombin, V factor, MTHFR genes. Conversely, a PLTs reduction and an increase in PT/INR and aPTT could be responsible for a prohemorrhagic condition [37,38].

In general, when dealing with coagulation alteration an interview with a coagulation specialist is suggested. However, if the infertility specialist is adequately trained, some conditions can be personally managed. In particular we suggest the following:

1. If a mutation is found on MTHFR, where the two assessed are C677T and A1298C, we suggest the following action. A single heterozygosis mutation does not require adjustment. A double heterozygosis or a single homozygosis requires that the woman take an active folic acid, especially when associated with higher levels of homocysteine.
2. A single high value on aCL, B2GP1, and LAC should be repeated in 3 months to confirm it. If the value is confirmed, a coagulation specialist interview is suggested. Two values higher than the threshold can be sufficient for requiring the coagulation specialist interview.
3. Generally, a coagulation prothrombotic condition is strictly linked with an increase in the estrogen levels during hormone stimulation. For this reason, the principal therapies are enoxaparine or cardioaspirin.

Genetic profile assessment

A genetic screening consists of three exams: karyotype, cystic fibrosis, and hemoglobin profile. Moreover, the aforementioned gene assessments for prothrombin

(factor II), V factor Leiden, and methylene tetrahydrofolate reductase (MTHFR) are included.

However, with regard to an infertility assessment flowchart, the following exams should be requested as follows:

1) Cystic fibrosis: this should be prescribed as first-line assessment in every first interview. A normal gene assessment for both partners does not require further evaluation. However, if one of the partners carries one mutation (e.g., DeltaF508) a genetic interview should be prescribed. In particular, the genetic specialist will clarify the risks about having a child with a cystic fibrosis mutation or affected by it.
2) Karyotype: this should be prescribed as first-line assessment in case an IVF treatment is necessary. However, in case of intrauterine inseminations (IUIs), the karyotype analysis is not mandatory.

   A normal karyotype (46XX and 26XY) is expected in the majority of cases. If aneuploidies are detected, they could be responsible for infertility. However, a clear explanation requires a genetic interview, which should be prescribed to the couples. In particular, detected mutations can be transmitted to the offspring and partners must be aware of the risks. Moreover, a karyotype aneuploidy could warrant a preimplant genetic screening in case of IVF/ICSI cycles. For this reason, couples should be adequately educated in this technology, its limits and benefits. Not all couples will require this methodology, but counseling is necessary [39].
3) Hemoglobin profile: this test is not properly a genetic test, but it is correlated with alteration in hemoglobin genes. The hemoglobin profile assessment is not a first-line exam. For detecting possible hemoglobin alterations, a blood count with the mean red cells corpuscular volume is the mandatory exam. However, if an alteration occurs, the hemoglobin profile should be prescribed at least in one of the two partners. In particular, thalassemia must be considered with caution. If a partner is a carrier of a mutation, a genetic consultation is needed. Often, if one partner is a carrier and the other is healthy, children will have hardly any problems.

Generally, an infertility specialist should remember that a genetic alteration should be carefully considered, especially when proposing an assistive reproduction treatment. A genetic consultation is mandatory.

Serologic assessment

Every couple entering an IVF center should be prescribed the serologic panel. In particular, the main viruses researched are these:

- HIV
- hepatitis B virus (HBV)
- hepatitis C virus (HCV)
- syphilis
- rubeovirus: in case of a negative serologic assessment, women should be vaccinated. Moreover, adequate counseling should be performed about the necessity of avoiding a pregnancy in the 28 days after the last vaccine dose.

Normally, in case of a positive serologic panel, the woman should be referred to an infectious disease specialist and the tailored therapy.

Sonohysterosalpingography

A sonohysterosalpingography (SHSG) should be prescribed to every woman to assess tubal patency. This exam is necessary to decide if in a young woman, with a good ovarian reserve and a normozoospermic partner, intrauterine inseminations can be proposed [40]. A negative SHSG exam is diagnostic for tubal infertility factor. However, a single tubal occlusion should be considered suitable for IUI [41]. Additionally, in an IVF center an infertility specialist should also consider when an SHSG is really helpful. For example, a 40-year-old woman with poor ovarian follicle reserve is a candidate mainly for an IVF/ICSI cycle, and SHSG should be not assessed because it would be meaningless. For this reason, although mandatory, an experienced and skilled infertility specialist can give the SHSG the proper importance for every single woman.

Moreover, in case of suspected or known comorbidity (i.e., pelvic inflammatory disease, previous extrauterine pregnancy, or endometriosis), a laparoscopy could be the first-line diagnostic tool. Indeed, salpingocromoscopy added to laparoscopy could be useful to detect tubal patency and other pelvic disease.

3D transvaginal ultrasound

A 3D transvaginal ultrasound should be prescribed mainly when a morphologic anomaly of the uterus is suspected or previously diagnosed (e.g., didelfus uterus, septate uterus, T-shaped uterus, and others). Moreover, in cases where polyps or myoma are suspected, the 3D ultrasound can be helpful to diagnose their presence or their protrusion into the endometrial cavity. A 3D ultrasound is not easily available, especially in poor countries. For this reason, a diagnostic hysteroscopy could bypass this exam [42].

Hysteroscopy

A hysteroscopy (HSC) is often prescribed to a woman with infertility problems. Although, an HSC should be not performed as a first-line diagnostic step. Its prescription is mainly decided when a condition affecting fertility is suspected. In particular, when myomas impairing endometrial cavity are found during an ultrasound screening, HSC is required. A diagnostic HSC can detect

how much the myoma protrudes into the cavity. Often, an operative HSC is prescribed with the aim to eliminate it and restore the correct cavity. Moreover, if a polyp is diagnosed with transvaginal ultrasound, a diagnostic HSC must be performed to confirm it. Subsequently, an operative HSC is administered to eliminate the polyp (Salazar [43]). Moreover, when morphologic anomalies are found during an ultrasound, an HSC can help in detecting a septate uterus, or didelfus or bicornual uterus. Specifically, an HSC added to a previous ultrasound can be helpful to differentiate a septate uterus from an arcuate uterus [42]. Finally, an HSC can be useful to diagnose chronic endometritis (CE), which has been recently considered a factor for embryo implantation failure [7,8]. In this case, the infertility specialist must be aware of HSC markers such as red spot and micropolyps. Although not diagnostic, these markers can orient the diagnosis. However, to confirm it, an endometrial biopsy is needed. In particular, the histologic exam will search for CD 138 plasma cells, which are peculiar for CE diagnosis. If CE is found, an appropriate antibiotic therapy should be assessed, and subsequently, a new endometrial biopsy should be performed [44].

PAP test or HPV-DNA test

In the first interview a PAP test or an HPV-DNA test must be checked or prescribed. We routinely consider a valid test to be one conducted within the last 1.5 years, or less if requested by personal condition. In case of L-SIL or H-SIL a closer follow-up should be guaranteed.

Breast ultrasound or radiography

A breast ultrasound or radiography should not be routinely prescribed. However, in case an anamnestic personal or familiar history is suggestive for breast tumor, this exam should be assessed. Validation should be 1.5 years, or less if requested by a radiology specialist. If a nodule is detected without any diagnosis or oncologic ascertainment, a needle-biopsy is required (and should be prescribed).

Timing of exams

All the exams should be prescribed in the first interview, and they should be executed before the secondary interview. However, an ideal timing is given in this chapter, considering a normal menstrual cycle of 28 days, with 5 days of blood loss.

1) From first to fifth day: execution of the ovarian follicle reserve (hormone assessment and antral follicular count by transvaginal ultrasound).
2) From 6th to 12th day: hysteroscopy with endometrial biopsy for CE assessment and SHSG. PAP test could easily be performed in the same sessions. Moreover, breast imaging can be performed to group exams as much as possible.
3) From 21st day: serum P4 check, to assess a correct and spontaneous ovulation. Moreover, a decrease in normal luteal blood serum progesterone levels can suggest a luteal support insufficiency.

Every other exam (both blood exams and imaging) can be assessed freely. However, we suggest doing all the exams as closely grouped as possible, to reduce time loss as much as possible and have all the exams synchronized.

Secondary interview

The secondary interview is mandatory to check all the exams prescribed to the couple during the first interview and decide the correct management of infertility. Different causes can be diagnosed, and each one could require an individual and specific approach. Sufficient and adequate time should be given to the couple to describe the assisted reproductive technique that the fertility specialist has decided. The ideal approach should be as follows:

1) First, the exams executed by the couple should be carefully checked. If new insights are requested, they should be prescribed. Timing should be carefully evaluated since new exams are time-consuming and should be individually considered depending on the couple's clinical condition.
2) The therapeutical approach should be carefully described, and every question should be answered.
3) Informed consent for the treatment should be given to the couple. Similarly, a timing schedule for the treatment should be arranged.

At the end of the interview, the couple should have both a clear idea about the treatment and about the necessary steps to do in the near future.

Follow-up interview

A follow-up interview is a tertiary interview that is generally executed following a failed treatment. The main aim is to focus on the previous failure and find a new strategy. The couple should be adequately informed about the risks and the probability of success. Moreover, time should be spent in focusing on the new strategy and answering all the questions raised by the couple.

Conclusion

The infertile woman workup is still a complex and troublesome path, throughout which the gynecologist should try to bring clarity to the diagnostic process and shed light on the often shadowy causes. Different issues should be considered, regarding anatomy, endocrinology, infections, environmental hazard risks, and psychological factors. For this reason, a gynecologist should undergo a thorough and efficient training before becoming an infertility specialist. This chapter reassumes all the exams and steps required to define a correct infertility diagnosis. However, it must still be considered that in about 30% of cases a couple's infertility diagnosis is not available. In these cases, a second important point must therefore be considered: the infertility assessment is also the background before an ART treatment. This feature encompasses the main role of the new era of clinical practice, a more individualized and tailored medicine.

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CHAPTER

5

Work-up of male infertility

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Epidemiology

Infertility, defined as the inability to conceive after at least 12 months of regular and unprotected sexual intercourse, has been estimated to affect about 8%–12% of couples in the reproductive age [1,2]. In this context, a male factor infertility (MFI) can be identified in roughly 50% of cases [2,3]. The prevalence of infertility is increasing, and a Global Burden of Disease survey has reported that within 3 decades (1990–2017) the age-standardized prevalence of infertility has gradually increased by 0.29% in men and 0.37% in women, respectively, every year [4].

Infertility is associated with psychological and social distress within the couple [5] and imposes a considerable economic burden on patients and health-care systems [6]. Therefore, early diagnosis and appropriate management are of fundamental clinical and social importance. Of relevance, recent evidence has also depicted MFI as a proxy of the overall men's health, with infertile men showing higher risk of cardiometabolic disorders and cancer and a lower general health compared to age-comparable fertile counterparts [7,8,9]. In this context, an accurate investigation of men's fertility potential and the early detection of male subfertility offers the opportunity for identification and correction of medical conditions affecting not only fertility by itself, but also general health and wellbeing. As a whole, couple infertility may be due to male factors, female factors, a combination of both, or it can be either idiopathic or unexplained in its nature [10]; therefore, parallel evaluation of both partners is always required. In this context, just as all infertile women are treated by those with specialized gynecologic training and expertise, it is crucial that also all male partners belonging to infertile couples should undergo medical evaluation by a physician trained in male reproduction.

Etiology and risk factors

Overall, etiological factors in the context of male infertility could be segregated into i) congenital, ii) acquired (e.g., metabolic diseases, gonadotoxin exposure, etc.), and iii) idiopathic [10]. The most frequently reported congenital causes of MFI are bilateral anorchia, vas deference absence, Y chromosome microdeletion, cystic fibrosis, Kallmann syndrome, Klinefelter syndrome, Robertsonian translocation, and genetic endocrinopathies (e.g., Prader-Willy syndrome) [10,11,12,13,14,15,16]. Instead, among acquired and idiopathic cases, many intertwined factors come into play [10,17]. Among acquired causes, varicocele has been estimated to be prevalent in almost 40% of infertile men and in 25.4% of men with impaired semen parameters [18]. Of some potential pathophysiology reasons, varicocele has been reported to dysregulate spermatogenesis by impairing the venous drainage and by interfering with the counter current exchange of heat mechanism from the spermatic cord resulting in increased scrotal temperature [18,19,20]. Additional acquired causes of MFI are testicular trauma and torsion, testicular neoplasms, medications' use (e.g., chemotherapy, etc.), radiation therapy, and comorbid systemic diseases (e.g., diabetes, liver cirrhosis, kidney failure) [10,11,21].

Idiopathic infertility accounts for approximately 30% of infertile couples. These men have no previous history of diseases affecting fertility and normal findings on physical examination and endocrine, genetic and biochemical laboratory testing, although semen analysis may reveal pathological findings. As a whole, idiopathic causes are all linked with some risk factors that are believed to negatively impact the fertility potential of the male population [17,22,23,24]. To this regard, smoking, alcohol, recreational drugs, obesity, and even

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psychological stress have all been linked with infertility and lower sperm quality [21,25,26,27]. As such, these factors might play important roles in terms of oxidative stress and impairment of sperm DNA fragmentation (SDF) [28,29,30]. Moreover, a certain amount of couples are infertile because of unexplained male infertility, which is defined as infertility of unknown origin with normal sperm parameters and partner evaluation. Between 20% and 30% of couples will have unexplained infertility [10].

Diagnostic work-up

A focused evaluation of all male patients seeking medical help because of infertility must include i) a medical and reproductive history, ii) physical examination, iii) semen analysis, performed according to World Health Organization (WHO) recommendations [31], and, iv) hormonal evaluation. Additional investigations (e.g., genetic analysis and imaging) may be required depending on the clinical characteristics and semen parameters.

Patient's history

Infertility history

The first step in evaluating infertility is obtaining a thorough history (Table 5.1). First, the identification of those suffering from primary (i.e., no previous fertility) and/or secondary infertility (i.e., previously fertile, currently infertile) should always be done [10]; although the management and the diagnostic work-up is usually similar between the two categories, some relevant differences concern the baseline health conditions of the individual (e.g., the genetic profile).

Of great clinical relevance, partner's age and her gynecological history, including ovarian reserve usually evaluated by means of the anti-Müllerian hormone (AMH) levels, should be evaluated throughout the very first steps of the work-up of the infertile couple, since this might impact the timing of and the therapeutic strategies themselves (e.g., assisted reproductive technology (ART) vs. surgical intervention). The duration of infertility should also be always investigated because of its detrimental impact on semen parameters [32] and the need to accelerate the decision-making according to the age of both partners [10].

TABLE 5.1 Important components of history taking in the evaluation of men with infertility.

Infertility history: previous pregnancies and outcomes (primary vs. secondary infertility), duration of infertility, partner's age and fertility history, previous fertility treatment and investigations (including ART)
Medical history: Health comorbidities with specific focus on diabetes, cancer, cardiovascular disorders, neurological diseases, cryptorchidism and timing of treatment, anosmia (Kallmann syndrome), timing of puberty, history of testicular trauma/torsion infections (genitourinary and mumps orchitis)
Lifestyle factors and gonadotoxin exposures: Tobacco, alcohol, and recreational drug consumption, medications (endocrine modulators, antihypertensives, antibiotics, antipsychotics), environmental (pesticides, heavy metals), chemotherapy or radiotherapy
Surgical history: Orchidopexy, vasectomy, retroperitoneal or pelvic surgery, bladder, neck, or prostatic surgery
Family history: Cystic fibrosis, Y chromosome microdeletions, androgen receptor deficiency
Sexual history: Libido frequency and timing of coitus, erectile dysfunction, ejaculatory dysfunction, type of lubricants, sexually transmitted disease

Keys: *ART*, assisted reproductive technology.

Medical history

Medical history should investigate potential risk factors that could affect the male's fertility, such as comorbidities (including cardiometabolic diseases and tumors), genitourinary (GU) infections, and history of previous testicular surgery (any type).

Childhood medical conditions such as cryptorchidism, postpubertal mumps, and past testicular traumas/torsions should be carefully assessed. In this context, it has been demonstrated that all these conditions are associated with reduced sperm quality and decreased fertility potential. In particular, children with undescended testis not only harbor a higher risk of developing testicular cancer, but also an increased incidence of lower sperm counts, poor sperm quality, and decreased fertility rates [33,34,35,36]. Many GU infections have been associated with male and female infertility. It has been estimated that male GU infections can be prevalent in up to 35% of the general population [33]. Likewise, an important study identified that 20% of men with male infertility were completely unaware of harboring a seminal infection [29,37]. Among patient's comorbid conditions, cardiometabolic diseases (e.g., obesity, diabetes, hypertension, dyslipidaemia, insulin resistance, and metabolic syndrome) are strongly associated with poor sperm quality [8,21,38,39,40,41,42]. It has been largely demonstrated that, among obese men, estrogen levels increase due to an augmented peripheral conversion of testosterone to estrogens by the aromatase enzyme. As a result, the hypothalamic-pituitary-gonadal (HPG) axis is dysregulated, resulting in lower sperm quality [16].

In terms of overall men's health, several studies have shown than infertile men are overall less healthy than the fertile counterpart (i.e., higher burden of comorbidities) [8,42]. In this context, published data showed that patients with a decreased general health status have lower sperm concentration, lower testosterone levels, and higher follicle stimulating hormone (FSH) values than fertile counterparts, thus confirming that poor health status appears to be associated with a malfunctioning male reproductive system [8].

Lifestyle factors

Lifestyle factors should be carefully investigated, including alcohol, tobacco, and recreational drug use. A large meta-analysis involving 20 studies and 5865 patients demonstrated that smoking detrimentally worsens semen parameters [43]. Moreover, another meta-analysis involving 15 studies revealed a negative association between alcohol consumption and semen analysis [27]. The concomitant use of cigarette smoking and alcohol consumption was found to have a greater detrimental effect on semen parameters than isolated recreational habits [44]. Recreational drugs have also been found to alter the fertility potential of a male individual. Cannabis, the most frequently used recreational drug, negatively affects spermatogenesis, sperm function, and HPG axis [45,46].

Likewise, a number of commonly used medications have been found to interfere with spermatogenesis. In this context, endocrine modulators, antihypertensives, antibiotics, and antipsychotics have all been linked with poor sperm quality [16,46,47]. Likewise, chemotherapy and radiotherapy may result in temporary, long-term, or permanent gonadal toxicity in male patients [48,49,50]. As such, all urological guidelines advise cryopreservation before any oncologic treatment is started [10,51,52].

Surgical history

Past vasectomies, vasectomy reversals, orchiectomy, retroperitoneal or pelvic surgeries, and prostatic/bladder neck surgeries should always be investigated in infertile men because of the potential implication with fertility and sperm quality [11].

Family history

Family history is important when it comes to hereditary disorders. Cystic fibrosis (CF) is a well-known genetic disease associated with MFI. Patients with CF have serious systematic disorders in multiple organs, including chronic lung infection, inflammation, and pancreatic insufficiency, along with alteration of the genital tract. In fact, 97%–98% of male CF patients are infertile because of congenital bilateral absence of vas deferens, which results in obstructive azoospermia [53,54].

MFI can also be present in men with the CF transmembrane conductance regulator (CFTR) gene mutation. These men can display semen impairment without any other clinical manifestation of CF as they are only carriers of the mutated gene [55]. Lastly and interestingly, clinically affected CF patients present a spectrum of genital phenotypes ranging from normal fertility to severely impaired spermatogenesis and congenital absence of the vas deferens. Other common genetic alterations in infertile men are microdeletions of the Y chromosome. Despite normal clinical phenotype, these men usually show a severely impacted fertility potential [56,57].

Sexual history

A couple's sexual practice, the timing of coitus, along with the man's erectile and ejaculatory functions should be investigated. It has been found that one in six men of an infertile couple suffers from some form of sexual dysfunction, including erectile dysfunction (ED), premature ejaculation, and low/reduced sexual desire (LSD) [58].

In terms of sexual frequency, intercourse is recommended every 48 h around the time of ovulation, to maximize the chance of fertilization [59]. This has been associated with psychological distress from both partners, resulting in lower chances of conceiving and reduced quality of life for the couple. Thus, sexual history always plays a fundamental role in these couples [58].

Physical examination

Physical examination is a key part of the baseline evaluation of the infertile man, including the presence of secondary sexual characteristics.

A comprehensive physical examination should include the following [10,11]:

General. Skin discoloration could be a sign of metabolic disorders. In this context, even if it is rare, iron overload syndromes cause infertility and manifest as diffuse, patchy hyperpigmentation. Moreover, Cushing syndrome manifests with thin skin, ecchymoses, purple

striae, and moon face. Loss of pubic or axillary hair and oily skin are signs of testosterone deficiency. Instead, reduced muscle mass, reduced facial and body hair, broad hips, tall stature, and long hands could be an indirect sign of Klinefelter syndrome. Gynecomastia and breast pain should also be evaluated in every infertile man.

Penis. The foreskin should be retracted to look for phimosis, short frenulum, nodules, ulcerations, scars, or signs of inflammation or of sexually transmitted infections. The amount and distribution of pubic hair is an important sign of secondary sexual characteristics development. Physicians must check the location of the urethral meatus (epispadias or hypospadias) and its aperture and for any suggestive discharge. Penile plaque and/or acquired penile curvature associated with La Peyronie disease may make vaginal intercourse difficult.

Testis and scrotum. The location, size, texture, and consistency of the testes must be evaluated. The presence of nodules or swelling should be excluded. Testicular volume is assessed by Prader's orchidometer in clinical practice. Despite the lack of uniform reference values in terms of Prader's orchidometer-derived testicular volume, a number of studies reported that the mean testis volume in the European general population is 20.0 ± 5.0 mL, whereas in infertile patients it is 18.0 ± 5.0 mL [60,61]. Moreover, testicular volume was positively associated with total testosterone levels and sperm quality in infertile men.

Epididymis. Shape and/or consistency for normal development should be identified to determine atresia that could be identified by the presence of a CFTR mutation. Induration and/or dilation could suggest obstruction. Epididymal cysts or spermatoceles may also lead to obstruction.

Spermatic cord. Large and palpable pampiniform plexus should be investigated. The presence and severity of varicocele is clinically evaluated at rest and during Valsalva maneuver.

Accordingly, in clinical practice varicocele is classified as follows [62]:

subclinical: not palpable or visible at rest or during Valsalva maneuver, but can be shown by special tests (Doppler ultrasound [US]);
grade 1: palpable during Valsalva maneuver;
grade 2: palpable at rest;
grade 3: visible and palpable at rest.

Vas deferens. Shape and/or consistency for normal development and contour should be confirmed to rule out agenesis as may be seen in the presence of a CFTR mutation or aberrant Wolffian duct embryogenesis. The presence or location of any vasectomy defect or granuloma should also be assessed.

Digital rectal examination. Midline prostatic cysts or dilated seminal vesicles may assist in the diagnosis obstruction.

Semen analysis

Semen analysis is a key step over the diagnostic work-up of any infertile individual (both complaining of primary or secondary infertility). Semen parameters give the physician important information about the overall sperm quality and the need of second-level diagnostic tests. Although this holds true, semen parameter values falling above or below the lower limit do not per se predict either fertility or infertility. Interestingly enough, a recently published study compared the semen and baseline characteristics of 1957 infertile men with 103 age-matched fertile controls and found that approximately 12% of infertile and only 41% of fertile men had normal sperm parameters in the real-life setting [63]. Ejaculate analysis should be standardized according to the most updated version of the WHO Laboratory Manual for the Examination and Processing of Human Semen (sixth edition) [64]. Overall consensus has been reached about following the after mentioned guidelines, and it is essential that the complete laboratory work-up is standardized according to reference values (Table 5.2). However, recent evidence has proved that more complex testing than "pure" macroscopic semen analysis may be required in men belonging to couples with unexplained male infertility, recurrent pregnancy loss from natural conception, or after ART [65]. In these patients there is a high risk of sperm DNA damage causing pregnancy failure; therefore additional tests such as the SDF index might be useful [10].

The most updated European Association of Urology (EAU) Guidelines on Sexual and Reproductive health suggests that a single test is sufficient in case of normal semen analysis, according to WHO criteria [10]. Conversely, a second semen analysis, performed after approximately 3 months from the first test, is required in case of sperm alterations, and if the results are abnormal on at least two tests, further andrological investigation is indicated.

Sperm alterations can be classified as follows [64]:

- oligozoospermia: < 16 million spermatozoa/mL;
- asthenozoospermia: $< 30\%$ progressive motile spermatozoa;
- teratozoospermia: $< 4\%$ normal forms.

When all three anomalies simultaneously occur the condition is defined as oligo-astheno-teratozoospermia syndrome.

In azoospermia, semen analysis presents with normal ejaculate volume but absence of spermatozoa after

TABLE 5.2 Lower reference limits (fifth centiles and their 95% CIs) for semen characteristics.

Parameter	Lower reference limit (range)
Semen volume (mL)	1.4 (1.3–1.5)
Total sperm number (10^6/ejaculate)	39 (35–40)
Sperm concentration (10^6/mL)	16 (15–18)
Total motility (PR + NP, %)	42 (40–43)
Progressive motility (PR, %)	30 (29–31)
Vitality (live spermatozoa, %)	54 (50–56)
Sperm morphology (normal forms, %)	4 (3.9–4.0)
Other consensus threshold values	
pH	>7.2
Peroxidase-positive leukocytes (10^6/mL)	<1.0
Optional investigations	
MAR test (motile spermatozoa with bound particles, %)	No evidence-based reference limits
Immunobead test (motile spermatozoa with bound beads, %)	No evidence-based reference limits
Seminal zinc (μmol/ejaculate)	≥2.4
Seminal fructose (μmol/ejaculate)	≥13
Seminal neutral glucosidase (mU/ejaculate)[a]	≥20

[a]CIs, *confidence intervals;* MAR, *mixed antiglobulin reaction;* NP, *nonprogressive;* PR, *progressive (a+b motility).*

centrifugation. A recommended method is semen centrifugation at 3000 g for 15 min and a thorough microscopic examination by phase contrast optics at ×200 magnification of the pellet. Azoospermia should always be confirmed by two consecutive semen analyses. The history, physical examination, and hormonal studies can help differentiate obstructive azoospermia from nonobstructive azoospermia (NOA). Men with azoospermia and small testes volume, elevated FSH, and normal semen volume are more likely to have NOA (due to impaired sperm production). Conversely, men with normal testis volume, low gonadotropins, and/or semen volume <0.5/1.0 mL most likely have obstructive azoospermia, especially if the proximal epididymis is enlarged on physical examination or the vasa deferentia are absent on exam.

Measurement of sperm DNA fragmentation index

SDF, or the accumulation of single- and double-strand DNA breaks, has been found to play a key role in the context of couple's infertility. Sperm DNA damage is more common in infertile men and in those with unexplained infertility compared to fertile controls [66,67]; furthermore, SDF has been identified as a key predictive factor of poorer outcomes following ART [68,69,70,71], including impaired embryo development [71], miscarriage, recurrent pregnancy loss, and birth defects [69,71,72,73]. Several conditions are known to increase SDF in clinical practice including aging, hormonal diseases, varicocele and cryptorchidism, chronic infection (including GU infections), and lifestyle factors (e.g., smoking, alcohol consumption) [29,74]. Several assays are currently used to measure sperm DNA damage. Terminal deoxynucleotidyl transferase mediated deoxyuridine triphosphate nick end labeling (TUNEL) and the alkaline comet test (COMET) directly measure DNA damage. Conversely, sperm chromatin structure assay (SCSA) and sperm chromatic dispersion test (SCD) are indirect tools for DNA fragmentation assessment [74]. The main limitation of SDF testing is the lack of a definitive cut-off value above which a sample is undoubtedly considered anomalous. Moreover, various SDF thresholds may be determined based on the predicted outcome measure (fertility/infertility, ART success/failure, etc.). A recent meta-analysis by Santi et al. compared the SDF results of four different assays (TUNEL, SCD, SCSA, and COMET) between 2883 infertile men and 1294 fertile men. The authors identified an SDF cut-off of 20%, which had a good predictive power in differentiating between fertile and infertile men, with a sensitivity of 79% and a specificity of 86% (area under the curve = 0.844) [67]. Furthermore, it is suggested that a threshold of 30%, as measured with SCSA, is associated with reduced pregnancy rates via natural conception or intrauterine insemination (IUI) [75]. Recently, the mean COMET score and scores for proportions of sperm with high or low DNA damage have been shown to be of value in diagnosing male infertility and providing additional discriminatory information for the prediction of both in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) outcomes [76]. Moreover, several studies have shown that testicular sperm have lower levels of SDF when compared to ejaculated sperm because of the loss of sperm chromatin integrity through the genital tract [77]. Consequently, clinical trials are now testing the value of using testicular sperm for ICSI in nonazoospermic men with raised SDF [78], but this practice is still considered experimental [10,67]. From a clinical standpoint, not all infertile men deserved to be tested for SDF. According to the American Urological Association/American Society for Reproductive Medicine (AUA/ASRM) Guidelines, SDF should not be routinely performed in the initial evaluation of the infertile male [52].

Conversely, the EAU Guidelines recommend SDF testing in couples with recurrent pregnancy loss or in men with unexplained infertility [10].

Hormonal evaluation

Hormonal profile is of paramount importance in the evaluation and management of infertile individuals. Despite that many physicians are used to testing hormonal values in every infertile man, international societies recommend limiting use to particular groups of patients, including men with oligozoospermia/azoospermia or impaired sexual function, or if endocrinopathy is suspected [10,52].

The basic hormonal evaluation should include FSH and total testosterone. In case of testosterone deficiency, a more thorough endocrine evaluation is recommended, including repetition of total testosterone and addition of luteinizing hormone (LH) assay to differentiate primary from secondary hypogonadism. Prolactin analysis is also recommended in men with hypogonadotropic hypogonadism or decreased libido. Testosterone measurements (taken between 7:00 and 11:00 a.m.) in the fasting state are recommended. Mass spectrometry is the gold standard of testosterone assays, but good-quality immunoassays provide fully acceptable results for clinical diagnosis [16]. In terms of cut-off values for the diagnosis of testosterone deficiency, the ASRM adopts the value of less than 300 ng/dL and the EAU recommends 230 ng/dL (8 nmol/L).

A single measure of total testosterone could be misleading in patients where sex hormone-binding globulin (SHBG) is increased (e.g., older men, men with thyroid disorders or diabetes) [79]. In these cases, measurement of free testosterone is recommended. The most accurate assay to measure free testosterone is equilibrium dialysis [80], but it is expensive and technically challenging. Alternatively, the calculated free testosterone (http://www.issam.ch/freetesto.htm) is consider a rapid, simple, and more clinically accurate method in assessing men with hypogonadal symptoms [81,82].

FSH is usually negatively associated with spermatogenesis, but in some cases of spermatogenic arrest at the level of spermatocyte or spermatid, FSH, LH, and testosterone concentrations might be normal [83].

Male hypogonadism is a common finding in infertile men. Hypogonadism for testicular failure, known as primary or hypergonadotropic hypogonadism, is characterized by high FSH and normal/high LH levels, with low/normal levels of total testosterone. Conversely, hypogonadism for a central disorder, also called secondary or hypogonadotropic hypogonadism, is characterized by low or normal levels of FSH and LH, with or without low levels of testosterone [16,84]. Recently, a new classification of hypogonadism in infertile men, which includes primary, secondary, and compensate hypogonadism (normal testosterone and elevated LH values), has been proposed with potential implications for further management and classification of testicular dysfunction [84].

Genetic testing

All clinicians working with infertile couples should have an understanding of the genetic abnormalities most commonly associated with infertility, so they can provide correct advice to couples seeking fertility treatment. Genetic abnormalities related to male infertility affect about 15% of men with infertility [85], and several genes and gene mutations related to spermatogenesis have been discovered [86]. The spermatozoa of infertile men show an increased rate of aneuploidy, defective spermatogenesis resulting in oligozoospermia or azoospermia, structural chromosomal abnormalities and DNA damage, carrying the risk of passing genetic abnormalities to the next generation [87]. Genetic mutations in embryos might lead to repeated ICSI failure and recurrent miscarriage; therefore, identifying genetic defects is crucial for diagnostic purposes and proper counseling before ART procedures.

Current routine clinical practice is based on the screening of genomic DNA from peripheral blood samples.

Chromosomal abnormalities

Chromosomal abnormalities can be numerical (e.g., trisomy) or structural (e.g., inversions or translocations). In infertile men the incidence of chromosomal abnormalities was found to be 5.8% (of which 4.2% were sex chromosome abnormalities and 1.5% were autosomal abnormalities) [88]; moreover, the frequency of chromosomal abnormalities increases as testicular deficiency becomes more severe. Patients with sperm count <5 million/mL have a 10-fold higher incidence of autosomal structural abnormalities compared with the general population [89,90]. Men with NOA are at highest risk (12%–15%), especially for sex chromosomal anomalies (e.g., Klinefelter syndrome) [91].

Karyotyping (also known as chromosomal analysis) detects numerical chromosomal defects and structural defects. Most scientific societies agree on recommending karyotype analysis for men with azoospermia or severe oligozoospermia (sperm count <5 million/mL) [52,92]. However, the EAU extended their guideline recommendations to include men with a sperm count of less than 10 million/mL or men with a family history of recurrent spontaneous abortions, malformations, or intellectual disability, regardless of the sperm concentration [10].

The most common karyotype defect is Klinefelter syndrome (also known as 47,XXY), followed by translocations, inversions, and deletions [93]. The phenotype of men with Klinefelter syndrome is the final result of a combination between genetic, hormonal, and age-related factors [94]. The phenotype varies from that of a normally virilized male to one with the stigmata of androgen deficiency. In most cases, the diagnosis of Klinefelter syndrome is done while seeking medical help for fertility purposes. At adolescence period, rising intratesticular testosterone levels are subsequently followed by an accelerating decline in germ cells, hyalinization of the tubules, degeneration of Sertoli cells, and hyperplasia of Leydig cells, resulting in the loss of testicular volume and a decrease in serum testosterone levels [95]. Adult men with Klinefelter syndrome usually have small firm testes along with features of primary hypogonadism. Besides spermatogenic deficiency, Leydig cell function is also commonly impaired in men with Klinefelter syndrome, so testosterone deficiency is more frequently observed than in the general population [15].

Klinefelter men have residual foci of preserved spermatogenesis, which are more frequently observed in mosaicism, 46,XY/47,XXY. In patients with azoospermia, testicular sperm extraction (TESE) are therapeutic options as spermatozoa can be recovered in up to 50% of cases [96,97]. Currently, there are no clinical, hormonal, or procedural factors that can predict positive sperm retrieval in this cohort of men [96,97].

Data from recent literature have not reported any difference in the prevalence of aneuploidy in children conceived using ICSI in Klinefelter syndrome compared to the general population; however, men with Klinefelter syndrome undergoing fertility treatments should be counseled regarding the potential genetic abnormalities in their offspring.

Testicular sperm extraction in peri-pubertal or prepubertal boys with Klinefelter syndrome aiming at cryopreservation of testicular spermatogonial stem cells is still considered experimental and should only be performed within a research setting [98]. The same applies to sperm retrieval in older boys who have not considered their fertility potential [99].

Men with Klinefelter syndrome are at higher risk of metabolic and cardiovascular diseases, venous thromboembolism, and malignancies compared with the general population; therefore, appropriate medical follow-up is advised in these men [95].

Autosomal abnormalities

Genetic counseling should be offered to couples with male partner having autosomal karyotype abnormalities (such as Robertsonian translocations, reciprocal translocations, paracentric inversions, and marker chromosomes). It is important to look for these structural chromosomal anomalies because there is an increased associated risk of aneuploidy or unbalanced chromosomal complements in the fetus. When IVF/ICSI is carried out for men with translocations, preimplantation genetic diagnosis or amniocentesis should be performed [100].

Cystic fibrosis gene mutations

CF is the most common genetic disease of Caucasians with an autosomal-recessive transmission [101]. Approximately 4% are carriers of gene mutations involving the CFTR gene located on chromosome 7p. It encodes a membrane protein that functions as an ion channel and influences the formation of the ejaculatory duct, seminal vesicle, vas deferens, and distal two-thirds of the epididymis. Approximately 2000 CFTR mutations have been identified, and any CFTR alteration may lead to congenital bilateral absence of the vas deferens (CBAVD). However, only those with homozygous mutations exhibit CF disease [102]. CBAVD is a rare reason for MFI, which is found in 1% of infertile men and in up to 6% of men with obstructive azoospermia [103]. Clinical diagnosis of absent vasa is easy to miss and all men with azoospermia should be carefully examined to exclude CBAVD, particularly those with a semen volume <1.0 mL and acidic pH < 7.0 [104,105]. In patients with CBAVD-only or CF, testicular sperm aspiration, microsurgical epididymal sperm aspiration, or TESE with ICSI can be used to achieve pregnancy. However, higher sperm quality, easier sperm retrieval, and better ICSI outcomes are associated with CBAVD-only patients compared with CF patients [102].

The most frequently found mutations are F508, R117H, and W1282X, but their frequency and the presence of other mutations largely depend on the ethnicity of the patient [106]. Routine testing is usually restricted to the most common mutations in a particular community through the analysis of a mutation panel. Men with CBAVD often have mild clinical stigmata of CF (e.g., history of chest infections). When a man has CBAVD, it is important to test also his partner for CF mutations. If the female partner is found to be a carrier of CFTR mutations, the couple must consider carefully whether to proceed with ICSI using the man's sperm, as the risk of having a child with CF or CBAVD will be 50%, depending on the type of mutations carried by the parents. If the female partner is negative for known mutations, the risk of being a carrier of unknown mutations is 0.4% [107].

Congenital unilateral absence of the vas deferens is usually associated with ipsilateral absence of the kidney and probably has a different genetic causation [108]. In these subjects, which are fertile, CFTR mutation screening is not indicated. Conversely, it is indicated in men with unilateral absence of the vas deferens with normal kidneys. The prevalence of renal anomalies is rare for patients who have CBAVD and CFTR mutations [109]. Abdominal US should be performed both in unilateral and bilateral absence of vas deferens without CFTR mutations.

Y microdeletions

Microdeletions on the Y chromosome are termed AZFa, AZFb, and AZFc deletions [110]. In each AZF region, there are several genes implicated in spermatogenesis. Clinically relevant deletions remove partially or completely one or more of the AZF regions and are the most frequent molecular genetic cause of severe oligozoospermia and azoospermia [111].

Y microdeletions are not found in normozoospermic men, proving there is a clear cause-and-effect relationship with spermatogenic failure, and have highest frequency in azoospermic men (8%−12%), followed by oligozoospermic (3%−7%) men [112]. AZFc deletions are most common (65%−70%), followed by Y deletions of the AZFb and AZFb+c or AZFa+b+c regions (25% −30%). AZFa region deletions are rare (5%) [113]. Lastly, the gr/gr deletion in the AZFc region, which removes half of the gene content of the AZFc region, confers a 2.5−8 fold increased risk for oligozoospermia [114]. The gr/gr deletion is a significant risk factor for impaired sperm production, and few reports have proposed an association between this mutation and testicular germ cell tumors [115].

Although sperm can be retrieved from the testes of men with azoospermia factor c deletions (50%−75% of cases), AZFa or AZFb deletions carry a very poor prognosis and sperm retrieval is not advised in such cases [10]. Importantly, Y chromosome microdeletions can be transmitted to male offspring, so counseling couples is recommended before ICSI.

Y chromosome microdeletion analysis is indicated for patients with azoospermia or oligozoospermia and a sperm count of <5 million/mL [10]. A meta-analysis showed that the majority of microdeletions occurred in men with sperm concentrations ≤1 million/mL, with <1% identified in men with >1 million/mL [116]. In this context, patients may be offered testing if sperm counts are <5 million/mL, but must be tested if ≤ 1 million/mL [10].

Measurement of oxidative stress

Oxidative stress is known to affect sperm quality, function, as well as the integrity of sperm [117]. Moreover, oxidative stress may lead to sperm DNA damage and poorer DNA integrity, resulting in higher risk of poor embryo development, miscarriage, and infertility [118]. Oxidative stress is associated with poor lifestyle (e.g., smoking, alcohol consumption) and environmental exposure, so antioxidant regimens and lifestyle interventions may reduce the risk of SDF and improve sperm quality [118]. Although oxidative stress can be measured by various assays (e.g., chemiluminescence or fluorescent techniques), routine measurement of testing should remain experimental [119].

Imaging

Scrotal ultrasound

In addition to physical examination, a scrotal US may be helpful in measuring testicular volume; assessing testicular anatomy and structure in terms of US patterns, blood flow, and testicular tumors; finding indirect signs of obstruction (e.g., dilatation of rete testis, enlarged epididymis with cystic lesions, or absent vas deferens); and grading varicocele severity [120]. Scrotal US is useful when Prader's orchidometer is unreliable, such as in case of large hydrocele, inguinal testis, epididymal enlargement/fibrosis, thickened scrotal skin, small testis, or where the epididymis is large in comparison to the total testicular volume [61].

Because of the known association between MFI and testicular cancer, scrotal US is widely used in everyday clinical practice in patients with oligozoospermia or azoospermia [121]. Men with infertility have an increased risk of testicular cancer (hazard ratio 3.3) compared with fertile controls and the risk increases along with the severity of sperm alterations [122]. In a recent systematic review, infertile men with testicular microcalcifications were found to have an approximately 18-fold higher prevalence of testicular cancer [123]. However, the utility of US as a routine screening tool in men with infertility to detect testicular cancer remains a matter of debate [121].

Important US criteria for detecting testicular tumors are size, vascularity, and echogenicity of the suspected nodule. Data suggest that the smaller the nodule is, the less likely that it is malignant, and lesions <5 mm could be monitored, as they have a low probability of malignancy [124]. Small hypoechoic/hyperechoic areas may be diagnosed as intratesticular cysts, focal Leydig

cell hyperplasia, fibrosis, and focal testicular inhomogeneity after previous pathological conditions. Previous studies have suggested that if a testicular lesion is hyperechoic and nonvascular on color Doppler US and associated with negative tumor markers, the likelihood of malignancy is low, and regular surveillance should be preferred over surgery. Conversely, hypoechoic and vascular lesions are more likely to be malignant [125], and they should be treated with open US-guided testicular biopsy, testis sparing surgery with tumor enucleation for frozen section examination, or radical orchidectomy. However, most lesions cannot be characterized by US (indeterminate), and histology remains the only certain diagnostic tool. A multidisciplinary team discussion, including invasive diagnostic modalities, should therefore be considered in these patients [10].

In the case of interval growth of a lesion and/or the presence of additional risk factors for malignancy (infertility, bilateral microcalcifications, history of cryptorchidism, testicular atrophy, inhomogeneous parenchyma, history of testicular tumor, history of contralateral tumor), testicular biopsy/surgery may be considered, although the evidence for adopting such a management policy is limited [10]. If intervention is to be undertaken in men with severe hypospermatogenesis (e.g., azoospermia), then a simultaneous TESE can be undertaken (termed onco-TESE), along with sperm banking.

Transrectal US

Patients with low seminal volume, acidic pH, and severe oligozoospermia or azoospermia, in whom obstruction is suspected, should be offered scrotal and transrectal US. This is a useful tool for detecting CBAVD, presence or absence of the epididymis and/or seminal vesicles (SV) (e.g., abnormalities/agenesis), and obstruction of the ejaculatory ducts (ejaculatory duct cysts), seminal vesicular dilatation, or hypoplasia/atrophy [120,126].

Other

If more detailed imaging of the genitourinary tract is required, MRI can be done. In men with infertility, hypogonadism, and elevated prolactin, cranial MRI can diagnose a pituitary pathology (most commonly prolactinoma) as an underlying cause of hyperprolactinaemia and hypogonadism.

Summary

Evaluation should proceed in parallel for both male and female partners of every infertile couple to optimize treatment success. A complete medical history, physical examination, and semen analysis are the essential components of male infertility evaluation. Semen analyses should be performed according to the WHO Laboratory Manual for the Examination and Processing of Human Semen (sixth edition) indications and reference criteria. In cases of oligozoospermia and azoospermia, a hormonal evaluation should be performed, including a serum total testosterone and FSH/LH. Genetic testing should be offered to azoospermic men and those with severe oligozoospermia. SDF testing should be performed over the assessment of couples with recurrent pregnancy loss from natural conception and ART or men with unexplained infertility. Ultrasound of the genitourinary tract can enrich the physical examination in specific cases, and it is useful when obstruction is suspected.

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CHAPTER

6

Prothrombotic gene polymorphisms and adverse reproductive outcomes in assisted reproductive technology

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Physiology of coagulation

The concept of blood coagulation (hemostasis), defined as arrest of bleeding, comes from the Greek words "heme" (blood) and "stasis" (to stop) [1–4]. The coagulation pathway is a complex chain of events leading to blood coagulation and clot formation [5]. Hemostasis is a dynamic equilibrium/balance between coagulation, anticoagulation, and hemolysis. This pathway enables prevention of spontaneous bleeding and allows stopping blood loss after injury.

The normal coagulation system consists of four compartments: the vessels, platelets, coagulation/anticoagulation proteins, and the fibrinolysis system [6,7]. Any event leading to a blood vessel trauma initiates interactions of all four compartments in a coordinated manner, which prevents excessive blood loss by a clot formation. The system works well when all the parts work in balance.

The process of coagulation includes primary and secondary hemostasis. Primary hemostasis is a process of thrombocytes adhesion and aggregation leading to plug formation at the site of injury. Secondary hemostasis includes the two main coagulation pathways, intrinsic and extrinsic. These two pathways originate separately but come across at a specific step, which is called the common pathway, to promote the fibrin activation process [5,8]. The common pathway ultimately activates fibrinogen into fibrin [2,5,9]. The purpose of the whole process is to stabilize the platelet plug with a fibrin net [5].

The intrinsic pathway is the longer pathway of secondary hemostasis and consists of factors I (fibrinogen), II (prothrombin), IX (Christmas factor), X (Stuart-Prower factor), XI (plasma thromboplastin), and XII (Hageman factor) [2,5,6,9]. The extrinsic pathway is shorter than intrinsic and consists of factors I, II, VII, and X. The common pathway depends on the involvement of factors I, II, V, VIII, and X [2,5,6]. Both pathways are activated by specific triggers: the intrinsic pathway through exposed endothelial collagen and the extrinsic pathway through tissue factor released by endothelial cells after external damage [2,5]. The common pathway begins at factor X and ends with fibrin monomers coming together to form fibrin ply, and factor XIII acts on fibrin strands to form a fibrin mesh that serves to reinforce the thrombocyte plug [2,4,5].

Coagulation factors' gene mutations

Damage of any of the coagulation components can lead to pathologies of the coagulation system, which are very diverse and could be divided in two major groups: disorders leading to hypercoagulation/thrombosis and disorders leading to hypocoagulation/bleeding [2]. In this chapter, we are discussing prothrombotic coagulation disorders. There is another

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terminology used to define this state—thrombophilia—as a disorder associated with an increased tendency to clot/thrombosis and venous thromboembolism (VTE) [10,11].

Coagulation factors are encoded in specific genes, mutation/polymorphism of which can lead to hereditary coagulation disorders: procoagulation conditions and rare bleeding disorders [1,12]. Numerous investigations have stressed the importance of genetic factors in the development of coagulopathy [1]. Understanding of the role of genetic variations in coagulation/anticoagulation factors involved in different pathologies gets much interest and has led to the identification of numerous mutations [13].

It is clear now that mutations in coding regions of genes and polymorphisms in regulatory regions of coagulation factor genes have an important impact on hemostasis due to their effect on the concentration of the specific proteins [1,14]. Mutations can be the following: (1) loss of function mutations, which include disorders affecting antithrombin, protein C, and protein S [15]; (2) gain of function mutations such as the factor V Leiden and the prothrombin gene 20210AG mutations [15]. Most congenital coagulopathies are inherited as autosomal recessive [12]. However, some cases of factor XI deficiency and dysfibrinogenemia are reported to be autosomal dominant [12,16]. Patients with inherited prothrombotic coagulation disorders very often have a family history of thrombotic events [17].

Activated protein C resistance (factor V Leiden mutation)

Factor V Leiden mutation is the most prevalent of thrombophilic syndromes and the most common cause of hereditary thrombophilia. The mutation was named after the city in which it was described [18]. According to different data, 3%–7% of the European Caucasian population are heterozygous. The point of mutation is in the factor V gene (R506Q) and results from a substitution of glutamine for arginine at position 506 in the factor V polypeptide. This mutation leads to resistance of plasma to the anticoagulant effects of activated protein C, so this mutation enhances thrombin generation. Diagnosis can be confirmed by DNA analysis for the mutant factor V gene [19].

Antithrombin deficiency

Antithrombin is a protein synthesized in the liver and appears to be one of the most important inhibitors of thrombin [20]. Antithrombin deficiency may appear in two types: (1) type I deficiency is due to reduced synthesis of biologically normal antithrombin, and (2) type II deficiency is characterized by normal levels of antithrombin with decreased functional activity [17]. Homozygous antithrombin deficiency is known to be a lethal disorder.

Protein C and protein S deficiency

Protein C is a natural anticoagulant [7,8,10]. Functionality of protein C is determined by levels of protein S. In the presence of protein S, protein C controls thrombin generation [10]. More than 160 diverse autosomal dominant mutations for the protein C gene have been described [21]. Protein S deficiency may be caused by more than 130 different mutations. The prevalence of these mutations is approximately 0.3–1.3 per 1000 individuals [21].

Prothrombin G20210A mutation

Prothrombin G20210A mutation is an abnormality in the prothrombin gene characterized by G to A transposition at nucleotide position 20210 of the prothrombin gene promoter region. The mutation results in increased levels of prothrombin, so increased thrombin generation that leads to excessive accumulation of prothrombin [18,22,23]. It is known that 1%–3% of the European Caucasian population are heterozygotes. Prothrombin levels are increased by 30% in heterozygous individuals and by 70% in homozygous individuals [18]. Homozygous individuals, or those who additionally have a G20210A mutation with a factor V Leiden mutation, are at a greater thromboembolism risk than heterozygous carriers [23–25].

Hyperhomocysteinemia

Mutation of the 5,10-methylene-tetrahydrofolate reductase (MTHFR) enzyme is the most common cause of increased homocysteine. A total of 34 rare mutations in MTHFR, as well as a total of nine polymorphisms have been reported [26]. The 677C→T (A222V) variant has been identified as the most common genetic cause of hyperhomocysteinemia [23,26]. The 677C→T mutation results in a thermolabile variant of MTHFR that can cause mild to moderate hyperhomocysteinemia. Inheritance of this disorder is autosomal recessive. In is also known that increased levels of homocysteine may result from deficiency of one of several enzymes involved in methionine metabolism and from dietary deficiencies of folic acid and vitamins B6 and B12 [27].

Plasminogen activator inhibitor type 1

Plasminogen activator inhibitor type 1 (PAI-1) is an important regulator of fibrinolysis. Polymorphisms in genes encoding for thrombin-activatable fibrinolysis inhibitor (TAFI) and PAI-1 are responsible for concentration of these fibrinolytic factors. Some polymorphisms in the gene promoter have been associated with slightly greater thrombotic risks [28].

Elevated factor VIII

Elevated levels of factor VIII are an independent marker of high recurrent thrombotic risk. However, levels can also be increased in numerous conditions as an acute phase agent, so its clinical use is controversial [29,30].

Blood coagulation in pregnancy

As was discussed before, the mechanisms of blood hemostasis are complex. In reality the process of hemostatic plug formation occurs on multiple levels with complex feedback systems. This process is even more intricated in pregnancy when multiple changes appear in the coagulation system through pregnancy progression [7,8]. Coagulation factor plasma concentrations change dramatically during pregnancy, with the largest changes appearing at term gestation. In particular, there is a significant increase of factors VII, VIII, X, von Willebrand factor activity, and fibrinogen concentration [7]. Thrombin generation markers are also increased. Furthermore, physiologic changes of coagulation in pregnancy are also accompanied with a significant decrease in anticoagulant activity: reduction of protein S levels and acquired activated protein C resistance [7,31]. Together with reduced fibrinolytic activity in the third trimester, when PAI-1 levels increase by fivefold and with increases in placentally derived plasminogen activator inhibitor type 2 (PAI-2), prothrombotic potential is created [7]. In general, all these discussed changes lead to approximately doubled coagulation activity of the blood if compared with the nonpregnant condition, and pregnancy is therefore known as a state of physiologic hypercoagulation developed through evolutionary metamorphoses to prevent postpartum bleeding [8]. However, these changes can predispose both the mother and fetus to thrombotic complications during pregnancy [32,33].

Due to physiologic changes in hemostasis, procoagulation markers increase during normal pregnancy [34]. Therefore, in cases of pathologic hemostasis in pregnancy, correct interpretation of coagulation test results and diagnosis of hereditary thrombophilia is difficult. It is possible to identify the DNA mutations to confirm factor V Leiden and prothrombin 20210AG, but can be problematic for antithrombin, protein C, and protein S [35]. Thus, it is recommended to postpone screening for hereditary thrombophilia until 6 weeks after the termination of pregnancy or loss of conception [36].

Prothrombotic hereditary coagulopathies and recurrent pregnancy loss

In spite of the advanced development of perinatal medicine, adverse pregnancy outcomes still remain a challenge for contemporary obstetrics [37]. A number of inherited thrombophilias and procoagulation conditions have been connected with pregnancy complications and specifically with recurrent pregnancy loss (RPL) [10]. Particular inherited thrombophilias linked to adverse obstetric outcomes and pregnancy loss include factor V Leiden mutation, protein C and protein S deficiencies, prothrombin gene mutation, and antithrombin III deficiency. Association of MTHFR mutations and hyperhomocysteinemia with procoagulation complications in pregnancy is still disputable. These mutations are no longer considered for routine assessment of thrombotic risks in pregnancy [36,38].

Some studies demonstrate no increased prevalence of hereditary thrombophilias in the RPL population [39]. The degree of risk for RPL and efficacy of treatment have been debated in the literature and in practice [40,41]. The real prevalence of hereditary hypercoagulation in women with RPL remains unclear.

Factor V Leiden mutation plays a major role in pregnancy complications related to hypercoagulability. Women who are heterozygous for factor V Leiden contribute for approximately 40% of thrombotic cases during pregnancy. Pregnant women who are homozygous without a personal or family history have a 1%–4% risk for venous thrombosis, while those with a family history have an approximately 17% risk [22,42].

A marked association between the prothrombin mutation and RPL was described in several studies [36,43]. They reported an overall twofold increase in risk of RPL in women with G20210A. Other researchers found an association between prothrombin mutation and RPL and between the mutation and RPL in first trimester of pregnancy in women with two or more pregnancy losses [44].

Protein C activity significantly increases throughout the first and second trimester of pregnancy [10]. There is a hypothesis that this increase in protein C activity might play a role in supporting early pregnancy through both anticoagulant and inflammatory regulatory pathways [10]. Inherited deficiencies of anticoagulant proteins (proteins C, protein S, and antithrombin) are less

common; they are more strongly associated with VTE than factor V Leiden and the prothrombin mutation. However, researchers reported no strong or significant association between these protein or factor deficiencies and RPL [44]. A more recent cross-sectional study on protein S and RPL did not find a difference in the frequency of the protein S variant between women with RPL and healthy controls [36,45].

In the past, several studies have suggested an association between adverse pregnancy outcome and homozygosity for the thermolabile mutation MTHFR causing homocysteinemia [7]. Furthermore, an association between 677C→T MTHFR and RPL has been reported by some reviews [46,47]. It is hypothesized that thrombophilia may cause placental insufficiency due to chorionic or placental vascular thrombosis [7]. However, more recent studies do not support this association, and overall the evidence of association between adverse pregnancy outcomes and MTHFR mutation is weak [36,44,48].

Overall, according to the recent guideline of the European Society of Human Reproduction and Embryology (ESHRE), there is either not an association or a weak association between RPL and hereditary thrombophilias [36]. The guideline does not recommend screening women with RPL for hereditary thrombophilia.

ART procedures in current practice

Infertility constitutes a significant challenge for contemporary reproductive medicine. Infertility is defined as a failure to conceive within 12 months of unprotected intercourse or therapeutic donor insemination in women younger than 35 years or within 6 months in women older than 35 years [22,49]. It is estimated to affect 8%–15% of reproductive-aged couples worldwide [50–54].

The management of infertility should precisely target the diagnosed cause. At present assisted reproductive technology (ART) plays an important role in the treatment of this condition. There is a huge progress in ART procedures improvement, resulting in successful treatment of previously untreatable cases [50,53,55,56]. ART includes, but is not limited to, intracytoplasmic sperm injection (ICSI), in vitro fertilization (IVF) and embryo transfer, embryo biopsy, gamete and embryo cryopreservation, and preimplantation genetic testing [57,58]. There are numerous reports about prognostic factors associated with the outcomes of IVF, such as maternal age and ovarian aging, diagnosis, and the ovarian reserve. Currently, relatively low attention has been paid to lifestyle and IVF outcomes. Smoking, alcohol consumption, bad nutritional habits, caffeine intake, exercise, and exposure to toxic bisphenols are evidently associated with lower rates of IVF success [52,57,59]. Data from the International Committee for Monitoring Assisted Reproductive Technologies reported over four million ART procedures worldwide for a period of 2 years (2008–10) [57]. Remarkable developments occurred in ART over the last decades, which substantially improved delivery rates from 26% in the 90s to about 40% nowadays [57]. In Europe and the United States, over 2% of all infants born result from ART treatments, and a conservative estimate indicates that worldwide over eight million babies were born from ART treatment [57,60].

However, we have to keep in mind side effects of medications used during the procedures in ART and significant complications of the ART procedures itself. The available literature focuses primarily on pregnancy-related and perinatal outcomes, such as gestational diabetes, pregnancy-induced hypertension, low birth weight, and preterm labor [56,58]. There are several complications, however, that are more germane to emergency medicine, particularly ovarian hyperstimulation syndrome (OHSS), ectopic and heterotopic pregnancy, ovarian torsion, and even malignancies [56–58,61–63]. The pathogenesis of OHSS is complex and may contribute to a hypercoagulable state, with an increased risk of venous thrombosis [56]. As a consequence of OHSS, there is a fluid shift into third space causing hemoconcentration, hypercoagulability, and electrolyte abnormalities [56–58]. Upper extremities venous thrombosis is the most common thrombotic event after ART reported in the scientific literature [56].

The utilization of ART for the purpose of fertility preservation is increasing, and special consideration should be given to these patients and the potential for unusual complications in light of their comorbidities [64,65]. For example, a patient with coagulation disorders undergoing ART for the purposes of fertility preservation will have a higher risk of adverse outcomes [58]. All patients undergoing ART should be considered to have a complicated pregnancy.

Prothrombotic gene polymorphisms and adverse reproductive outcomes in ART

Although the success rate of ART gradually increases over the years, it is far from optimal [48,51,57,66]. Since the highest success rate of ART is around 40% [57], more than half of couples seeking assisted fertilization have been left frustrated following multiple failed attempts. Whether hereditary thrombophilias have any impact on the success of ART is a point of debate among reproductive endocrinologists and reproductive immunologists [32,33,48].

The coagulation system plays an important part in the implantation process through the fibrinolytic system that plays a role in miscarriage and implantation failure [28]. However, the existing literature and some reports provide controversial results [28,67].

It has been suggested that a potential cause of implantation failure in ART cycles occurs in vascular bed of decidua as a microvascular occlusion due to thrombophilia. However, it is still unclear whether congenital or acquired thrombophilia is an underlying cause of implantation failure [44,48].

ART procedures such as IVF or ICSI often require ovarian stimulation, which has demonstrated to induce prothrombotic conditions through alterations of both coagulation and fibrinolysis pathways [28,48,68]. Some researchers suggest using low molecular weight heparins (LMWH) for prevention of RPL after ART [48,69].

During embryo implantation, progesterone should induce endometrial stromal cells to undergo decidualization. This process, being physiologic, protects against bleeding due to endometrial capillaries being invaded by the implanting cytotrophoblast [7]. There is a recruitment of factors to promote hemostasis including upregulated expression of tissue factor, the primary initiator of hemostasis through thrombin generation, and PAI-1, which inactivates tissue-type plasminogen activator, the predominant agent in fibrinolysis [7].

The role of hypercoagulation in recurrent implantation failure after IVF procedures is thought to be through mechanisms similar to those identified in RPL [7]. It has been hypothesized that normal invasion of syncytiotrophoblasts into the maternal vascular bed might be affected by localized thrombosis at the implantation site, leading to IVF failure [7,69].

In the study by Sticchi and colleagues, significant changes in fibrinolytic parameters during ovarian stimulation were found (clot lysis time $P = .003$; TAFI $P = .009$, and PAI-1 $P = .003$) [27]. Clot lysis time values and TAFI and PAI-1 concentrations significantly increased from baseline to day 5 (pb0.0001, $P = .01$, $P = .005$, respectively), and they decreased at day 7 but remained higher than those at mid-luteal phase of menstrual cycle. Significant differences of TAFI and PAI-1 concentrations during ovarian stimulation according to TAFI and PAI-1 polymorphisms were observed in this study [28]. The researchers concluded that mutations of TAFI and PAI-1 genes can lead to fibrinolysis changes during the ovarian stimulation cycle.

In another study investigating prothrombotic hereditary coagulation disorders, women with at least two failed IVF or ICSI procedures were screened for factor V Leiden, prothrombin gene mutation, or MTHFR C677T and excluded if acquired thrombophilias were detected [7,69]. Participants received treatment with LMWH from time of controlled hyperstimulation until the β-human gonadotropin (β-HCG) test. The only significant finding was that the pregnancy rate in women who were older than 36 years was higher in a group that received LMWH if compared to those who did not receive LMWH. This study did not find any significant difference between the presence or absence of thrombophilia, but only 32% of the study population had a thrombophilia marker, largely MTHFR C677T homozygotes (25%) [69]. The cited investigation proves that LMWH prophylaxis could reduce the risk of implantation and early pregnancy failure after ART.

Some researchers retrospectively analyzed 594 women who underwent ART and had a thrombophilia workup [70]. None of the common thrombophilias identified were found to be significantly associated with the number of prior failed ART cycles or with lower fertility [48,70]. According to this study, thrombophilia carrier status was not associated with poorer reproductive outcomes. Data from this large retrospective study confirm that screening for factor V Leiden mutation is not indicated in couples undergoing ART.

Later, a meta-analysis done by Bates, which analyzed data from eight case-control studies, showed controversial results: threefold increased risk of ART failure in patients with the factor V Leiden mutation (OR 3.08, 95% CI 1.77−5.36) [71]. However, there were no other congenital thrombophilias (P2 mutation, AT, protein C, or protein S deficiency) found to be associated with an increased risk of ART procedure failure [71].

An association between MTHFR mutation and ART procedure outcomes was evaluated in many studies [48,72,73], and no association was found between MTHFR carrier status and ART outcomes [48].

In sum, the association of congenital thrombophilias with ART outcome is still disputable. Based on the available evidence, testing for and treatment of congenital thrombophilia are not suggested in patients undergoing ART if there is no personal or family history of venous thrombosis [7,44,48].

Preconceptional and prenatal diagnostic approach

Due to physiologic changes in the coagulation system in pregnancy, prothrombotic markers will be increased even during physiologic pregnancy [7,8,34]. Therefore, correct interpretation of results and diagnosis of hereditary thrombophilia during pregnancy is difficult and inaccurate. Testing for coagulopathies should be performed when results could be used to improve or modify management [15]. Testing has been suggested to assist with secondary prevention and for hereditary disorders.

It is possible to test for the DNA mutations to identify factor V Leiden and prothrombin 20210A genes

polymorphism [36,44]. However, it can be problematic for antithrombin, protein C, and protein S. Therefore, it is recommended to perform screening for hereditary thrombophilia as a preconception investigation or after delivery or miscarriage [36].

As was mentioned before, current guidelines do not recommend screening for hereditary thrombophilia patients without family history of venous thrombotic events [36,44]. The recommendation to not perform screening for hereditary thrombophilia in women with RPL is similar to the suggestions of the guideline on thrombophilia, antithrombotic therapy, and pregnancy of the American College of Chest Physicians [36,74]. The screening could be considered only if there are other risk factors present or known family history of thrombotic events.

According to other recently published study results, tests to confirm thrombophilia should be carried out in women with adverse pregnancy outcomes in the past medical history [37]. Those with confirmed prothrombotic changes in the coagulation system and planning pregnancy are recommended to start anticoagulant prophylaxis. The study of Dłuski et al. supports the hypothesis that tests for thrombophilia should be done for women with a history of adverse pregnancy outcomes [37].

For patients with a history of adverse pregnancy outcomes due to the prothrombotic coagulopathy, preconception counseling and testing is recommended to prevent a recurrence [41]. This is the reason for most of guidelines to advise investigations in women with RPL. However, it is still unclear when to perform investigations for risk factors in couples with RPL [41]. Furthermore, there are no proven effective treatment methods currently available if abnormal test results have been received.

Possible management options

There is no consensus whether peri-implantation heparin administration in ART procedures improve live birth and clinical pregnancy rates [15,48,75]. Heparin was used to improve outcomes, but it had side effects such as bruising and bleeding, and no conclusion could be made regarding its safety because none of the studies reported comparative data on adverse effects [75].

Decisions on whether to use prophylactic anticoagulation during pregnancy to prevent thrombotic events depend on the benefit-risk ratio [23]. The rate of clinically relevant maternal bleeding due to the LMWH use is about 2% [23]. However, in a systematic review of nine case-control studies with 2526 patients that included considering the association between thrombophilia and pregnancy-associated thrombosis, it was found that the highest risk was associated with homozygous factor V Leiden or prothrombin G20201A mutations [23,76]. For most inherited thrombophilias, except homozygotes factor V Leiden or prothrombin G20201A, the risk increases to approximately 4% in pregnancy [23].

Treatment with LMWH is widely accepted in thrombophilia carriers with implantation failure despite the absence of evidence-based proof of effectiveness [48]. Heparin might improve implantation rates in patients undergoing ART through mechanisms not related to anticoagulation, but by improving endometrial receptivity and decidualization of endometrial stromal cells, as well as trophoblast adhesion and invasiveness [48].

It is still questionable whether empirical LMWH could enhance pregnancy rates in women with unexplained recurrent implantation failures in ART. One study reported that significantly higher pregnancy rate in patients with previous ART implantation failures was observed with administration of LMWH [77]. These study results have proven no association between hereditary thrombophilia and pregnancy rate in patients with previous IVF implantation failures. However, the authors suggest confirming the findings by randomized controlled trials before use of LMWH for ART cycles [48,77].

To answer the same query, Urman, in the randomized trial, included 150 women with two and more failed ART procedures [78]. Participants in this study underwent controlled ovarian stimulation and were randomly allocated to receive LMWH or no treatment in addition to routine luteal phase support. LMWH was continued up to 12 weeks of gestation in participants who got pregnant [48,78]. In this study, higher live birth rates were observed in the group treated with LMWH (34.7% vs. 26.7%). However, the difference was short of statistical significance. Therefore, the quality of evidence is moderate at best, and it cannot be justified to recommend heparin administration to all women suffering from recurrent ART cycles failure [48].

Conclusions

Women with inherited prothrombotic coagulation disorders are at high risk of developing early and late complications in pregnancy. Thrombophilia testing is performed very often for pregnant patients, and in many cases its frequency cannot be justified based on available evidence. The majority of such testing is not of benefit to the patient and may be harmful as it pushes physicians to make unnecessary prescriptions. Up to now, routine screening for thrombophilic defects has not been recommended in women without previous pregnancy complications. However, prevention of

implantation complications, especially in ART-achieved conceptions, remains a major challenge. Talking about management, apart from the prevention of RPL and VTE in antiphospholipid syndrome, there is currently insufficient evidence to suggest LMWH administration for women with inherited thrombophilia and pregnancy complications as well as for those women with ART-related implantation failure.

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CHAPTER

7

Endocrinological causes of female infertility

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Introduction

The probability of healthy young women achieving pregnancy in one menstrual cycle is 25%–30% [1].

Nowadays, approaching endocrine disorders in reproduction has been deeply influenced by the central role of assisted reproductive technology. Hormonal dysfunctions constitute 25% of infertility issues [2].

Currently available treatments in women require an understanding of the hormonal bases of folliculogenesis (FG). From this perspective the endocrine and molecular events in FG are crucial for acquiring oocyte competence, a factor of fetal viability. This publication develops a practical pathophysiological and therapeutic approach to the endocrine causes of female infertility. Key aspects to consider are patient background, age, ovarian factors, spermatic factors, expectations, decisions, and treatment accessibility. The need for early access to in vitro fertilization (IVF) must contemplate the availability of genomic and embryologic testing to shorten time to pregnancy, avoid multiple pregnancies, and other additional risk factors.

Older patients make ovarian aging (OA) and endocrine disturbances key concepts within reproductive health. The debate regarding which hormones to evaluate before treatment has been present throughout history. This chapter will develop a guide of endocrine disturbances focused on women in the context of infertility consultation.

Anovulation

The ovarian follicle is considered the functional unit of the ovary and is involved in oocyte production and endocrine homeostasis. The endocrine activity of developing ovarian follicles is crucial for the process of follicular selection and the production of competent oocytes. In this regard, folliculogenesis comprises all the stages of ovarian follicle development, the release of the mature oocyte, and the formation of the corpus luteum [3]. Follicular development starts as early as fetal life and is continuous until menopause. Three phases can be described according to the developmental stage of the follicle and its gonadotropin dependence: (1) follicular growth from primordial to secondary stages, (2) transition from preantral to early antral stage, and (3) the development of the preovulatory follicle, which ends in the release of a mature oocyte and the consequent formation of the corpus luteum. The first phase is gonadotropin-independent, and the second one gonadotropin-responsive. The third and last stage depends on pulsatile gonadotropin secretion [4].

The preantral-early antral transition is the most susceptible to follicular atresia [5]. This step is mainly control by intraovarian paracrine or autocrine regulators, like gonadal steroids, cytokines, and growth factors. For example, oocyte-derived growth differentiation factor 9 (GDF-9) stimulates growth and survival of the follicle by suppressing granulosa cell apoptosis [4]. Only a few early antral follicles escape apoptosis, due mainly to the survival action of follicle-stimulating hormone (FSH), whose concentration increases during the perimenstrual period. This gonadotropin is responsible for granulosa cells survival and proliferation, estradiol production, and luteinizing hormone (LH) receptor expression [3]. Of this cohort of follicles selected for their high responsiveness to FSH, one will grow faster and will produce more estradiol and inhibin A [5]. This is evidenced in part because the follicular fluid of the dominant follicles has more estrogen and less androgen than the follicular fluid of atretic subordinate follicles [6].

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The estradiol and inhibin A produced by the dominant follicle are responsible for negative feedback on the pituitary FSH [7]. In addition, during this process, FSH and estradiol increase LH sensitivity of the dominant follicle granulosa cells. Therefore, the dominant follicle becomes less dependent on FSH and more responsive to LH. This fact allows the dominant follicle to survive in spite of falling FSH concentration, while all the other antral follicles become atretic [6].

At midcycle, a characteristic event takes place: the LH surge. This is the result of the activation of the positive feedback mechanism by the high amounts of estradiol secreted by the dominant follicle. This steroid sensitizes the pituitary to gonadotrophin releasing hormone (GnRH) [8]. The dominant follicle responds to the rise in LH releasing the mature oocyte for fertilization. The remaining granulosa and theca cells become the corpus luteum and are responsible for the high concentrations of progesterone found during the luteal phase of the menstrual cycle [9].

All these crucial events are finely regulated. However, failure of any of these processes could provoke one of the most relevant alterations observed in infertile patients: anovulation. Albeit oligomenorrhea and amenorrhea are the most frequent symptoms, most infertile women have regular cycles with diverse endocrine alterations being the causes of anovulation.

Ovarian aging

Ovarian aging is a preponderant factor in nonfertile patients, with maternal age being one of the most relevant prognostic factors within reproductive potential. As patients approach the fourth decade of life, anovulation becomes more frequent [10]. It corresponds to a stage-denominated menopausal transition. Multiple genes have been identified as causing this stage, which also relate with the age of menopause.

Menopausal age is defined by multiple factors, genetic and environmental being the most important [11]. At birth, there are approximately one million primordial follicles (PFs); however this number decreases as age increases. Around 25,000 PFs are left at 37 years of age, and after that the rate of recruitment increases sharply, leading the total number of PFs to around 1000 at 50 years of age and accelerating atresia.

Ovarian reserve

Ovarian reserve (OR) is defined by the number of oocytes present within the ovary at each moment of the woman's lifecycle [12]. The total number of PFs in the ovaries, each constituted by a germ cell and a somatic cell crown, represents OR available in each patient. Decrease in these numbers is genetically determined. OR assessment resides in the possibility to predict clinical response to gonadotrophic stimulation and pharmacologic dosing. Basal plasma concentrations of FSH, LH, anti-Müllerian hormone levels (AMH), and plasmatic estradiol are measured on days 1–3 of the cycle to study this aspect [13].

AMH is an OR marker that belongs to the TGF-β growth factor family. Genetically located within chromosome 19, it plays a key role in follicular development inhibition and preventing the recruitment of a dominant follicle [12]. Even though serum levels drop with age, it is readily dosable throughout the menstrual cycle, acting as a tool that allows categorization of response to ovarian stimulus, yielding different protocols in ovulation induction.

In patients with AMH <1 ng/mL, potentially low response could exist, with AMH 1–3.5 ng/mL = normoresponse, and AMH >3.5 ng/mL may expect high ovarian response. Values exceeding 4.7 ng/mL suggest the presence of polycystic ovary syndrome (PCOS) with a sensitivity (SS) of 82% and specificity (SP) of 79.4% [14]. We must keep in mind young patients with AMH >3.5 ng/mL have a higher risk for ovarian hyperstimulation syndrome (OHSS).

Although AMH is considered a marker of OR in an infertile population, it does not possess predictive value in the assessment of a live-born embryo, and it is not an appropriate screening method for fecundability in fertile women [14,15].

Primary ovarian insufficiency

This pathology known either as primary ovarian insufficiency (POI) or premature ovarian failure (POF), which roughly reaches 4% of the population, is defined as an early cease of menstruation [16]. Clinical presentation is both fluctuating and heterogenous, leading to amenorrhea and hypoestrogenism associated to hypergonadotropic hypogonadism.

It responds to an alteration in ovarian function with a decrease in quantity and quality of follicles and oocytes as well as a reduced number in PFs. Even though there are several etiologies, the mechanisms behind it are either follicular depletion or dysfunction [17].

This decrease in PFs is linked to an increase of follicular atresia with a failure in follicular recruiting. It can present itself as a hidden ovarian insufficiency [18] or completely lacking in menstrual cycle anomalies and signs of hypoestrogenism, with amenorrhea being the extreme setting. Once identified, its etiology is idiopathic in 63% of cases or because of exposure to

chemotherapy/radiation or surgery. Other causes are genetic, infectious, autoimmune, or linked to systemic illness.

Amongst genetic causes are aneuploidy, chromosome X translocations and/or deletions, and premutation of FMR 1 gene has been described. Therefore, genetic counseling is a good complement to bear in mind.

POI diagnosis is made in oligo/amenorrheic patients who are <40 years old with the following:

- high FSH (>40 UI/mL) during early follicular phase in at least two separate measurements (4 week separation between samples)
- low estradiol (<30 pg/mL)
- diminished ovarian volume detected by ultrasound

Complementary assessments are karyotype, FMR1 premutation detection, bone densitometry, and antithyroid antibodies.

Patients at risk for POF who will undergo fertility interventions (surgery regarding ovarian masses, endometriosis, maternal history of early menopause or autoimmune disease) should be advised to take preventive measures and carry out oocyte vitrification [19,20]. This procedure allows proper maintenance of reproductive potential as ultrafast temperature drop accomplishes oocyte survival rates higher than 80%. Oocytes in metaphase II arrest are required, narrowing selection of candidates to patients with normal FSH and LH levels. Other interventions, such as ovarian cortex fragments cryopreservation, are alternative methods. Nevertheless a specific program and professional experience are needed to carry it out appropriately [21].

The most effective treatment of POF is egg donation, either fresh or frozen [22,23]. Oocyte donation has evolved for over 3 decades, sparking controversies linked to ethics, social and regulatory change. It has also contributed to knowledge of the implantation window in humans, implantation dynamics, and fertility preservation, new strategies in the establishment of an ovulatory peak, and the optimization in transfer cycles of cryopreserved oocytes [24].This model allows the understanding of endometrial receptivity showing how implantation can be achieved even in patients with gonadal failure by administrating a tailored exogenous protocol of estradiol/progesterone [25]. Moreover, implantation effectiveness in patients over 40 years of age that receive oocytes from donors under the age of 35 shines a light on oocyte competence as a crucial factor.

Polycystic ovary syndrome

PCOS is a complex and heterogeneous endocrinometabolic dysfunction, characterized by chronic oligoovulation and hyperandrogenism [26].

Several phenotypes exist, but diagnostic certainty is achieved through identification of chronic ovulatory dysfunction, clinical or biochemical hyperandrogenism, and ultrasound-detected polycystic ovary. Oligomenorrhea or secondary amenorrhea constitutes 70% of cases. It can manifest itself as normogonadotrophic normoestrogenic amenorrhea (WHO group 2), presenting normal serum FSH and estradiol, either normal or high LH. Weight gain, insulin resistance, hyperinsulinemia, spontaneous abortion, gestational diabetes, and increased cardiovascular and oncologic risk can be associated [27–29]. This adverse metabolic event negatively impacts the oocyte quality and endometrial receptivity, therefore affecting the reproductive outcome. A high OR is frequent, which leads to an increased risk of complications such as multiple pregnancies and OHSS after IVF protocols [30,31].

Treatment approach is individual to each patient, considering general measures such as diet and exercise, and weight loss if body mass index (BMI) is > 30. Then specific measures for each issue can be addressed: treat hyperandrogenism and hirsutism if present, correct metabolic aspects (dyslipidemia and hyperinsulinemia), as well as treatment of anovulation. Metformin can be used at 500–1500 mg per day for its insulin-sensitizing properties. Certain patients are candidates for a metformin-clomiphene citrate regimen with ultrasound monitoring for ovulation. If patients are known to be clomiphene resistant, consider gonadotropin-mediated ovulatory induction [32].

Hypothalamic-pituitary axis pathology

It is characterized by altered GnRH pulses, which affects secretion of pituitary gonadotropins. Said pulses can be of low frequency or nonexistence, clinically manifesting as hypothalamic amenorrhea. This is produced as a consequence of a state of hypogonadotropic hypogonadism that shows low levels of FSH, LH, and plasmatic estradiol. Because of this inadequate secretion, folliculogenesis is affected, causing anovulation [33].

Most hypothalamic amenorrhea in women of reproductive age is functional. Amongst the most frequent etiologies that have been described, we can find weight loss, anorexia nervosa, caloric restriction, excessive exercise, bariatric surgery, and severe obesity. Infertile patients can have varying degrees of ovarian dysfunction, stress being one of the most frequent causes of hypothalamic-pituitary function alterations with a neuroendocrine response listing ACTH as the primary culprit, stimulating secretion of cortisol by the adrenals. Diagnosis is achieved via FSH, LH, and estrogen dosage, with either normal or diminished levels being

possible findings. All of these findings are known as WHO group I anovulation disorders. It is possible to improve spontaneous ovulation with moderate exercise and increasing BMI if it is < 19. Treatment is done with hygiene-dietetic measures, psychotherapy, and gonadotropin-induced ovulation [34].

Hyperprolactinemia

It is described as a cause of anovulation in 5%−10% of infertile patients. Prolactin (PRL) is a hormone with a role in both lactation and reproduction. High PRL levels can be pharmacologically produced by drugs (dopamine receptor antagonists, metoclopramide, alpha methyl dopamine) or by stress and hypothyroidism (TSH screening is recommended during patient work-up, as it can stimulate PRL release). It can be clinically associated to oligo- or amenorrhea in 8% −36% of cases; it might also be asymptomatic or galactorrhea can also present within menstrual anomalies. Laboratory values will show slightly low or normal FSH and estradiol shows a tendency to drop. Diagnosis can be made through PRL dosage in blood. As normal values lie below 20 ng/mL, values between 20 and 40 are indefinite and imply the need to repeat blood dosage. And, values higher than 40 ng/mL require imaging studies to rule out central nervous system tumors. First-line treatment relies on prolactin inhibition by dopaminergic agonist administration (bromocriptine or cabergoline) [35].

Hyper- and hypoandrogenism

The adrenals, ovaries, and testes are the main sources of androgens [36]. In the ovary, androgens participate in steroidogenesis and folliculogenesis.

During the follicular phase of the menstrual cycle, the thecal cells synthesize androgens and are sensitive to LH through LH receptors. These androgens can only be aromatized to estrogens by the granulosa cells. These cells have FSH receptors that stimulate the aromatase enzyme necessary for this conversion [6].

The most common cause of an increase in androgenic secretion in patients undergoing infertility work-up is PCOS. Differential diagnosis should be established between hypertrichosis and congenital adrenal hyperplasia, the latter presenting itself at a younger age accompanied by a deficit in 21α hydroxylase. Androgen-secreting tumors should be ruled out. In counterpart, androgen level decrease could hinder fertility by diminishing sexual desire or altering folliculogenesis. However, the results of previous publications remain inconclusive [10].

Therapeutical intervention

Pharmacologic interventions in infertility require a comprehensive patient work-up. In this context it is imperative to only order tests that have potential clinical value. Infertility patients are already under considerable psychological distress, and untimely anxiety brought along by pointless testing must be avoided. A high number of patients require assisted reproductive technologies, where hormonal evaluation optimizes IVF outcome. It may be useful to predict the response to ovulation induction to reduce both emotional and economic costs of treatment. In that sense, day 3 FSH >15 mU/mL is an extremely poor prognostic factor for any treatment with a woman's own eggs.

Estrogen antagonists

Clomiphene citrate treatment: A α and β estrogen receptor competitive antagonist could be used as an ovulation inductor. By avoiding negative feedback at the hypothalamus, it modifies the GnRH secretion pattern, inducing an increase in plasma FSH and LH. Follicular maturation and ovulation are triggered at the ovarian level. An antiestrogenic effect can be seen in some patients at the endometrium and cervical mucus. Recommended dose stands at 50 mg/day with a maximum dose of 150 mg/day.

Determinant patient risk factors such as OHSS and multiple gestations must be carefully considered before indication, while others less severe include hot flashes, visual alteration, mastalgia, and nausea [37].

Letrozol is a potent, specific, nonsteroidal aromatase inhibitor. It has good tolerance and constitutes another therapeutic option, as used in PCOS and reporting cumulative pregnancy rates of 27% in anovulatory patients. Ovulatory stimulation initial doses are 2.5 mg, indicating human chorionic gonadotropin (hCG) when the follicle reaches 20 mm during late follicular phase. Just as clomifene citrate, it can be used in association with intercourse or intrauterine insemination.

Gonadotropins treatments

Gonadotropins are glycoproteins with an alpha subunit of 92 amino acids (common to LH and FSH), and a beta subunit that grants specificity [38].

These can be obtained via urinary origin or genetic engineering, the latter known as recombinants. FSH can induce recruitment, selection, and follicular dominance. LH on the other hand participates in follicular maturation, ovulation, meiosis resumption, and progesterone secretion by the corpus luteum [39]. Gonadotropin ovarian hyperstimulation is applied at different

treatment levels: intrauterine insemination, IVF, and intracytoplasmic and preimplantation genetic diagnosis. Dosing and protocols are variable and adapted regarding treatment complexity, inducing multiple follicular development.

The challenge of implementing gonadotropins in anovulatory patients is the achievement of fetal viability with the lowest complication (such as OHSS or multiple pregnancies) rate possible, so individualization of patients and risk factor assessment is mandatory, and categorization of follicular response dimension in each patient is needed [40].

Gonadotropins can be a therapeutic option in patients with simple anovulation (normo- or hipogonadotrophic) who have failed previous treatments. In this scenario, monofollicular responses or <3 follicles and initial daily doses of 37.5 IU up to 75 IU are required to avoid multiple gestation. On the other hand, they become first-line therapy in IVF or ICSI patients who require procurement of a greater number of oocytes; in these cases combined protocols with antiestrogens could also be applied. Drug choice depends on availability and attainability.

To induce ovulation, LH surge could be replicated using urinary hCG, recombinant hCG, or eventually GnRH analogs; this last strategy is effective in preventing OHSS risk in assisted reproductive technology treatments [39].

Preventing complications

Ovarian hyperstimulation syndrome

A complication that stems from pharmacological stimulation of ovulation is an increased permeability and size in both ovaries. Its severity lies in the presence of renal alterations, hypovolemia, hypercoagulability, oliguria, and/or dyspnea. It holds a mortality risk of one in 400,000 cases [41].

Etiology is nuclear, responding to the presence of proinflammatory mediators, particularly vascular endothelial growth factor (VEFG) [42,43]. Presentation depends on hCG administration that induces VEFG messenger RNA and VEFG type II receptor expression. Increased vascular permeability with arteriolar and capillary dilation are characteristic findings. Third spacing can bring about hypovolemia, hemoconcentration, and hyponatremia. Low weight, early age, polycystic ovary (PCO), and AMH >3.6 ng are all risk factors.

OHSS is the most serious complication of ovulation induction and prevention must be the most important feature of the management. Nowadays, in "OHSS-free" clinics, patient risk can be reduced using GnRH agonists to induce final oocyte maturation in IVF [44,45].

Multiple gestations

Multiple gestations are another complication in younger patients with normal-high OR and the presence of PCO. If more than four follicles are detected by ultrasound, cycle cancellation should be considered or engaging in assisted reproductive technologies with oocyte recovery and single embryo transfer in fresh or frozen cycles [46].

Final considerations

Endocrine causes of infertility frequently have a genetic base, exceeding the scope of this publication. Patients could benefit by having a molecular diagnosis followed by appropriate care and counseling. Considerations of patient's age, clinical and surgical background, semiology, menstrual cycle characteristics, drug use, and emotional and nutritional state are recommended.

In addition, evaluation of FSH and LH levels, plasma estradiol and progesterone test, PRL dosage, thyroid function tests, testosterone and DHEA levels, AMH, as well as chromosomal and genetic studies would be necessary to complete a diagnosis associated with female infertility. When expectant management is low, IVF may be considered as a new treatment option. Experience shows that with prolonging treatment of ovulatory stimulation, negative results or exposure to complications generates emotional distress that will discourage patients who ultimately abandon treatment. Discussing risks and benefits with patients is always appropriate while keeping age, OR, sperm quality, and potential endometrial receptivity in mind for future patient reference. Worldwide contrast in ethical, financial, and regulatory aspects influences patient management and decision-making to this day.

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CHAPTER

8

Management of tubal factor

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Tubal infertility

Infertility is a common health problem affecting one in nine couples, and tubal subfertility represents 20%–30% of different infertility factors [1]. Hence, it is crucial to assess tubal function in the initial infertility work-up. In the past few decades, the introduction of assisted reproduction technologies has entirely changed the tubal pathology management practice.

These changes were significant and have led some authors to state that "tubal surgery was dead and only obituary remains" [2]. Currently, a more balanced approach is highly demanded, and it is time to establish the "state of the art" on this topic.

Etiology

There are three main etiologies that contribute to the tubal pathology:

- congenital malformations
- postinflammatory disease (PID)
- endometriosis

Most frequently pelvic inflammatory disease is considered the common cause of tubal damage, followed by endometriosis. Congenital anomalies are a separate entity that will be discussed in more detail below. In both postinflammatory pathology and endometriosis, adhesions are reciprocally found and consequently will reflect on the tubal pathology prognosis and treatment.

Tubal lesions

It is essential to identify the tubal lesions accurately and precisely, to ensure providing appropriate treatment and imposed intervention. This is often very confusing in many literatures.

The main two types of organic tubal pathologies are distal tubal lesion and proximal tubal lesion, where the latter representing more than 90% of cases.

Proximal

The proximal segment of the tube consists of interstitial and isthmic portions. Usually, the proximal lesions are obstructive lesions apart from cornual polyps where the exact mechanism of infertility is not well acknowledged. The obstructive lesions are either **organic** related to PID or endometriosis or, most frequently, **functional** due to tubal spasm and mucous plug. The intervention will be customized accordingly. Another subcategory that should be considered is iatrogenic obstructions related to tubal sterilization, where, also, the management will be distinct.

Distal

The distal part of the tube comprises the tubal ampulla and the fimbria. It is again a site of occlusion lesions. The occlusion is either complete or incomplete, where the former can induce hydrosalpinx or hematosalpinx in the case of endometriosis, the later is called phimosis. The prognosis of these lesions once operated is radically different because when obstruction is incomplete, usually the mucosa is of good quality, whereas in the case of hydrosalpinx, tubal mucosa may be very damaged. So the prognosis of treatment depends on the quality of tubal mucosa.

It is vital to differentiate between the two lesions prior to any intervention.

Congenital lesions

Congenital tubal lesions are illustrated by tubal agenesis and tubal duplication. Exclusively unilateral

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agenesis is recognized, which is typically associated with renal agenesis. Bilateral tubal agenesis is a lethal malformation. Tubal duplication is sparse, so its impact on fertility is not well understood.

On the other hand, the genitalia deriving from the Müllerian and Wolffian ducts are found to have numerous embryonic remnants that in present day are known as a **subtle and congenital tubal abnormalit**ies. These abnormalities were until recently considered to have no impact on fertility. Several publications have recently challenged this assertion and it seems important to pay attention to them [3,5].

What are the subtle tubal lesions?

There are a large number of minor anomalies, which are considered embryological remnants either from Müllerian or Wolffian ducts. Some are considered to be a simple anatomic variation, such as **appendix vesiculosa** (a small cyst attached to the ampulla or the fimbria of the tube, less than 5 mm in size), whereas larger cysts are called **hydatid of Morgagni** (or paratubal cyst), the most common anomaly. In addition, there are **accessory tubes**, **intrafimbrial adhesions**, **ampullary sacculations,** and **diverticula** (Figs. 8.1–8.4). Subtle lesions of the fallopian tubes have been described for a long time, but their impact on fertility has not been fully addressed.

Diagnosis

The detection of tubal pathology needs to use varying diagnostic tools of varying value [4].

It is admitted that three parameters should be addressed to have a good tubo-peritoneal investigation: tubal patency, quality of tubal mucosa, and tubo-peritoneal environment (i.e., presence of adhesions that may impair the tubo-ovarian relationship and the ovum pick-up mechanism).

Diagnostic tools may be noninvasive or invasive:

Noninvasive approach is by hysterosalpingography (HSG) and hysterosonography (USG). These methods should be used as an initial assessment. They allow to detect distal or proximal obstructive tubal pathologies. However, they can produce too many false negatives and false positives (30% and 15%, respectively, in the Mol,Swart meta-analysis) [6], which makes them unreliable tools. Although they are increasing in accuracy, USG in particular, they still lack value in detecting adhesions, which are of great influence in fertility.

Invasive approach is a gold standard tool to address the three parameters described above. In practice these methods are based on endoscopy. Classically this is exhibited by **laparoscopy**, a less invasive method by transvaginal endoscopy, originally described by Gordts (Trans Vaginal Endoscopy (TVE)) [7], which we have referred to as **fertiloscopy** [8], and which has shown in the past few years to be basically as good as laparoscopy in detecting tubo-peritoneal pathology [9]. Whatever

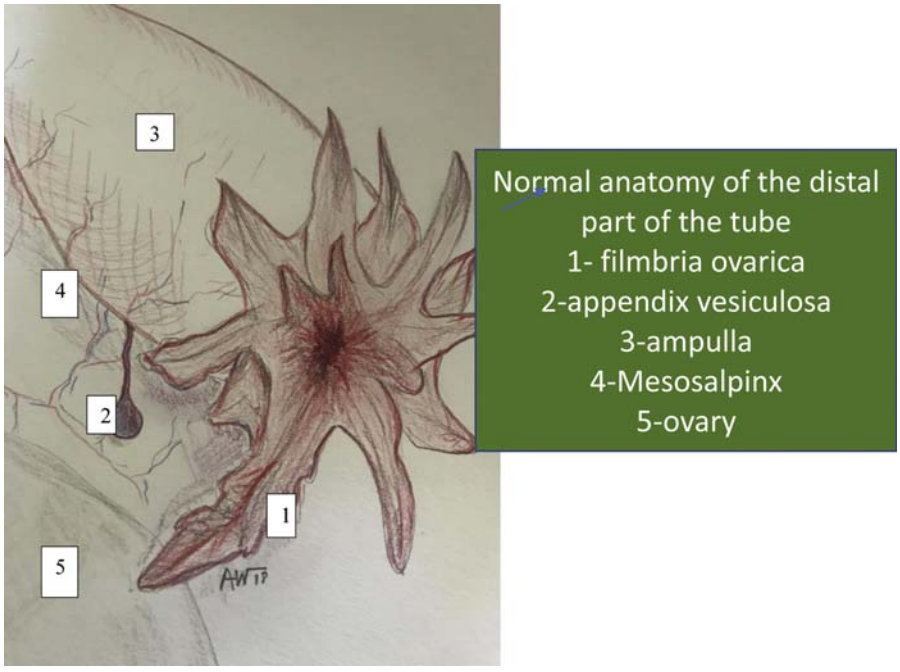

FIGURE 8.1 Normal fimbria.

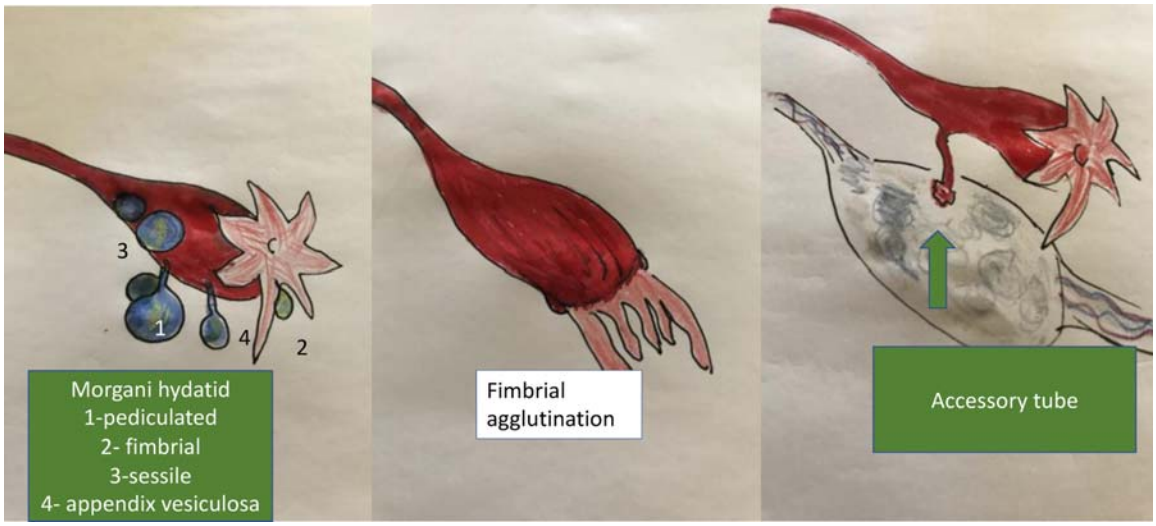

FIGURE 8.2 Subtle tubal abnormalities.

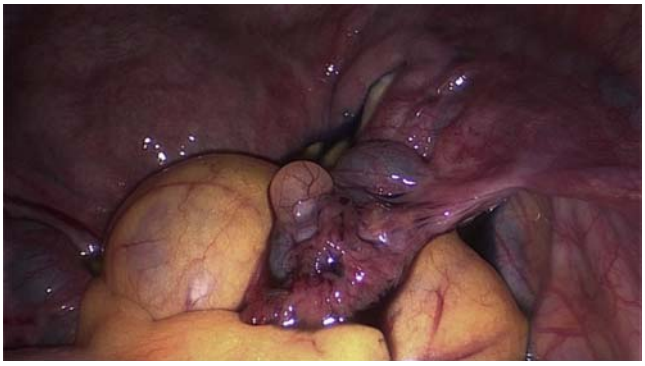

FIGURE 8.3 Hydatid of Morgagni (laparoscopy).

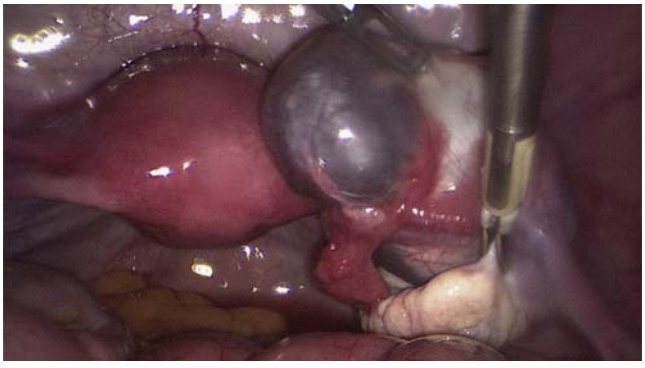

FIGURE 8.4 Sacculation (laparoscopy).

procedure is chosen, it is crucial not to judge on the tubal factor until a complete evaluation by endoscopy has been done, including an evaluation of the tubal mucosa at the fimbria level and if possible at the ampulla level through salpingoscopy. Salpingoscopy is easily performed through fertiloscopy [10] but is more technically challenging by laparoscopy, and it needs two optics, two light sources, irrigation, etc. Regrettably, many patients are referred for vitro fertilization (IVF) without having had this proper pelvic evaluation.

Therapeutic options

Tubal flushing

Usually all diagnostic tools, invasive or not, comprise a patency test. In case of HSG, this test is performed through a medium contrast, usually a water-soluble contrast medium and sometimes oil soluble contrast medium (OSCM), whereas in case of endoscopy the dye test usually uses methylene blue or indocarmine. Therefore a tubal flushing is made and may flush out tubal debris and dislodge mucus plugs.

This can be attended through any examinations involved in assessing the tubal patency by injecting contrast material or dye. About 10% of subfertile patients successfully get pregnant spontaneously following "tubal flushing" procedure whatever the method used. This figure even can be raised to 20% during HSG [11], when the procedure is performed using an OSCM, which in addition enhances fertility, perhaps through antiinflammatory effects on the otherwise damaged tube. Therefore, when it is possible, using them as a first line of treatment is highly recommended. Studies are underway to see if OSCM can also be used during USG).

Tubal surgery

General principles

Currently, tubal surgery is performed by laparoscopy. Its results are equivalent to those obtained by conventional microsurgery by laparotomy provided that the principles of microsurgery as described in the 1980s by

Winston and Gomel are respected [12,13]. These principles are represented by the use of magnification, precise but sparing hemostasis, irrigation of the operating field to avoid desiccation, which is a source of adhesions, and use of microsutures. All this is perfectly achieved by laparoscopy, except for proximal surgery, where conventional microsurgery gives generally better results (see below).

Surgery of pelvic adhesions

Tubal lesions are very often associated with pelvic adhesions, and their treatment is always the first step of all tubal surgery. Peritoneal adhesions occur as a result of surgical tissue trauma and healing, infection, radiation, ischemia, and foreign body reactions. One of the most important consequences of adhesions is infertility, leading to distorted adnexal anatomy, and they may affect the ovum pick-up by the fimbria.

There is good evidence to suggest that adhesiolysis improves fertility. Among infertile women with adhesions, the pregnancy rates are higher in those who are treated versus those who are not. In following women over a period of time, after tubal surgery, pregnancy rates are inversely correlated with adhesion scores assigned according to the ASRM classification system for adhesions.

Prevention of adhesions as a result of surgery in the first instance is important. It has been shown that postsurgical adhesions increase with number of previous laparotomies and complexity of the surgery. It is controversial as to whether laparoscopy reduces adhesion formation when compared with laparotomy. A review of nine trials suggests a comparable or reduced adhesion formation in women who undergo laparoscopic procedures [14]. However, one large epidemiological study of 24,046 women suggests that laparoscopy is only less adhesiogenic in the simple procedures (e.g., tubal sterilization) [15]. The ASRM Practice Committee has stated that despite the belief that laparotomy results in higher rates of adhesion formation, laparoscopy itself does not result in fewer adhesions; it is the extent of tissue injury, not the surgical approach that is the determining factor. Some of the aspects of laparoscopy that lend it to less tissue trauma include smaller anterior abdominal wall incisions, less tissue handling, no contamination from fibers from surgical packs, less tissue desiccation, and less postoperative infection [16].

Pharmacological agents have been suggested as adjuvant therapy to prevent adhesion formation in laparoscopic surgery. Antiinflammatory agents (corticosteroids), progesterone, preoperative and postoperative gonadotropin-releasing hormone agonists, fibrinolytic agents, heparin, and antibiotics have all been examined, in either animal or clinical models or both. Studies are limited, and the consensus is that further research is required before any can be recommended in the clinical setting [17].

The most popular antiadhesion practice at present is the use of barrier adjuvants. Theoretically, inert physical materials, which are able to prevent mechanical contact between serosal surfaces for longer than 3 days, have the potential to be helpful in adhesion prevention. Barrier adjuvants developed include solid barriers such as omental grafts, oxidized regenerated cellulose (ORC) and nonabsorbable barriers, or intraabdominal instillates, such as glucose polymers, hydrogels, and fibrin sealants. A Cochrane review [18] was performed to assess the effect of the commercially available solid barriers in gynecological surgery on reformation of adhesions, pregnancy rates, and pelvic pain. The review found that the ORC barrier (Interceed, Johnson and Johnson, Cincinnati, OH, USA) did reduce adhesion formation and that another inert barrier (Gore-Tex, W. L. Gore and Associates, Elagstaff, AZ, USA) was superior to ORC, but was not absorbable so required a further operation to be removed. An absorbable adhesion barrier comprising sodium hyaluronate and carboxymethylcellulose (Seprafilm, Genzyme, Cambridge, MA, USA) has been examined in nongynecological abdominal surgery and has been shown to be effective in reducing the incidence, extent, and severity of postoperative adhesions. However, its use in laparoscopic surgery is noted to be very difficult. Despite the reduction in adhesions, there were limited data to support the use of solid barriers to improve pregnancy rates.

Isotonic solutions remain in the abdomen for only a few hours, whereas icodextrin (Adept, ML Laboratories, Leicester, UK) is a glucose polymer in an electrolyte solution that has been developed as an intraperitoneal instillate, which remains for several days. A randomized controlled pilot study of its safety and effectiveness observed reduced adhesion [19] formation, although a Cochrane review concluded insufficient evidence for its use in adhesion prevention [20]. There is no evidence that it improves fertility or pregnancy rates. Because of its ease of use, Adept is widely used as an adjuvant to good surgical technique in laparoscopic gynecological surgery.

There is no single modality that reduces adhesion formation and improves pregnancy and fertility rates. The main factor is probably a nontraumatic approach that is provided by using the microsurgical principles mentioned above.

Proximal pathology

As we have shown, the lesions are either functional (spasm, mucosal plug) or organic (PID,). In the former it is useful to try a selective catheterization of the tubes, which can be carried out in a radiology department.

In recent studies, between 52% and 47% of patients had patency after tubal cannulation [21,22]. If the result was revealed to be negative with still no tubal spillage, organic obstruction can be concluded. In this instance, the patient can be sent directly to IVF or given an option for tubal-interstitial anastomosis by laparotomy microsurgical. Despite the pregnancy rate obtained by microsurgery being 45%−60%, it still is seldomly practiced. Invasiveness of the procedure and lack of expert, well-trained surgeons are the main factors behind that. In practice anastomosis is only proposed if for any reason IVF is not possible or accepted by the patient. However, the use of the robotic surgery could be an alternative, where the cost effectiveness is a major concern, so a majority of practitioners refer patients directly to IVF.

In case of juxtauterine blockage of the tube, the only operation possible is the reimplantation of the tube proposed by Ehrler in the 1970s [23], but the results are poor with 80% reobturation, so this operation should be abandoned.

However, proximal tubal surgery is still a valid option in case of tubal recanalization after sterilization procedure. This indication is quite frequent in countries where sterilization is performed on patient request regardless of a patient's age. In this instance, it has been shown that surgery should always be proposed as a first line of treatment, which can be accomplished by excising the sterilization site followed by turbo-tubal anastomosis. All obstructive lesions in the proximal segment of the tube should be resected. The larger the resection is, the lower is the postoperative pregnancy rate.

Therefore, when performing a tubal sterilization, it is important to consider the patient's possible regret and to destroy the smallest portion of the tube. In practice the use of Filsie or Hulka clips is certainly the method that destroys the smallest portion of tube. Other sterilization techniques such as Yoon ring placement or bipolar coagulation are more extensive, and the desterilization procedure is less successful. The tubal anastomosis will be either isthmo-isthmic, isthmo-interstitial, or isthmo-ampullary depending on the location.

The anastomosis should be done in a microsurgical fashion in two layers: the first layer is muscular, avoiding if possible involving the mucosa. Usually four stitches with 7×0 or 8×0 monofilament placed at 6, 9, 12, and 3 o'clock (in this order) are performed after approximation of the meso using a 6×0 suture (Fig. 8.5). Then a second serous layer is done with the same thread to have a perfect approximation. Therefore the other techniques proposed such as the "one-stitch technique" should be abandoned due to the poor results obtained. This can be performed by laparoscopic, robotic, or conventional microsurgical laparotomy.

FIGURE 8.5 Tubo-tubal anastomosis (the two layers).

The outcome end results from such a procedure are variable depending on the technique used as well as the patient's age [24]. The best results are obtained by using robotic surgery or conventional microsurgery with a rate of tubal permeability reported up to 90% compared with laparoscopy surgery approach where the pregnancy rate is closer to 70%. This mandates that patients be thoroughly informed prior to the surgery and different options be stated clearly.

Distal pathology

Distal pathology accounts for almost 90% of obstructive tubal disease, and diagnosis of each subtle tubal lesion is crucial as each condition has a different approach in the management of, for instance, phimosis and hydrosalpinx. In the former, **fimbrioplasty** is indicated. This can be accomplished by enlarging the tubal ostium using gentle divergent tractions at the level of the fimbria. In the latter, **salpingoneostomy** is required by creating a new tubal ostium.

The prognosis factors for conservative treatment of hydrosalpinx are thin tubal wall and healthy tubal mucosa. The quality of tubal mucosa is critical as demonstrated by several works [25,26]. Even if the tubal scoring system is rarely used in routine (Fig. 8.6), it is important to evaluate the tubal mucosa: if the tubal folds are absent or, worst, in case of intratubal ampullary, the nonconservative option (i.e., salpingectomy) should be preferred.

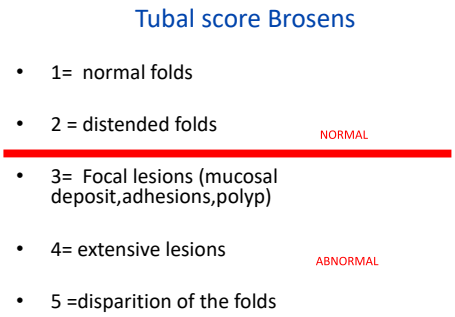

FIGURE 8.6 Tubal mucosa evaluation.

Depend on the tubal wall condition, a cuff-neostomy can be performed. If the wall is thin and non sclerotic (Fig. 8.7), a racket form neostomy must be performed (Fig. 8.8) by navigating to find the old ostium then making radial incisions to recreate tubal flaps that will be everted subsequently. The eversion must be maintained by microsutures (in practice the use of 5×0 or 6×0 monofilament sutures is recommended).

On the other hand, maintaining the eversion with CO_2 laser or bipolar coagulation should be avoided. This technique, known as the "flower effect," although being spotless, exposes a ring of sclerosis at the base of the fimbria.

The prognosis of these different techniques is very different. Fimbrioplasty is performed on tubes of fairly good quality and allows a pregnancy rate of about 60% to be obtained [27]. While in salpingoneostomy, if cuff salpingoneostomy is possible, the pregnancy rate in our retrospective studies is 52%, and in racket form neostomy the pregnancy rate is only 21% (Fig. 8.9). It is therefore very clear that cuff salpingoneostomy should be preferred, and if only racket neostomy is possible, it is probably preferable to refer the patient directly to IVF after having performed a salpingectomy [28].

If a salpingectomy is decided, it is highly recommended to be done as close as possible to the tube to preserve the ovarian vascularization by avoiding disturbing shared vascularization that might lead the ovarian reserve to be diminished or worse to ovarian failure.

In case of hydrosalpinx, the decision to use conservative (salpingoneostomy) or radical (salpingectomy) treatment must be shared with the patient. The pros and cons of each method must be clearly addressed to the patient. The risk of failure (this time leading to the obligation to perform a salpingectomy during a second laparoscopy) and the risk of an extrauterine pregnancy in the case of conservative must be highlighted in particular.

Subtle tubal lesions

Minimal tubal lesions can be considered a new chapter of tubal pathology, as their treatment is recent.

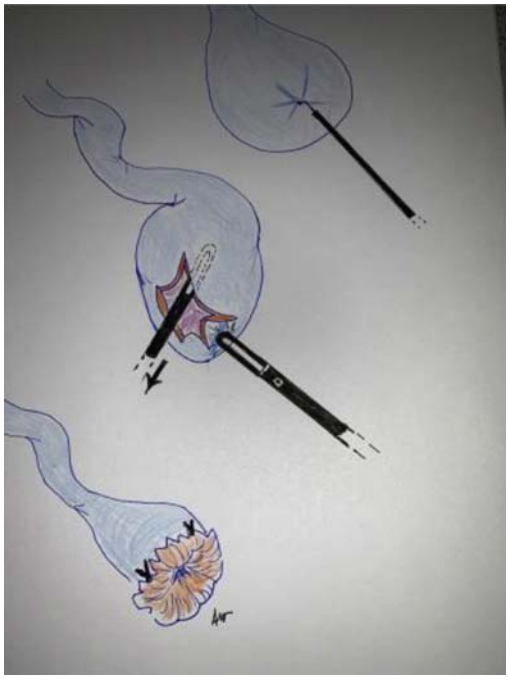

FIGURE 8.7 Cuff salpingoneostomy

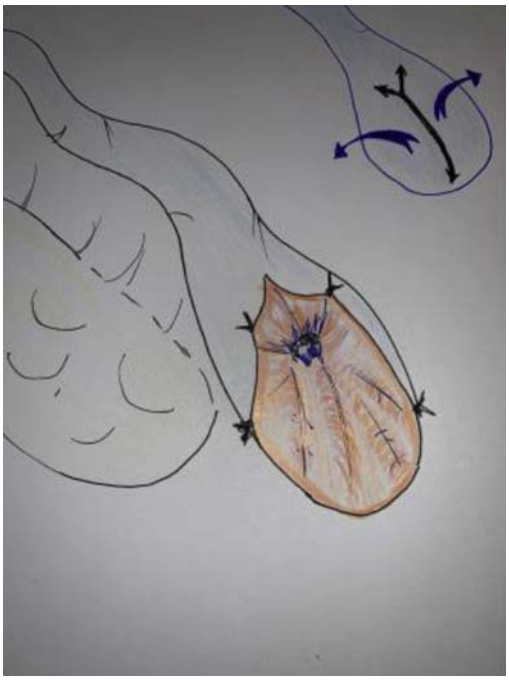

FIGURE 8.8 Racket form neosalpingostomy

Indeed, until recently, these lesions were considered to have no impact on fertility and consequently were neglected.

A growing number of studies [3,29] show that treatment of minimal anomalies is followed by a pregnancy rate of around 50% in patients with a long

	N	Pregnant	Miscarriage	Ectopic
Racket salpingoneostomy	113	28(24.8)	14(12.3)	9(7.9)
Cuff salpingoneostomy	367	210(57.2)	38(10.3)	10(2.7)
Total	480	238(49.5)	52(10.8)	16(3.3)

FIGURE 8.9 Results of salpingoneoastomy according to the technique (personal series of 480 cases 1998–2018).

history of unexplained subfertility. The anomalies are multiple, and they usually affect the distal part of the tubes.

For instance, **paratubal cysts** (or **hydatid of Morgagni**) removal significantly increases the pregnancy rate [5]. In the case of bridge adhesions, their section, followed by suture of the resulting eversion, is necessary. The **accessory tubes** if any need to be removed. There remains the problem of sacculations where the muscular tunic of the tubal ampulla has disappeared. Their treatment depends on the size and location of the sacculation. When it is distal and may be extensive, an incision should be made on the antimesial edge of the tube, and the tubal mucosa should be poured and fixed in the same way as for neosalpingostomies. If the sacculation is medio-ampullary, the hernia must be reduced by suturing the edges of the tube, which has the effect of "erasing" the muscular dehiscence.

Conclusion

The advent of assisted reproduction techniques has profoundly changed the indications for tubal surgery. The indications have of course decreased and have been reevaluated according to the results obtained by IVF techniques. Nevertheless, at the same time, tubal surgery has made great progress, becoming almost exclusively a laparoscopic technique, often ambulatory. In this respect, it is still indicated because of its cost effectiveness compared to IVF. In addition, when a successful result is obtained, it allows several pregnancies, which is a crucial advantage. The two techniques should therefore not be opposed but considered complementary. The most important issue is to assess the tuboperitoneal state accurately and precisely through endoscopy. In conclusion: desterilization, selective catheterization, fimbrioplasty and neosalpingoneostomy remain the intervention of choice providing relatively healthy tube. They should continue to be taught in medical schools.

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CHAPTER

9

Cervical and uterine congenital anomalies

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Introduction

Cervical and uterine anomalies are a very important clinical entity for women, since they can affect not only their reproductive capacity, but also their quality of life. Although it is true that their diagnosis is usually made after a study or evaluation for infertility, repeated abortions, or obstetric complications, there are a series of symptoms and consequences at a nonreproductive level that can seriously affect the quality of life of women, such as pelvic pain and prolonged or abnormal bleeding. In the same way, the impact on fertility generates a direct impact on the life of the woman, both physical and emotional, due not only to the inability, in some cases, to carry a pregnancy, but also to the association of these anomalies with infertility, recurrent abortion, prematurity, and fetal malposition.

Embryology: the origin of anomalies

Cervical and uterine malformations originate as a consequence of a failure in embryogenesis, during the process of formation of the female reproductive organs. During embryonic development, the Müllerian ducts will give rise to the cervix, the uterus, the fallopian tubes, and the upper part of the vagina through a dynamic process that can be divided into five phases, which follow each other:

Differentiation Phase: It occurs around the 6th week of gestation and consists of the appearance of the Müllerian ducts as two longitudinal invaginations in the coelomic epithelium, in the external part of the urogenital crest.

Phases of Migration and Fusion: They occur around the 9th week of gestation and consist of the caudal growth of the Müllerian ducts until they reach the mesonephric ducts laterally and cross them anteriorly to join both in the midline and form the uterine primordium. At the same time, the primordium contacts the invagination of the urogenital sinus and fuses.

Channeling and Reabsorption Phases: They occur around the 10th week of gestation and consist of the already fused Müllerian ducts being channeled inside, giving rise to two channels divided by a septum that is later reabsorbed in a caudo-cranial direction, finishing this process around the 20th week of gestation and finally giving rise to the formation of the uterus, cervix, fallopian tubes, and upper third of the vagina.

Thus, the cranial portions of the Müllerian ducts give rise to the fallopian tubes. The caudal portions of the Müllerian ducts, after completion of the fusion and canalization phases, give rise to the endometrium, the innermost layer of myometrial muscle, the cervix, and the upper third of the vagina. It is important to note that the middle and outer layers of the myometrium are of mesenchymal origin, as well as the supporting ligaments of the uterus.

Pathogenesis

Understanding the process of formation of the uterus as a dynamic process helps us to understand that any failure that occurs in this process of embryogenesis will give rise to an anatomic alteration of the uterus and/or cervix. The type of anomaly that will result will be defined by the exact moment in which the embryonic development of the internal genitalia is altered. There are three main mechanisms of abnormal uterine development:

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Failures in the differentiation process: They will give rise to uterine agenesis or agenesis of one of the horns.
Failures in the migration and fusion process: They will give rise to didelphys and bicornuate uteri.
Failures in the channeling process: They will give rise to nonfunctioning rudimentary uteruses.
Failures in the reabsorption process: They will give rise to uterine or uterocervical septa, or arcuate uterus.

In-utero exposure to diethylstilbestrol (DES), a synthetic estrogen that was used between 1949 and 1971 to prevent spontaneous abortion, premature labor, and other obstetric conditions, has also been associated with the development of cervical and uterine abnormalities. The mechanism of action, in addition to its action as an endocrine disruptor, has been established through the blockade of the epithelium of the Müllerian duct [1]. All this has placed DES as the only scientifically confirmed transplacental carcinogen in humans responsible for the development of uterine, cervical, and vaginal abnormalities in female fetuses exposed in utero.

Etiology

Although it remains unknown to this day, most authors postulate a multifactorial origin, since although the most frequent cases are sporadic in appearance, a risk of familial recurrence has been estimated between 1% and 5%. To date, certain genes have been identified associated to uterine malformations, although generally as part of a syndrome and not as an isolated malformation, among these are HNF1B, WNT4, WNT7A, and HOXA13 [2]. The karyotype of the patients is normal in most of cases (46 XX).

Prevalence

Currently, the true prevalence of uterine malformations in the general population cannot be accurately estimated. The two main reasons are as follows: the underdiagnosis that occurs of asymptomatic anomalies for which the woman does not consult (since these cases are not diagnosed), and the lack of a uniform criterion or universal classification that allows comparison with accuracy of data from different researchers. Perhaps the most accepted reference at the present time is the one published by Chang et al., which includes 94 observational studies, with a total of 89,861 participants determining that the prevalence of uterine malformations was 5.5% in the general population, 8.0% in women with infertility, 12.3% in women with history of spontaneous abortion, and up to 24.5% in women with a history of spontaneous abortion and infertility [3].

Within the different uterine anomalies, attempts have also been made to estimate their prevalence. Although multiple authors have reached varying conclusions, all of them agreed that the uterine septum is the most common uterine anomaly, and Mayer-Rokitansky-Küster-Hauser syndrome is the least common. Among important studies that report on the prevalence of different anomalies, we found Grimbizis [4], who reported that the most prevalent anomaly was septate uterus (34.9%), followed by bicornuate uterus (26%) and arcuate uterus (18.3%); Simón [5], who reported a prevalence of the uterine septum of 90% within uterine anomalies; and Raga [6], who reported that 65% of uterine malformations were septate or arcuate uteri, thus establishing that most of the uterine malformations can be treated hysteroscopically.

Other associated anomalies

Patients with congenital uterine anomalies have a higher risk than other patients of having associated anomalies: renal, skeletal, or abdominal wall. Among them, renal anomalies are the most frequent, since they can be found in 20%–30% of patients with Müllerian defects [7,8]. The double collecting system, the horseshoe kidney, the pelvic kidney, and unilateral renal agenesis stand out, taking into account that when there is a renal anomaly, it is generally ipsilateral to the associated uterine malformation. Due to this high prevalence of associated renal anomalies, it is recommended to always include the study of the renal system when making a diagnosis of congenital uterine anomaly.

Clinical presentation

As we have explained, a large number of women with uterine malformations are asymptomatic and stay undiagnosed, so it is impossible to estimate the prevalence of congenital uterine anomalies. However, symptomatic women may present with pelvic pain, abnormal vaginal bleeding, and infertility, among others, and these symptoms will depend on the type of abnormality present.

Classification

Although there are several classifications developed with the aim of organizing the different types of uterine anomalies, to date there is still not an accepted "universal" classification that allows all to codify the symptoms, treatments, and outcomes in the same way, especially for research and comparison of outcomes.

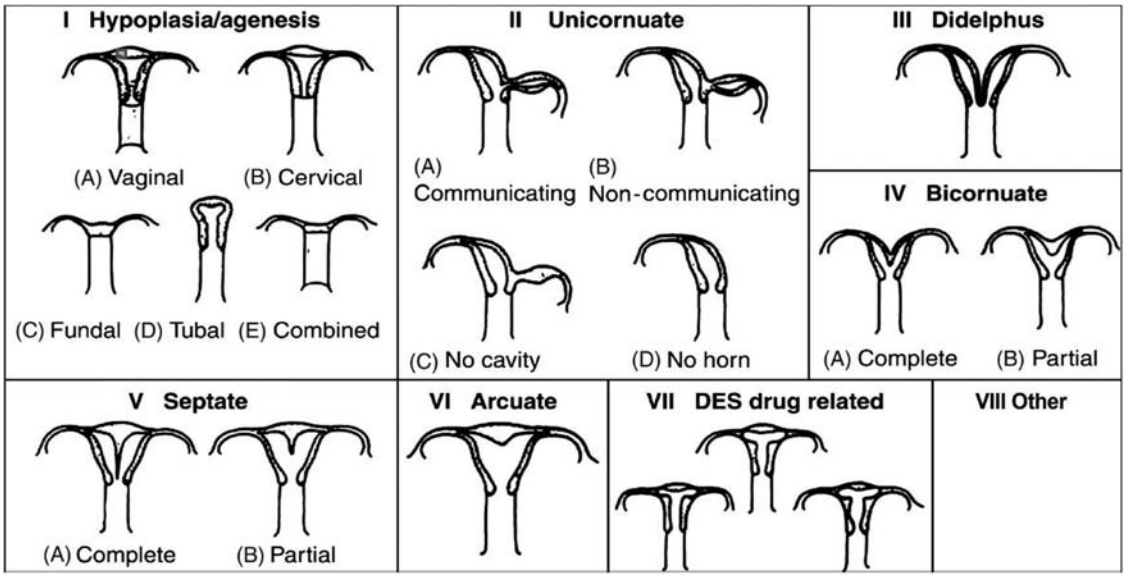


FIGURE 9.1 AFS classification of Müllerian anomalies, 1988. *Adapted from the original by Buttram and Gibbons.*

As for the classifications developed to date, we can highlight that of Buttram and Gibbons from 1979, which was later revised and adopted by the American Fertility Society (AFS), now the American Society for Reproductive Medicine (ASMR) in 1988 [9]. This is a widely used classification that classifies uterine malformations based on hysterosalpingography and divides uterine malformations into seven categories. Although it is an easy classification to use, it has some disadvantages, such as the absence of classification of vaginal and cervical anomalies. It also makes it impossible to classify uteri with multiple anomalies (Fig. 9.1). This classification has recently been revised and updated by the ASMR in 2021 [10], 33 years later, which uses the descriptive terminology instead of the previous numerical system. It includes three additional groups: longitudinal vaginal septum, transverse vaginal septum, and complex anomalies, thus establishing nine categories (Fig. 9.2). As a comment to this new classification, we can point out that it does not contemplate the dysmorphic uterus, which is increasingly important in patients with infertility.

Another widely used classification is that of the European Society for Gynecological Endoscopy (ESGE) together with the European Society for Reproduction (ESHRE). Both, jointly, published in 2013 a new classification system based on anatomy, which allows cervical and uterine anomalies to be subclassified in different sections [11]. This classification divides uterine malformations into six types and leaves the arcuate uterus out of the classification, which is included in the AFS and ASMR classifications. However, it does include the dysmorphic uterus or U1, which includes the T-shaped uterus and the hypoplastic uterus (Fig. 9.3).

Although there are proposals for classifications of other groups [12,13], or subclassifications of some of the existing ones, such as the subclassification of dysmorphic uterus [14], the AFS/ASMR and ESGE/ESHRE classifications are those internationally accepted.

Diagnosis

For a correct diagnosis of a uterine malformations, it is essential to know both the external contour of the uterus and the interior of the uterine cavity. That is why the diagnostic procedures that allow a correct identification of these two contours have greater diagnostic accuracy.

The classic tools in the diagnosis of uterine malformations are two-dimensional ultrasound, hysterosalpingography, hysteroscopy together with laparoscopy, 3D ultrasound, and magnetic resonance imaging (MRI).

Classically, it has been considered that the combination of hysteroscopy-laparoscopy is the method of choice for the diagnosis and classification of uterine malformations, since hysteroscopy offers a perfect view of the cavity and makes up for its main deficiency, which is the impossibility of examining the outer contour of the uterus when performed together with laparoscopy. The problem with this diagnostic method is that it is invasive, and it must be performed in the operating room with the patient under general anesthesia. Currently, 2D ultrasound and hysterosalpingography (HSG) are good screening methods, while the use of both 3D ultrasound and MRI can achieve a diagnostic accuracy similar to that of hysteroscopy-laparoscopy.

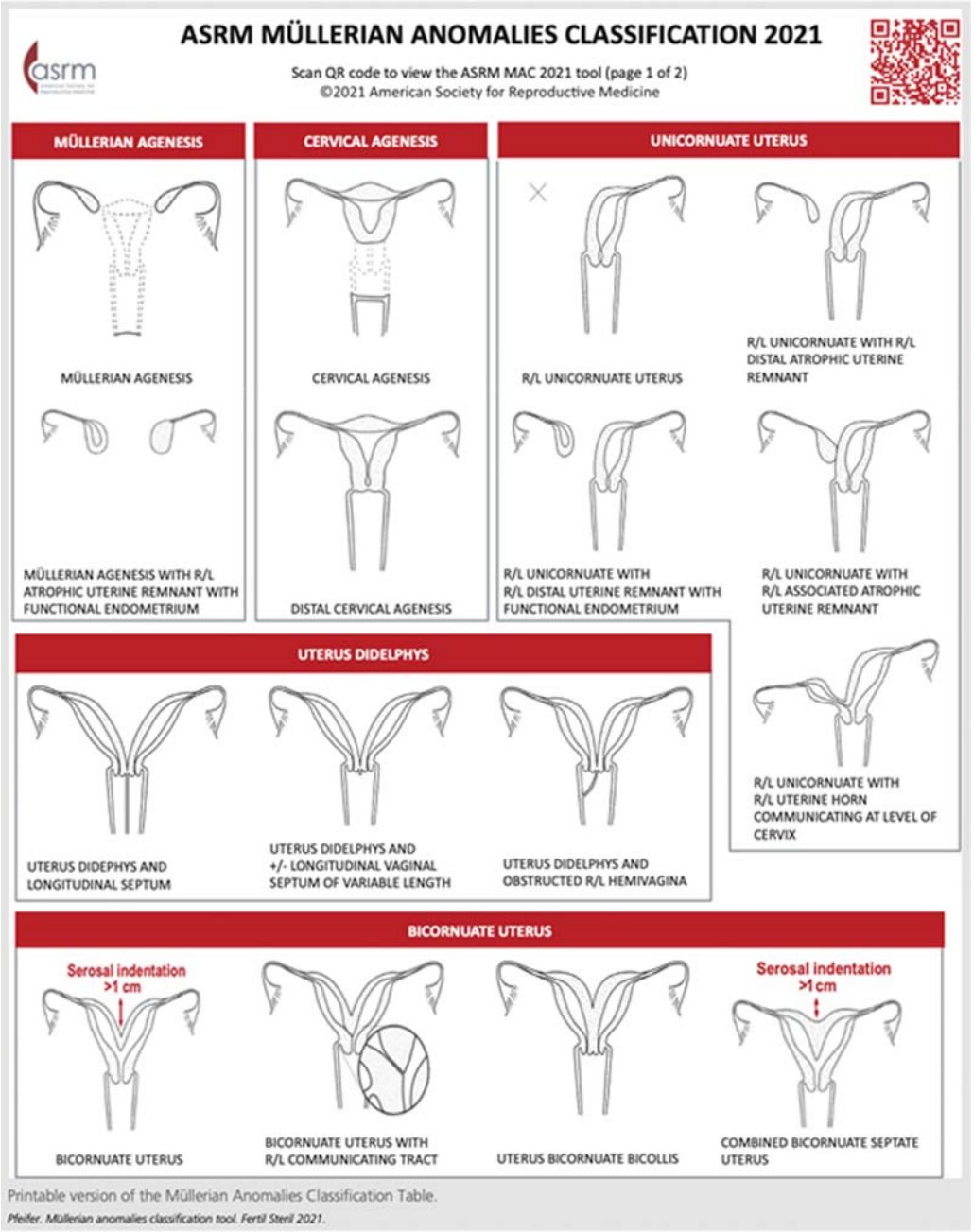
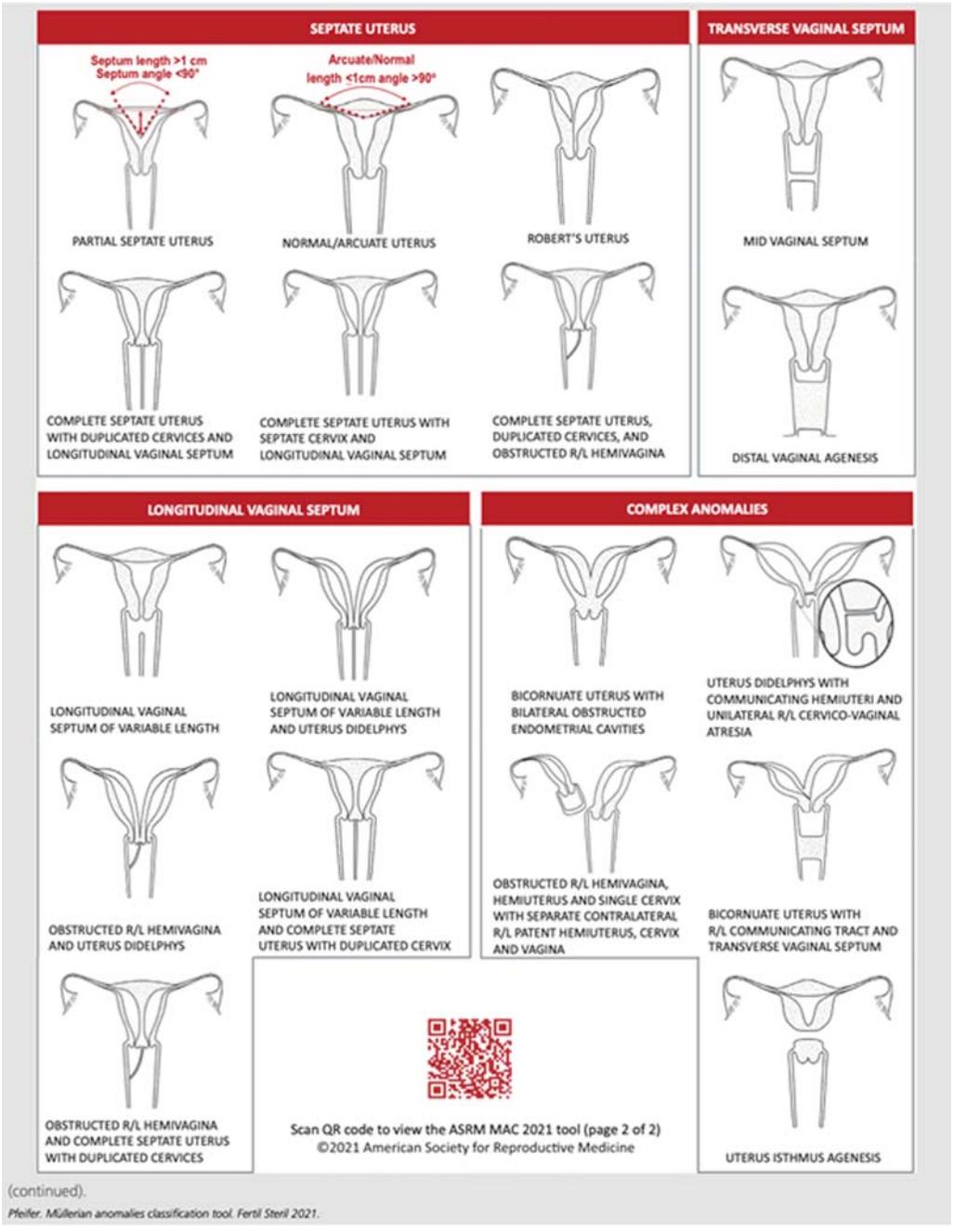


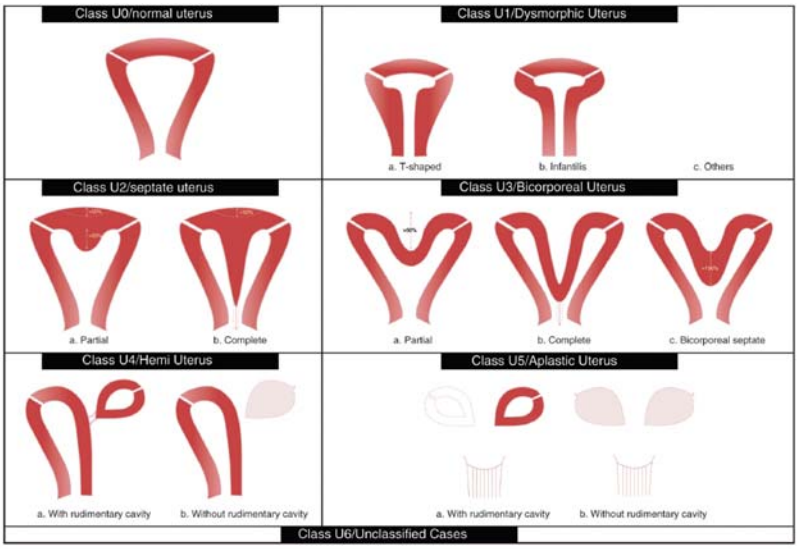
FIGURE 9.2 ASRM classification of Müllerian anomalies, 2021.



(continued).

Pfeifer. Müllerian anomalies classification tool. Fertil Steril 2021.

FIGURE 9.2 Continued.



	Uterine anomaly		Cervical/vaginal anomaly	
	Main class	***Sub-class***	***Co-existent class***	
U0	Normal uterus		***C0***	*Normal cervix*
U1	Dysmorphic uterus	**a.** T-shaped **b.** Infantilis **c.** Others	***C1***	*Septate cervix*
U2	Septate uterus	**a.** Partial **b.** Complete	***C2***	*Double 'normal' cervix*
U3	Bicorporeal uterus	**a.** Partial **b.** Complete **c.** Bicorporeal septate	***C3***	*Unilateral cervical aplasia*
U4	Hemi-uterus	**a.** With rudimentary cavity (communicating or not horn) **b.** Without rudimentary cavity (horn without cavity/no horn)	***C4***	*Cervical aplasia*
U5	Aplastic	**a.** With rudimentary cavity (bi- or unilateral horn) **b.** Without rudimentary cavity (bi- or unilateral uterine remnants/aplasia)	***V0***	*Normal vagina*
			V1	*Longitudinal non-obstructing vaginal septum*
			V2	*Longitudinal obstructing vaginal septum*
			V3	*Transverse vaginal septum and/or imperforate hymen*
			V4	*Vaginal aplasia*
U6	Unclassified malformations			
U			***C***	***V***

Associated anomalies of non-Müllerian origin:

Drawing of the anomaly

FIGURE 9.3 ESGE/ESHRE classification of Müllerian anomalies, 2013.

2D ultrasound

It is the simplest and cheapest test, in addition to having the advantage that it is widely available. Its main advantage is that it is a noninvasive and inexpensive technique, and most gynecologist are familiar with it. It allows the visualization of the external and internal uterine contour, being recommended to be carried out during the secretory phase of the menstrual cycle to obtain a correct visualization of the internal uterine contour, since in this period there is a better visualization of the endometrium. The visualization of two cavities in the cross-section, at the level of the uterine fundus, is

indicative of the presence of a uterine malformation [15]. Its great limitation is the impossibility of obtaining the coronal plane (frontal plane), which is the most useful when diagnosing most malformations.

The instillation of liquid in the cavity, also known as hysterosonography, allows a better visualization of the contour of the cavity, thus increasing the diagnostic accuracy.

Studies comparing the diagnostic accuracy of 2D ultrasound with hysteroscopy show that ultrasound has a sensitivity of less than 60%, while its specificity is close to 100%. This suggests that although 2D ultrasound can only diagnose slightly more than half of uterine malformations, its diagnosis capacity is very accurate. Sonohysterography has high precision both when diagnosing uterine malformations and in classifying them.

Hysterosalpingography

It is useful as a screening method, and it has been widely used until the appearance of ultrasound since it allows visualization of the contour of the uterine cavity and can be useful to assess the size and characteristics of a uterine septum; however, it has the drawback of not providing information on the external uterine contour, so hysterosalpingography offers little precision in differentiating between a septate uterus and a bicornuate uterus. It has been suggested that the existence of an angle less than 75° between the uterine horns is suggestive of a septum, while the existence of an angle greater than 105° indicates a bicornuate uterus [16]. In addition, we must not forget that it is a more invasive test than 2D ultrasound, it is uncomfortable or painful for the patient, and it cannot differentiate the different malformations (Fig. 9.4).

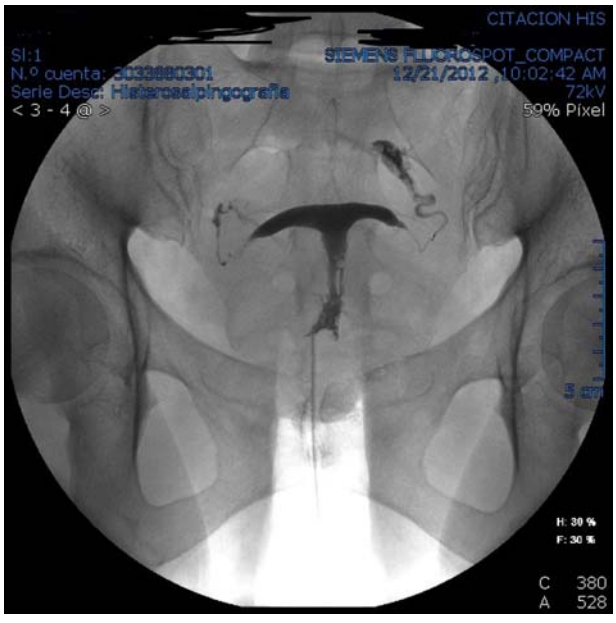


FIGURE 9.4 T-shaped uterus by hysterosalpingography.

Hysteroscopy

It is the only diagnostic procedure that allows direct visualization of the vagina, the cervix, the cervical canal, and the uterine cavity, and for this reason it is the gold standard in the diagnosis of intracavitary pathology. It is therefore a very precise technique in the diagnosis of uterine malformations, the only disadvantage being the impossibility of evaluating the external uterine contour, which limits its diagnostic precision in certain malformations.

Hysteroscopy offers the possibility of a diagnostic approach performed in office. In addition, it reduces the discomfort and infectious risk classically associated with hysterosalpingography and adds the option to study the physiology and microbiota of the endometrium, allowing to obtain biopsy in patients who require it, thus improving therapeutic planning and reducing surgical times.

It is noteworthy that the combination of hysteroscopy together with laparoscopy has been considered the gold standard for the diagnosis of uterine malformations, also offering the possibility of concurrent treatment of the pathology found during the examination.

3D ultrasound

It allows the visualization of the uterus in the three planes of space and offers the possibility of obtaining uterine volumes to be able to study them in more detail once the exploration with the patient has been completed.

The possibility of obtaining a coronal plane (frontal plane) is extremely important when defining the type of uterine malformation since it shows in a single image the uterine cavity, the myometrium, and the external contour of the uterus, and it is, without a doubt, the great advantage of this novel technique when compared to two-dimensional ultrasound (Fig. 9.5). Currently, not all ultrasound machines have this technology, although it is present in most of the newer machines, so it will be a matter of time before its implementation is widespread in all ultrasound machines. It will also be a small learning curve for those gynecologists who are not used to it, but once it is performed, it is a very easy, reproducible, and fast test to perform.

Various published studies agree in defining 3D ultrasound as a technique with very high precision in the diagnosis of uterine malformations. Sensitivity and specificity have been established as high as 91.6% in the study of the outer uterine contour and 100% in that of the uterine cavity [17].

Magnetic resonance

It is a noninvasive test that accurately defines both the contour of the cavity and the outer contour of the uterus.

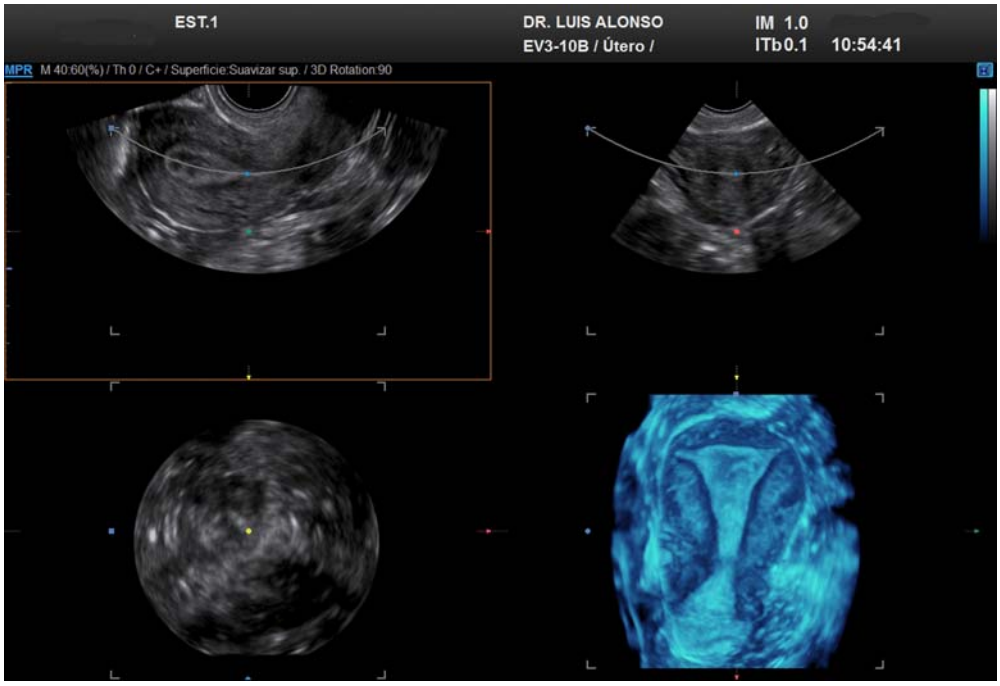


FIGURE 9.5 Three-dimensional ultrasound for uterine evaluation.

Some authors have given it high precision and have even suggested that it could replace the combination of laparoscopy-hysteroscopy for the definitive diagnosis of uterine malformations. Its great limitation, and therefore the great difference with respect to the aforementioned 3D ultrasound, lies in its poor accessibility for most gynecologists, as well as its higher cost.

Uterine abnormalities

Septate uterus

It occurs as a result of a failure of the reabsorption process of the medial septum that forms after the fusion and canalization of the Müllerian ducts. The degree of septation or size of the septum will depend on the reabsorption failure of this midline septum between the Müllerian ducts, so the anomaly may vary from the persistence of a small septum in the uterine fundus, to a complete separation of the uterus into two cavities and may also have a double cervix and vaginal septum.

Classically it has been divided into complete and partial or subseptum. In the complete septum form, the cavity is completely divided by a septum that runs from the uterine fundus to the internal cervical os, while in the subseptum or partial septate uterus, the septum does not reach the internal cervical os. The uterine septum can present differences in terms of its length, its width, and its internal structure.

Diagnosis

To establish the correct diagnosis, we must differentiate between septate and bicornuate uterus, and within the uterine septum, differentiate whether it is a complete septum, subseptum, or arcuate uterus. With hysterosalpingography, it is possible to differentiate the presence of two symmetrical cavities of somewhat smaller size than normal, and with hysteroscopy, it is possible to document the presence of two hemicavities separated by a septum (Fig. 9.6). However, the external uterine contour can only be assessed with 3D ultrasound, MRI, or the combination of hysteroscopy-laparoscopy.

The correct determination of the outer uterine contour and the shape of the fundus is essential to be able to distinguish between a septate uterus and a bicornuate uterus, since this will determine which is the most appropriate surgical approach for its correction. The appearance of the uterine fundus can be convex, flat, or slightly indented (this indentation being less than 1 cm).

Clinical relevance

The presence of a uterine septum is associated with poor reproductive outcomes and a high incidence of obstetric complications, including recurrent miscarriages, intrauterine growth restriction, premature delivery, and fetal malpositions. Grimbizis [4] observed a recurrent abortion rate of 44.1%, premature delivery of 22.3%, and full term delivery of 32.9% with a combined live birth rate of 50%. Regarding the role of the septum

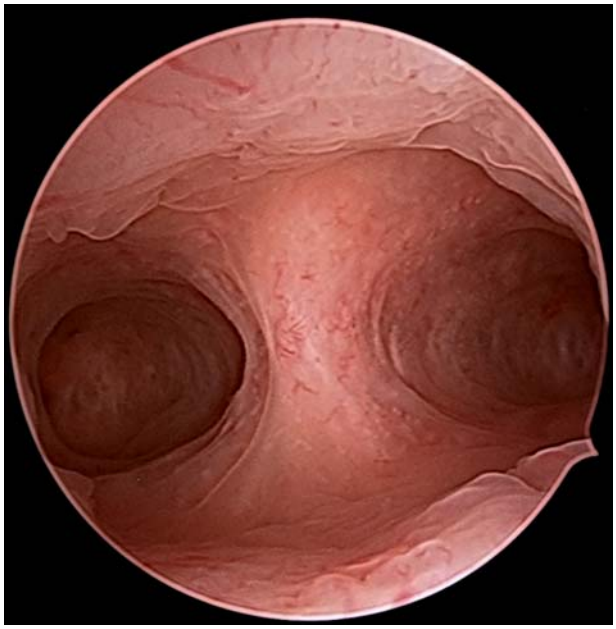


FIGURE 9.6 Hysteroscopic view of a uterine septum.

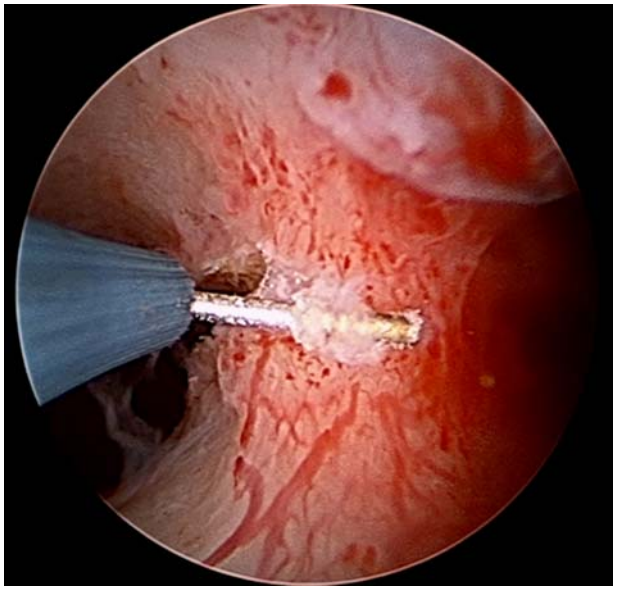


FIGURE 9.7 Septal incision with miniresectoscope.

in infertility, there is controversy since there are studies that report poor fertility outcomes in patients with septate uterus, while other studies fail to demonstrate this correlation.

Surgical treatment

What will indicate the need for treatment will be the clinical history of the patient, not the size or length of the septum. Surgical correction in case of a septate uterus is indicated in symptomatic cases, the main indication being the existence of a poor obstetric history.

The surgical approach to the septate uterus has evolved from the classic abdominal approach to current endoscopic techniques. The abdominal techniques of Jones and Tompkins were associated with acceptable obstetric results, but they were aggressive techniques, with a longer recovery period and the existence of a scar at the uterine level, which made it necessary to prolong the safety interval to look for a pregnancy after surgical correction. In 1974, Edstrom described for the first time hysteroscopy-guided section of a uterine septum, and this was the starting point of hysteroscopic metroplasty, a technique that has completely displaced correction surgery via the abdomen.

Hysteroscopic metroplasty is actually a section of the uterine septum rather than a resection of it. This incision should be made in the middle of the septum in the midpoint between the anterior and posterior walls. The visualization of the tubal ostia during the section guides the hysteroscopist to maintain the adequate plane, thus avoiding injury to healthy myometrium (Fig. 9.7).

There are two ways to perform hysteroscopic metroplasty: the thinning technique and the shortening technique. In the first technique, longitudinal incisions are made on each side of the septum, from its base to the apex. The objective is to decrease its width and transform the initial septum into a fundic remnant that can be easily sectioned from one cornual recess to the other. In the shortening technique, the septum is incised transversely, from the apex to the base. This incision in the center of the septum retracts the tissue toward the anterior and posterior walls.

The classic criterion for deciding when the metroplasty is completed, and no additional septum incision is needed, is obtaining a panoramic view of the cavity allowing to visualize both tubal ostium and when, in addition, the tip of the hysteroscope can move freely from ostium to ostium. After the study by Fedele [18], it is accepted that the existence of a residual septum of less than 1 cm after hysteroscopic metroplasty does not affect reproductive results.

The possibility of uterine perforation occurring during a metroplasty has a risk of 6.78%, which is similar to that of endometrial ablation or hysteroscopic myomectomy, and which of course will be closely related with the skill and experience of the surgeon.

Results after hysteroscopic metroplasty

Surgery to correct the septate uterus significantly decreases abortion and preterm birth rates [4], in addition to improving fertility in infertile women with this type of malformation. It is important to note that those women who have undergone hysteroscopic metroplasty to correct a septate uterus do not have a higher risk of

adverse situations during childbirth compared to the general population.

Various studies have evaluated the role of hysteroscopic metroplasty in improving perinatal outcomes. Most of the studies are observational studies, so the results are still questioned. Most of these retrospective studies conclude that resection of the uterine septum significantly decreases abortion and preterm birth rates and also improves fertility in women with a septum and infertility of unknown origin [19]. It has also been observed that it also has an impact on the pregnancy rate in those patients who undergo *in vitro* fertilization (IVF).

Of note is the systematic review and meta-analysis carried out by Valle [20] that included 2528 women (in 37 observational studies) in the systematic review and 2074 women (in 29 studies) included in the meta-analysis. All of these women had a septate uterus and a history of recurrent abortion, infertility, spontaneous abortion, or preterm birth. The 29 studies included in the meta-analysis were subsequently decided, eliminating in the second those with inconsistent results or with inconsistent follow-up data, thus a new meta-analysis was performed on the 19 studies with complete data. In this group of 19, an overall pregnancy rate of 63.5% (95% CI 56.6 to 69.9) was found as well as a live birth rate after metroplasty of 50.2% (95% CI 43.3 to 57.1). The author concludes that careful review of the published data supports this type of treatment in those cases in which the uterine septum adversely affects normal reproductive function.

A multicenter international cohort study has recently been published in a period between August 2018 and January 2000 and included 21 centers distributed between the Netherlands, the United Kingdom, and the United States. Data from 257 women were included, of whom 151 underwent septal resection and 106 constituted the control group [21]. The result of this work concluded that the resection of the uterine septum was not associated with an improvement in obstetric outcomes compared to expectant management in women with uterine septum, although there is a great debate due to the important methodological limitations and methodological errors present in the study.

Finally, a systematic review and meta-analysis recently published by Carrera et al. concludes that corrective surgery for septate uterus significantly decreases spontaneous abortion rates in both patients with complete and partial septate uterus [22].

Complete septa with double cervix

The first documented case of this malformation was published by McBean in 1994, and since that publication, the cases published in the literature do not exceed 300 (Fig. 9.8). It is caused by a complete failure in the reabsorption process of the medial septum, causing the septate uterus to be associated with a cervical duplicity and even a vaginal septum, which is called U2bC1V1 according to the ESGE/ESHRE classification, and as a septate intrauterine variant in the latest ASRM classification.

The existence of a double cervix can be associated with different uterine anomalies such as uterus didelphys, bicornuate uterus, and septate uterus. The existence of a complete septate uterus in cases of double cervix is probably the most frequent association, followed very closely by the didelphys uterus; much less frequent is the bicornuate uterus. And although a separation of both cervixes of more than 1.5 cm is more

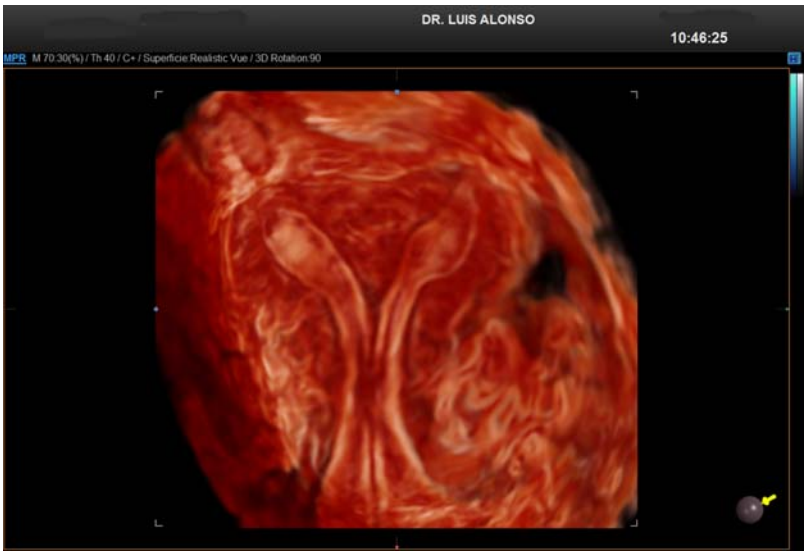


FIGURE 9.8 3D ultrasound showing a complete septum with cervical duplication.

frequent in cases of uterus didelphys, this is not a valid rule, and a more complete study must be carried out to determine exactly the type of associated malformation.

The largest series of patients with complete septate uterus, vaginal duplicity, and vaginal septum corresponds to Heinonen [23], who compared the reproductive results, clinical implications, and consequences of this variant of septate uterus in a descriptive study of 67 patients. In this study it was observed that this malformation was not related to primary infertility. Regarding obstetric results, the spontaneous abortion rate was 27%, the preterm birth rate was 12%, and the live birth rate was 72%. Only four of these women underwent metroplasty, three hysteroscopically and one using the Jones technique.

In the treatment of this anomaly, there are authors in favor of preserving the cervical septum and others in favor of performing a section of it.

The surgical technique with preservation of the cervical septum was described by Rock [24] in a series of 21 patients. The description of the technique is as follows: after cervical dilation, a Foley catheter or a dilator is inserted into one of the cavities that serve as a guide for sectioning the body part of the septum. Subsequently, the resectoscope was introduced with a Collins loop into the other cavity, and the intrauterine septum was incised at the supracervical level. Once the unification of the cavities had begun, the procedure was as in any other metroplasty. The classic arguments that have been considered to preserve the cervical septum are that it is a vascular structure whose section could result in massive intraoperative bleeding, and that the section of the cervical septum could cause cervical insufficiency, which would require performing a cerclage in case of pregnancy, as well as a special control during the course of it.

Probably the first reference that we can find regarding the section of the cervical septum is that of Vercellini [25] who performed the section of the cervical septum with Metzenbaun scissors in seven patients in whom they had great difficulties in creating the initial communication between the two cavities. Subsequently, they compared the results of these patients with another group of nine patients in whom this intracervical septum was preserved. They had no intraoperative or obstetric complications related to the section of the cervical septum. No cerclage was performed in any of the patients.

There are few randomized controlled trials comparing the results of complete septal uterus surgery with cervical duplicity. Parsanezhad [26] compared the results of 28 women with this malformation and who had a history of poor obstetric outcomes or infertility. The patients were assigned to two groups: in one the section of the intracervical portion of the septum was performed, while in the other group the cervical septum was preserved. Both surgical time and fluid deficit were greater in the group in which the cervical septum was preserved. In addition, two cases of pulmonary edema and three cases of massive bleeding occurred in these patients. However, there were no significant differences in obstetric outcomes. Four of the 15 patients in the group in which the septum was sectioned had uterine cerclage, while in the group in which the cervical septum was preserved, two of the 13 patients underwent cerclage.

In view of these results, the authors recommend sectioning the cervical septum in all cases of complete uterine septum since it makes the procedure safer, faster, and with similar obstetric results in both groups.

Unicornuate uterus

It occurs as a result of an alteration in the development process of only one of the Müllerian ducts, with the other duct developing normally. This unilateral developmental defect may be complete or partial.

Classically and according to the AFS classification, four different subtypes of unicornuate uterus have been distinguished, depending on how affected the development of the Müllerian duct is: a) with functioning and communicating rudimentary horn, b) with functioning and noncommunicating rudimentary horn, c) with nonfunctioning rudimentary horn, and d) without rudimentary horn (Fig. 9.1). However, the new ASRM classification includes a new subtype: with noncommunicating uterine horn and distal to the uterus (Fig. 9.2). The ESGE/ESHRE classification includes this anomaly in group U4, distinguishing only between the presence of a uterus with a rudimentary cavity U4a (communicating) and the absence of a rudimentary cavity (with or without a cavity) U4b (Fig. 9.3).

Diagnosis

It is usually due to a casual or accidental finding since the patient is normally asymptomatic. She will only present symptoms if in the anomaly she presents there is a noncommunicating rudimentary horn (with endometrial cavity), manifesting in this case secondary dysmenorrhea to hematometra produced by accumulation of menstrual flow, from menarche, within that rudimentary horn.

When visualizing a unicornuate uterus by hysteroscopy, especially in cases in which the woman has not had any pregnancy, a uterus of tubular morphology is observed in which only one of the tubal ostia is visualized with the presence of concentric muscular rings with little endometrial development (Fig. 9.9). Whenever the diagnosis of this uterine anomaly is made, the

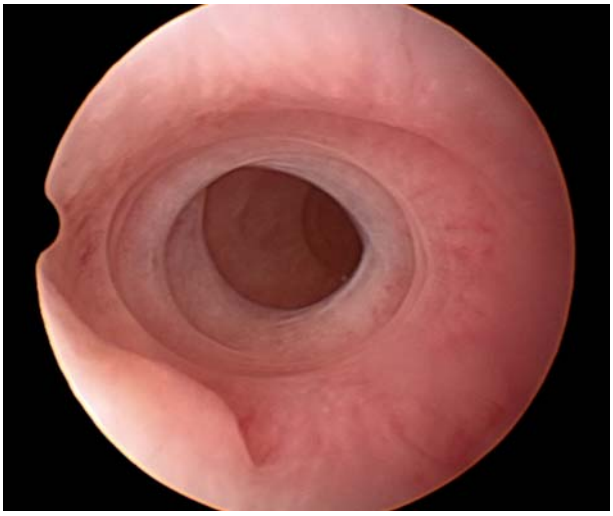


FIGURE 9.9 Hysteroscopic view of a unicornuate uterus.

presence of an associated rudimentary uterus must be investigated, as well as the existence of vaginal septa, since it could be present generating a bicorne-bicollis uterus. In the event of a rudimentary uterus, the possible communication existing at the isthmic level in the contralateral wall to that of the present tubal ostium should be sought.

Clinical importance

The presence of a unicornuate uterus is associated with poor reproductive outcomes such as first-trimester abortion (24.3%), preterm delivery (20.1%), second-trimester abortion (9.7%), or ectopic pregnancy (2.7%), in addition to presenting a higher risk of fetal breech presentation during pregnancy. The obstetric problems associated with unicornuate uterus are due to the difficulty of expansion of an abnormal uterine cavity, so it seems that the main fertility problem of patients with unicornuate uterus lies more in maintaining the pregnancy than in the fact of becoming pregnant. In addition, in patients with a unicornuate uterus and rudimentary horn that functions and communicates with an endometrial cavity (IIa of the AFS classification), there is the possibility of a pregnancy occurring at that level, with the risk of its rupture if the pregnancy progresses, which usually occurs in the second trimester in 80%–90% of cases, constituting a real emergency situation [27].

For all these reasons, patients with this anomaly must be strictly controlled due to the risk of premature rupture of the membranes, premature birth, and cervical incontinence, requiring periodic cervical length checks and even prophylactic uterine cerclage.

Added to the above is the additional risk of developing endometriosis in patients in whom the unicornuate uterus is associated with a remnant of a functional, but noncommunicating, rudimentary horn, due to the impossibility of vaginal evacuation of the endometrial tissue of the rudimentary horn [28].

Associated anomalies

The prevalence of renal anomalies associated with unicornuate uterus is high (40.5%), the most common being renal agenesis contralateral to unicornuate uterus, which occurs in 16% of cases, followed by the presence of an ectopic kidney or the existence of a pyelocaliceal duplication [29].

Ectopic or undescended ovary is found in 42% of cases of unicornuate uterus. This occurs as a consequence of the absence of descent of the gonad in the pelvis, which in normal situations occurs in the third month of gestation, at which time the ovary, from a position close to the kidney, reaches its final location in the pelvis. Undescended ovary is a difficult situation to detect, in which MRI has proven to be the best method for diagnosing both ovaries in abnormal positions and associated malformations.

Surgical repair

The hysteroscopic technique proposed for enlarging the cavity of unicornuate uteri is the "transcervical uterine incision" [30], which consists of making a shallow transverse incision over the narrowest fundic portion of the unicornuate uterus, thus creating a new fundus of about 2 cm, and subsequently by making a vertical incision of about 4 cm along the entire lateral wall opposite to the ostium, approximately 1 cm deep, until reaching the level of the isthmus. In this way, the uterine cavity is enlarged.

In the case of a rudimentary communicating and functioning horn, the treatment is surgical removal as soon as it is diagnosed, to prevent dysmenorrhea and the possibility of pregnancy in the rudimentary uterus. The same procedure is followed in cases of noncommunicating functioning cavity to treat dysmenorrhea and associated hematometra, preventing the development of secondary endometriosis.

Results after hysteroscopic metroplasty

Transcervical uterine incision appears to improve obstetric outcomes in women with a unicornuate uterus by reducing first-trimester miscarriage rates and increasing term birth rates. Although the results are promising, more studies are needed to determine the usefulness of this new technique [30].

Dysmorphic uterus

The ESGE/ESHRE classification is the only one that contemplates the category of dysmorphic uterus or U1

(Fig. 9.3). Within this is the uterus in "T" or A1, which is defined as a uterus with normal external contour but with thickening of the lateral walls that suppose the existence of a hypoplastic uterine cavity, presenting a 2/3 body ratio uterus and 1/3 cervix.

The first proposal of the existence of a dysmorphic uterus was made in 1930 by doctors K. Menge and Kv Oettingen [31], who already clearly defined two types of uterus different from the normal one, and with an abnormal development that affected the uterine size and the morphology of the uterine cavity. On the one hand is the hypoplastic uterus, which showed a normal relationship between cervical length and the length of the uterine body of approximately 1:2 (which in the ESGE/ESHRE classification corresponds to U1a), and on the other hand is the infantile uterus, with an abnormal relationship between the cervix and the uterine body, settling in 1:1 or 2:1 (corresponding to U1b) (Fig. 9.10).

The cause of this type of uterine malformation remains unknown, and although there is a clear relationship with in-utero exposure to DES, the cases observed today cannot be related to this drug since it was withdrawn at the beginning from the 1970s, which makes it very difficult to find a DES-related T-uterus today.

Diagnosis

Through the combined use of hysteroscopy and 3D ultrasound, three subtypes of dysmorphic uterus can be observed that meet the ESGE/ESHRE criteria with different morphology of the uterine cavity, which are called uterus T, Y, and I [14,32] (Fig. 9.11).

T-shaped uterus presents a thickening of the lateral walls while the fundus is normal (without the presence of a septum or subseptum), with normal or increased interostium distance, and very pronounced narrowing at the level of the middle 1/3 of the endometrial cavity.

Y-shaped uterus presents a thickening of the lateral walls (with very pronounced narrowing at the level of the middle 1/3 of the endometrial cavity), and subseptum-type fundic indentation with a normal or reduced interostium distance.

I-shaped uterus presents a thickening of the lateral walls and a very marked reduction in the interostium distance, which gives a tubular appearance to the entire cavity, observing a generalized narrowing.

Although there is still no defined and accepted criteria worldwide, in our experience to obtain a good 3D ultrasound-hysteroscopy correlation in the diagnosis of this type of uterine malformation, the measurement of

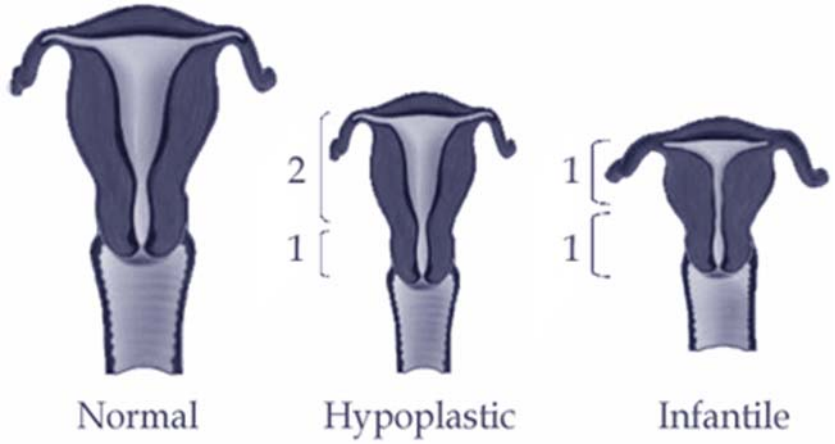


FIGURE 9.10 Normal, hypoplastic, and infantile uterus.

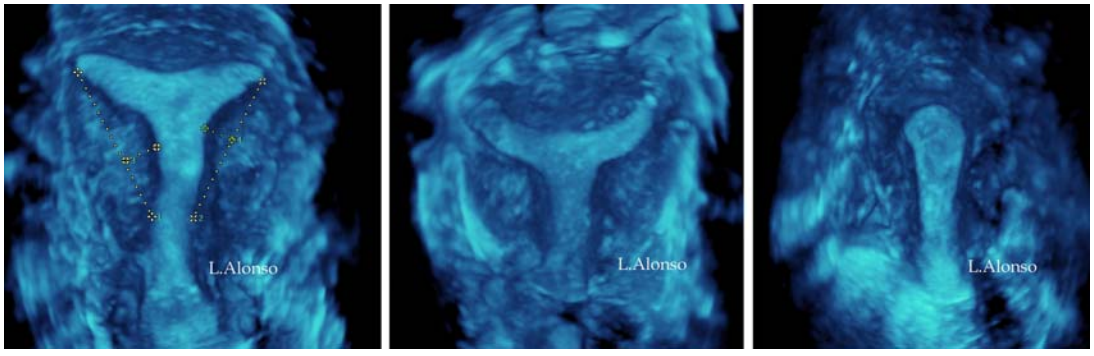


FIGURE 9.11 T-shaped, Y-shaped, and I-shaped uteri.

the cavity at the middle 1/3 of the uterus in the coronal plane obtained by 3D ultrasound is less than 10 mm.

Clinical importance

The dysmorphic uterus has been associated with very poor obstetric outcomes, with full-term live birth rate below 2%, and with high rates of infertility and spontaneous abortion, making this type of malformation the malformation that is probably associated with worse obstetrical outcomes. It is noteworthy that an increased ectopic pregnancy rate is also observed compared to the general population.

Surgical repair

The technique proposed for its correction is hysteroscopic metroplasty, which consists of making two incisions in the lateral walls, sectioning the myometrium, from the isthmus to the ostium, thus achieving an enlargement of the uterine cavity. The intervention is considered complete when both tubal ostia are visible from the uterine isthmus, which is usually achieved with a lateral incision about 6–7 mm in depth [33,34] (Fig. 9.12).

Results after hysteroscopic metroplasty

In different published case series [33–37], a significant improvement has been described after hysteroscopic repair, observing an increase in the number of live births, as well as a decrease in the spontaneous abortion rate. The improvement observed after the procedure is a consequence of the remodeling carried out in the uterine cavity, as well as the improvement in uterine distensibility and vascularization.

The systematic review and meta-analysis by Garzon et al. [38] on reproductive outcomes after surgery for T-uteruses concluded that hysteroscopic correction of this type of uterus was associated with high rates of live birth and low rates of spontaneous abortion in both those patients with primary infertility as well as those with a history of recurrent abortion. This meta-analysis also observed high rates of spontaneous pregnancy after surgery, which even reached 32.4% in patients with a history of failed IVF. In addition, obstetric complications related to the procedure were negligible, except for a higher rate in the number of cesarean sections performed.

Bicornate uterus

It occurs as a result of a failure in the process of fusion of the Müllerian ducts. The degree of separation of the hemiuteri will depend on the embryological moment in which the defect occurs. The earlier this failure occurs, the more complex the resulting malformation will be. Bicornuate implies the existence of an abnormal outer uterine contour in which an indentation can be seen at the fundic level that exceeds 50% of the thickness of the uterine wall. This indentation can totally or partially divide the uterine cavity.

Clinical importance

The importance of this type of uterus lies more in its relationship with poor obstetric outcomes than with infertility. Although the existing data is limited, an increase in the rates of preterm birth and abortion has been observed when compared to the control group, so the abortion rate is estimated at 36% and the preterm birth rate at 23% [4], being higher in cases of complete bicornuate uterus than in partial bicornuate uterus. However, the existence of a bicornuate uterus does not seem to affect fertility [6].

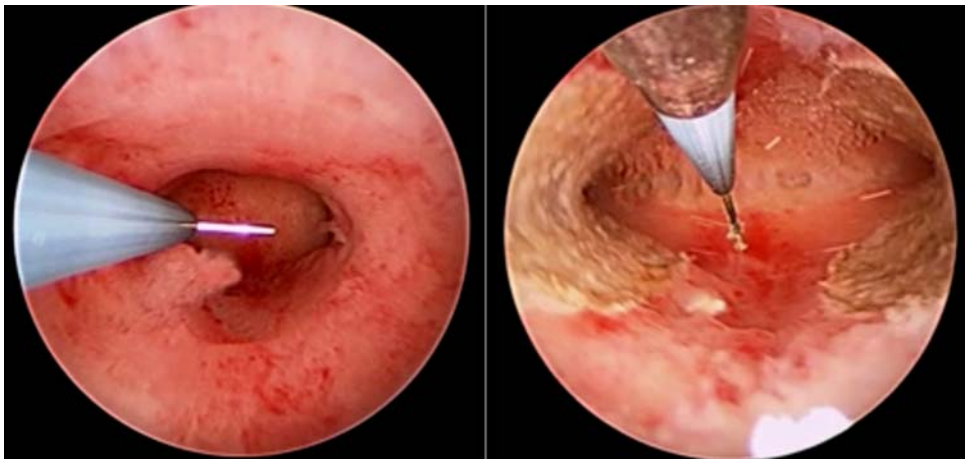


FIGURE 9.12 Hysteroscopic metroplasty for a dysmorphic uterus.

Diagnosis

The 2D ultrasound image in the sagittal section and with the probe scanning from side to side shows the existence of two uterine bodies with a greater or lesser degree of separation between them, as well as two endometrial cavities. In the cross-section at the fundic level, it shows two endometrial cavities and two uterine horns separated by a central area in which no myometrial tissue is seen.

The 3D ultrasound shows in the coronal plane two well-shaped uterine horns with a convex fundus in each of them [39] that may or may not join at some point along the path. The external uterine contour shows an indentation in the fundus greater than 10 mm in depth.

The hysteroscopic view is very similar to that of the septate uterus with two separate tubular uterine cavities showing the muscular rings of the internal myometrial layer. The division point can be found at different levels depending on the type of bicornuate uterus.

MRI offers an image similar to the one seen with 3D ultrasound with an outer uterine contour with an indentation greater than 10 mm and a divided uterine cavity.

Surgical repair

In principle, the bicornuate uterus is not a candidate for hysteroscopic correction, and the best surgery in these cases is the classic Strassman metroplasty, which consists of the unification of the two uterine hemicavities and is usually performed by laparotomy or laparoscopy.

However, in cases of septate bicornuate uterus or U3c, in which a fusion problem coexists with a reabsorption problem, a partial resection of the septum is possible, performing in most cases a unification of approximately the lower 2/3 of the uterine cavity. This type of corrective surgery is usually performed under laparoscopic guidance to try to avoid uterine perforation.

Results after surgery

Surgical reconstruction of the bicornuate uterus is limited to selected women with poor reproductive outcomes in whom other etiologies have been ruled out. Although the data available are very scarce, the rate of live births after Strassman surgery reaches up to 80% in the largest published series [40]. There are no data on the outcome of surgery on fertility.

Robert's uterus

It is an asymmetric variant of the septate uterus that is characterized by having a complete uterine septum that divides the uterine cavity asymmetrically from the fundus to the internal cervical os, resulting in a noncommunicating hemicavity and another with the appearance of a unicornuate uterus, all this in a uterus with a normal external morphology (Fig. 9.13).

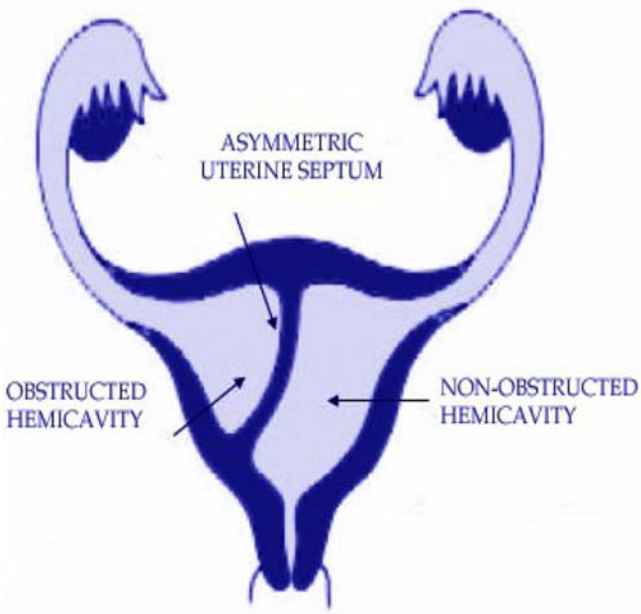


FIGURE 9.13 Robert's uterus.

It was first described by Hélène Robert under the name "asymmetrical bifiditis with unilateral menstrual retention" [41], a term that very well describes the pathogenesis that can be associated with this uterine anomaly: hematometra, hematosalpinx, and due to the existence of retrograde menstrual flow, there may be associated endometriotic foci.

Included in the ESGE-ESHRE classification, it is defined as U6 or unclassified uterine malformation, although some groups have defined it as complete septate uterus (U2b) with unilateral cervical aplasia (C3) and normal vagina (V0) [42]. The new ASRM classification has also included this type of uterine anomaly, and it does so within the septate uterus group (Fig. 9.2).

According to the characteristics of the existing hematometra in the blind hemicavity at the time of diagnosis, three types of Robert's uterus have been described [43]: Type I, with large hematometra; Type II, without hematometra; Type III, with small hematometra.

Diagnosis

It is not easy to perform, since it is a complex malformation, which means that in some cases it can be confused with a unicornuate uterus with a rudimentary noncommunicating horn. This happens because 2D ultrasound does not have a high diagnostic sensitivity since it usually gives an impressive image of a unicornuate uterus. On hysterosalpingography, the typical

fusiform image that is seen in cases of unicornuate uterus is also seen, with visualization of a single tube. That is why MRI in the coronal section is the best imaging modality for the diagnosis of Robert's uterus, since this reveals the septum, the hematometra in the blind cavity, and the existence of a normal uterine contour. Currently, 3D ultrasound offers results similar to those of MRI.

The gold standard for diagnosis of Robert's uterus is the combination of hysteroscopy-laparoscopy since hysteroscopy allows visualization of the unicornuate uterus, and laparoscopy allows the visualization a normal external uterine morphology or with an indentation of less than 1 cm, while the unicornuate uterus in laparoscopy would be visualized with an indentation greater than 1 cm, the external morphology being similar to a bicornuate uterus.

Clinical importance

Robert's uterus is associated with poor reproductive outcomes, since the hemiuterus that does have communication with the vagina behaves like a unicornuate uterus, so the clinical presentation is usually infertility and recurrent pregnancy rate. In the event that the blind hemicavity presents hematometra, both this and the associated dysmenorrhea tend to be of increasing intensity as time passes, due to the increase in size and tension that occurs in the hemicavity that does not have drainage.

Surgical repair

Two techniques have been proposed to repair this uterine anomaly: on the one hand, performing a hysterotomy of the dilated hemicavity with drainage of the blood content and subsequent endometrectomy to prevent recurrence of the hematometra, and the other surgical alternative is metroplasty with communication of the two hemicavities, which can be performed laparoscopically after hysterotomy of the blind hemicavity dilated by the hematometra, or transcervically, performing a hysteroscopic metroplasty [44].

Unfortunately, due to errors in diagnosis and confusion with a rudimentary noncommunicating uterus, many times these patients are subjected to a total resection of the noncommunicating hemiuterus, with the functional repercussions that this entails.

Control after hysteroscopic surgery

Although there is currently no consensus on protocols to follow after surgical correction of uterine anomalies, most authors recommend performing a follow-up hysteroscopy to assess the final anatomy after the performed metroplasty, and also at the same time, assess the healing process and the appearance of possible intrauterine adhesions, since the earlier they are diagnosed, the more easily they can be resolved. All this takes into account that the endometrium takes between 30 and 90 days to reestablish itself [45].

Regarding the prevention of intrauterine adhesions after hysteroscopic surgery, two main strategies are contemplated, combined or not with each other: the use of hormonal therapy and the use of mechanical barriers after the procedure.

The objective of the use of hormone therapy is the stimulation of the endometrium to favor its growth and the re-epithelialization of the entire cavity. However, we do not have studies that guide us on the most effective hormonal combination, the recommended dose, or the ideal length of use.

The contribution of mechanical barriers in preventing the formation of intrauterine adhesions would be the prevention of the uterine walls from contacting each other, either with the use of a nonhormonal intrauterine device, the use of an intracavitary Foley catheter (Fig. 9.14), or a Cook's balloon. Although there are no randomized studies that show the advantage of using a mechanical barrier, it is recommended to maintain it intracavitarily for 1 or 2 weeks, in addition to combining it with hormonal treatment.

In addition to intrauterine mechanical barriers with a device in the cavity, there are also physical barriers in gel format whose main component is hyaluronic acid, and which act not only by preventing contact with the uterine walls, but also by promoting tissue healing. Although a priori it was expected to obtain promising results with its use, currently, in the absence of randomized studies, the latest reviews do not show significant differences in terms of the reduction of adhesions after its use.

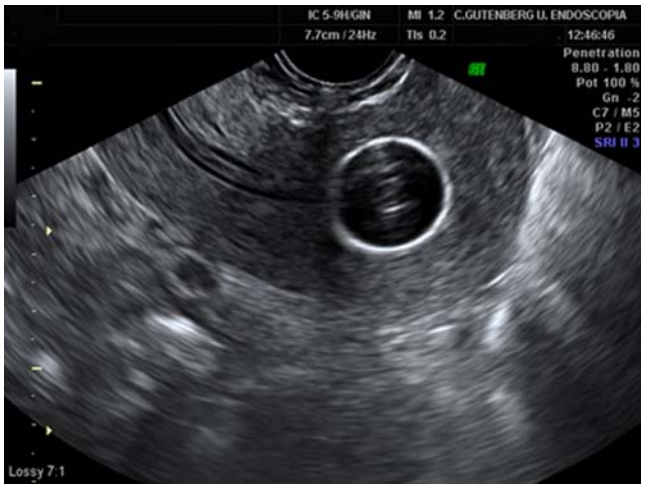


FIGURE 9.14 Intrauterine Foley's catheter.

Recommendations and conclusions

Hysteroscopy is the gold standard in the study of the uterine cavity and plays an important role in both the diagnosis and treatment of uterine malformations, which represent a real challenge for the gynecologist. It is important to obtain an accurate diagnosis to select the corrective surgery with the maximum guarantee of success. Likewise, it is recommended that this type of surgery be performed only by well-trained and experienced hysteroscopists, since an incomplete or improperly performed surgery can result in significant complications and even irreparable reproductive damage.

Currently, the use of 3D ultrasound allows a quick and cheap diagnosis with a sensitivity and specificity of almost 100%. It is important to know the different surgical techniques as well as when each of the different techniques is considered complete.

Preliminary data in the study of posttreatment reproductive results are encouraging and suggest that the surgical management of these malformations not only manages to remodel and recover the normal anatomy of the uterus, but more importantly, its function. That is why the infertility and reproduction societies include in the updates of their guidelines and protocols the performance of a hysteroscopy and the screening of uterine malformations in patients with infertility.

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CHAPTER

10

Uterine fibroids and infertility

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Introduction and definition

Infertility is an important social and economic problem because many couples plan their families much later in life now than couples did 3 decades ago. With increasing age, women have fewer chances of natural fertilization and the maintenance of pregnancy. Consequently, many couples need assisted reproductive technology (ART). However, a large number of women undergoing *in vitro* fertilization (IVF) suffer from infertility in the form of recurrent implantation failure [1].

Infertility has been diversely defined from clinical, demographic, and epidemiological viewpoints. It has also been viewed as a disability. By clinical definition, infertility is a disease of the reproductive system defined by the failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse [2].

Human reproduction is an inefficient process, because only about 30% of conceptions result in a live birth. Although exact percentages are impossible to access, it has been estimated that approximately 30% of embryos are lost at the preimplantation stage, while 30% are lost after implantation in the uterus and only detected by a positive serum human chorionic gonadotropin (hCG) test in the absence of ultrasound findings. Ten percent are clinical miscarriages, including abortion and stillbirth [3]. Most pregnancy wastage is caused by the embryo itself. In about 70% of cases, significant chromosome abnormalities are responsible for sporadic abortions. The problem of early abortion became known in the era of IVF treatment because the exact date of embryo transfer and expected implantation can be predicted in IVF treatment. Hence, recurrent implantation failure became a clinically identifiable phenomenon.

The development of a pregnancy is a multifaceted process. It can be influenced and hindered by various systemic and local factors, such as maternal age, oocyte and sperm quality, parental chromosomal abnormalities, genetic or metabolic abnormalities of the embryo, poor uterine receptivity, and immunological imbalance at the implantation site. Gynecological conditions that could influence implantation rates include endometriosis, uterine fibroids, hydrosalpinges, and endometrial polyps. Finally, factors such as lifestyle, smoking, alcohol, drugs, and obesity causing insulin resistance might impair the success of reproduction [4–6].

In the following, we discuss uterine fibroids as a possible cause of infertility, their investigation, and treatment options.

Uterine fibroids, also known as uterine leiomyomas or fibroids, are benign smooth muscle tumors of the uterus that affect women of reproductive age. Fibroids have both smooth muscle and fibroblast components, in addition to a substantial fibrous extracellular matrix, all of which contribute to the process of pathogenesis. Fibroids are extremely heterogeneous in terms of pathophysiology, size, location, and clinical symptoms [7]. While some women have no symptoms, others experience dysmenorrhea or hypermenorrhea. The symptoms and their severity may differ, depending on the size and location of the fibroids. The most common presenting symptom is heavy menstrual bleeding, which may lead to anemia, fatigue, or painful periods. Other possible symptoms include lower back pain, pelvic pressure or pain, and pain during intercourse. In the presence of fibroids beyond a certain size, pressure on the bladder or bowel may result in increased micturition frequency or retention, pain, or constipation. Uterine fibroids may also be associated with reproductive problems such as infertility, recurrent pregnancy loss, and adverse obstetric outcomes [8,9].

Uterine fibroids are the most common neoplasm in women, and reported to occur in more than 70% of women at the onset of menopause [10]. Their incidence in women of reproductive age is 5.4%–77%, depending on biological, demographic, reproductive, and lifestyle factors [11–13]. The frequency of fibroids appears to

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be threefold higher in Negroid than in Caucasian women [14]. Other factors influencing the incidence of fibroids include premenopausal state and age. As regards the latter, fibroids were reported to be especially common in women in their fifth or sixth decade of life compared to those in their third decade [14]. This effect did not persist beyond the sixth decade, which reflects the protective effect of menopause. A positive family history in patients has confirmed the genetic aspect of fibroids. Hypertension, food additives, and soybean milk consumption were found to increase the frequency of fibroids in single-center studies [15,16]. One of the many protective factors is parity. A single-center study revealed that, compared to nulliparity, parity was associated with a fivefold lower risk of uterine fibroids requiring surgical treatment [17]. Further protective factors include oral contraception and a low BMI, which is frequently associated with lower estrogen levels.

Traditionally, fibroids have been classified by their location in the uterus. They may be divided into cervical, submucosal, subserosal, and intramural fibroids. The International Federation of Gynecology and Obstetrics (FIGO) uses the following classification: Intracavitary lesions are attached to the endometrium by a narrow stalk (≤10% or the mean of three diameters of the leiomyoma) and are classified as type 0, whereas types 1 and 2 require a portion of the lesion to be intramural, with type 1 being less than 50% of the mean diameter and type 2 at least 50%. Type 3 lesions are entirely intramural, but also around the endometrium [18]. Type 3 is formally distinguished from type 2 by hysteroscopy, using the lowest possible intrauterine pressure needed for visualization. Type 4 lesions are intramural leiomyomas entirely within the myometrium, with no extension to the endometrial surface or the serosa. Subserosal (types 5, 6, and 7) leiomyomas are the mirror image of the submucosal leiomyomas, with type 5 being at least 50% intramural, type 6 less than 50% intramural, and type 7 attached to the serosa by a stalk that is also ≤10% or the mean of three diameters of the leiomyoma [18]. Transmural lesions are categorized by their relationship to the endometrial

TABLE 10.1 The International Federation of Gynecology and Obstetrics (FIGO)—classification of fibroids.

	Type	Location
Submucosal	0	Pedunculated intracavitary
	1	<50% intramural
	2	≥50% intramural
Intramural	3	Contact with the endometrium, 100% intramural
	4	Intramural
	5	Subserosal ≥50% intramural
	6	Subserosal <50% intramural
Subserosal	7	Subserosal pedunculated
	8	Other (e.g., cervical, intraligamentous)
Hybrid (contact with the endometrium and the serosal layer) The numbers are listed separately with a hyphen. The first refers to the relationship with the endometrium, and the second refers to the relationship with the serosa	2–5	Submucosal and subserosal, each with less than half the diameter in the endometrial and peritoneal cavities, respectively

and serosal surfaces. The endometrial relationship is noted first, and the serosal relationship ranks second (e.g., types 2–5). Type 8, an additional category, is reserved for leiomyomas that do not relate to the myometrium at all; this category includes cervical lesions and those that exist in the round or broad ligaments with no direct attachment to the uterus [18]. Table 10.1 provides an overview of types and respective locations of the fibroids.

Uterine fibroids and infertility

Uterine fibroids are the most common tumors in women, and their prevalence is high in the presence of infertility. Fibroids may be the sole cause of infertility in 2%–3% of women [19,20]. Depending on their location in the uterus, fibroids have been implicated in recurrent pregnancy loss as well as infertility.

Implantation is a process by which the embryo attaches itself to the endometrium, migrates via the luminal epithelium, and invades the deep layer of the endometrium to become embedded in the deeper layer. The process involves a complex sequence of cellular and molecular changes. Implantation has a well-defined starting point and then proceeds rather slowly for several weeks; the time of its conclusion cannot be predicted in advance. Clinically, implantation is considered to be successful when there is ultrasonic evidence of an intrauterine gestational sac, which usually forms at about 5 weeks of gestation. In contrast, implantation failure is defined as the absence of an intrauterine gestational sac on ultrasound. Implantation failure may occur in the rather early stages of attachment or migration. The absence of objective evidence of pregnancy is a negative hCG test. Implantation failure may also occur later on, after successful migration of the embryo through the luminal surface of the endometrium. hCG, which is produced by the embryo, can be detected in a blood or urine test. However, the process may be disrupted before the emergence of an intrauterine gestational sac; this condition is known as a biochemical pregnancy [21].

An evaluation of outcomes in women with infertility revealed that those with fibroids in any location had significant lower rates of clinical pregnancy, implantation, ongoing pregnancy, and live birth rates compared with controls. In addition, the spontaneous abortion rate was significantly higher in women with fibroids. No difference was noted in regard of preterm delivery rates [22].

In the following, fibroids are divided according to their location and their impact on fertility:

a. **Submucosal fibroids** (with and without distortion of the cavity): Compared to infertile women without fibroids, women with submucosal fibroids have significantly lower clinical pregnancy rates, implantation rates, ongoing pregnancy/live birth rates, and significantly higher spontaneous abortion rates. No difference was observed in regard of preterm delivery rates. Distortion of the uterine cavity had no impact on clinical pregnancy rates [22,23].
b. **Subserosal fibroids:** None of the aforementioned outcome measures differed in women with subserosal fluids compared to those without fibroids [22].
c. **Intramural fibroids:** Women with intramural fibroids had significantly lower clinical pregnancy rates, implantation rates, ongoing pregnancy/live birth rates, and significantly higher spontaneous abortion rates. No difference was registered in preterm delivery rates [22].

Women with subserosal fibroids did not differ from those without fibroids in regard of implantation rates, clinical pregnancy rates, live birth rates, and abortion rates. Thus, subserosal fibroids do not seem to affect fertility [22]. In contrast, submucosal and intramural fibroids that distort the endometrial cavity are associated with lower pregnancy, implantation, and delivery rates in women undergoing IVF compared to infertile women without fibroids [24,25]. Furthermore, there is a higher risk of infertility when the endometrial cavity is distorted by submucosal fibroids [26,27]. Pregnancy and delivery rates appear to be improved after resection of submucosal fibroids, especially when fibroids are the sole identifiable cause of infertility [24,27,28]. The exact pathomechanism as to how intramural fibroids affect the overlying endometrium and influence receptivity is not fully understood. Fibroids may affect implantation by several mechanisms, including increased uterine contractility, deranged cytokine profile, abnormal vascularization, and chronic inflammation [29]. In the following, we will address the pathophysiology of intramural fibroids.

Pathophysiology

HOXA 10 is a homeobox-containing transcription factor that is essential for embryonic uterine development as well as proper adult endometrial development during each menstrual cycle [30]. HOXA 10 expression is necessary for endometrial receptivity [31–33]. *Glycodelin* is a secretory glycoprotein that affects cell proliferation, differentiation, adhesion, and motility [34]. Glycodelin is responsible for promoting angiogenesis and suppressing natural killer cells during implantation. Normally, HOXA 10 and glycodelin are reduced during the follicular phase and increased during implantation. In cases of intramural fibroids, both HOXA 10 and glycodelin are reduced during implantation, which may lead to embryo implantation failure and cause infertility [33].

The *uterine junctional zone* is the inner third of the myometrium and the layer that immediately abuts the endometrium. The layer differs architecturally from the rest of the myometrium and appears to be the origin of myometrial contractions. Thickening or disruption of the layer by intramural fibroids may also contribute to a poor reproductive outcome, including infertility or early pregnancy loss [33,35]. In contrast to the rest of the myometrium, the junctional zone changes under the influence of estrogen and progesterone. During the window of implantation, at about 5–7 days after ovulation, myometrial contractions are limited to a minimum; decidualization of the endometrium and the junctional zone occurs. Uterine natural killer cells (uNK) and macrophages are responsible for the differentiation of tissue during decidualization. uNK cells are the most abundant and important immune cells in the uterus at the time of implantation. An alteration of uNK cell numbers has been associated with implantation failure [35,36].

The presence of fibroids appears to influence the number of *uNK cells and macrophage cells*. Kitaya et al. analyzed those cells in samples obtained after hysterectomy; the authors compared cell counts near fibroids with cells on the contralateral side of the uterus, far away from fibroids. In the mid-secretory phase, uNK cells were significantly reduced and macrophage cells significantly increased in the endometrium near fibroids compared to endometrium away from the fibroids, and also significantly reduced compared to healthy controls [37]. Regrettably, the study provides no data about the location of the fibroids. Furthermore, the mean age of women with fibroids as well as healthy controls was 40 years. They were candidates for hysterectomy, but not representative of the typical patient population suffering from infertility and recurrent pregnancy loss.

A *physical disruption* of the junctional zone, caused by intramural fibroids, may also lead to implantation failure or early pregnancy loss [38]. The expression of *estrogen and progesterone* as well as their receptors was reported to be altered at the junctional zone. However, this aspect needs further investigation [39,40].

Uterine myometrial peristalsis

Cine-mode magnetic resonance imaging (MRI) permits analysis of myometrial contractions in the uterus [41]. The frequency of contractions appears to increase from menses to the mid-ovulatory phase of the cycle, and the contractions progress from the cervix to the fundus. The frequency is reduced after ovulation and especially during the time of implantation. The direction of peristalsis is also reversed in the luteal phase [42]. Compared to healthy controls, women with intramural and submucosal fibroids had increased myometrial peristalsis during the mid-luteal phase and decreased peristalsis in the peri-ovulatory phase [43,44]. Fifteen patients with intramural fibroids and a high frequency of uterine peristalsis in the mid-luteal phase were followed in a retrospective study. After myomectomy, peristalsis returned to normal in 14 of 15 patients; a pregnancy rate in excess of 40% was observed in the course of 1 year after surgery [45].

Leiomyomas are surrounded by a fibroid pseudocapsule (PC) that can be best identified during surgery, at the time of myomectomy. It consists of a bundle of smooth muscle cells and a vascular capsule responsible for blood supply. The PC is rich in neurotransmitters and neurovascularization. Endoglin and CD34, markers of neovascularization, are upregulated in the PC compared to the fibroid itself and the surrounding myometrium. The thickness of the capsule varies according to fibroid type and location. Submucosal fibroid PCs are significantly thicker than intramural myoma PCs, and intramural PCs are significantly thicker than subserosal PCs. The thickness also increases when the fibroid is located closer to the cervix [33,46]. The latter PCs are marked by higher expressions of enkephalin and oxytocin. These neuropeptides may alter fertility by inducing abnormal uterine contractions [47]. Furthermore, the intramural fibroid PC has been associated with increased levels of neurotensin, neuropeptide tyrosine, and the protein gene product 9.5 [47], all of which may induce muscular contractions. Large intramural fibroids might cause premature uterine contractions and disrupt early pregnancies, or cause preterm delivery [33,48].

Diagnosis

Ultrasonography, preferably by the transvaginal route, is the first-line diagnostic imaging procedure for

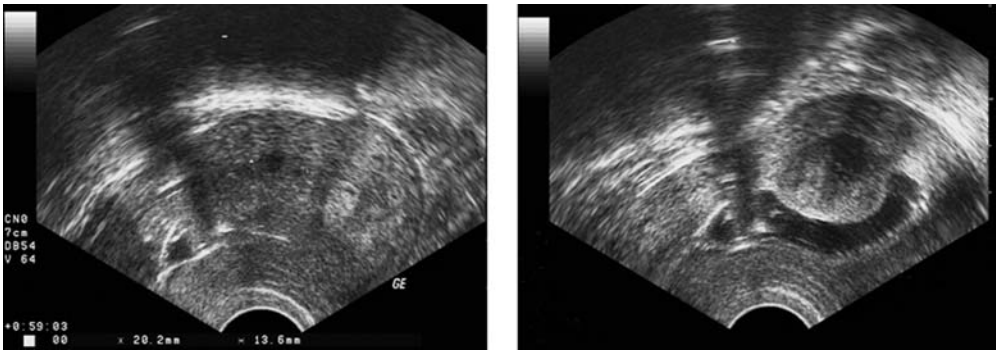

FIGURE 10.1 (A and B): Presentation of an intramural fibroid affecting the uterine cavity (FIGO 2), with regular 2D vaginal ultrasound on the left hand side and with hysterosonography on the right hand side.

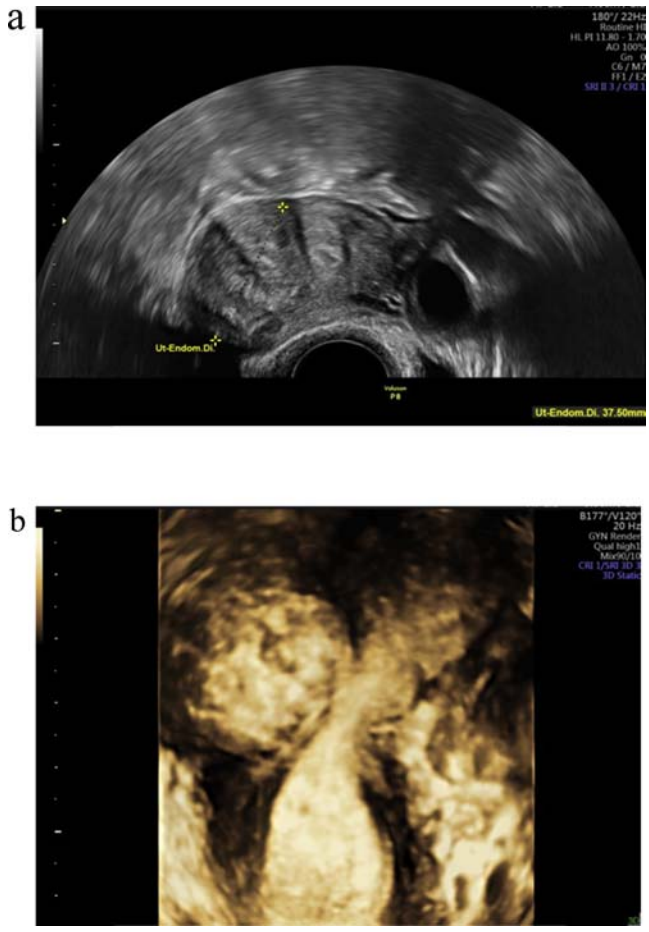

FIGURE 10.2 (A and B): Presentation of an intramural myoma, affecting the cavum uteri, with regular 2D vaginal ultrasound on the left hand side and with 3D vaginal ultrasound on the right hand side.

the detection of fibroids. It is a widely available, economical, noninvasive, and painless means of investigating the uterine cavity. Ultrasound is known for its high sensitivity and specificity in identifying fibroids. The size, exact location, and potential presence of fibroids in the uterine cavity can be assessed. After infusion of saline into the uterine cavity, transvaginal ultrasound is able to demonstrate submucosal fibroids and indicate the proximity of intramural fibroids to the cavity [49].

Fig. 10.1A and B show a fibroid on 2D ultrasound and hysterosonography

A "normal" 2D transvaginal ultrasound may be supplemented with a 3D transvaginal ultrasound. The latter permits reconstruction of the coronal plane of the uterus and thus demonstrates the exact location of the fibroid and distortion of the cavity due to submucosal fibroids [50,51].

Fig. 10.2A and B show a myoma on 2D and 3D vaginal ultrasound.

On ultrasound examination, a uterine fibroid is typically seen as a well-defined round lesion within the myometrium or belonging to it, frequently with shadows at the edge or an internal fan-shaped shadow [52]. Doppler ultrasound reveals circumferential flow around the fibroid. Fibroids are usually hypoechoic or isoechoic. The echogenicity varies, depending on the level of calcification and the quantity of fibrous tissue. Sometimes a fibroid has anechoic components due to advancing necrosis. The size of the fibroid is estimated by measuring its three largest orthogonal diameters. Additionally, the minimum distance from the fibroid to the serosal surface and the endometrium of the uterus is measured [52].

The differential diagnosis of uterine masses is of crucial importance. Adenomyosis, endometrial polyps, or solid tumors of the adnexa are some of the most common misdiagnosed pathologies. Adenomyosis may be difficult to diagnose. A distinction is made between diffuse and focal adenomyosis, which are differentiated from adenomyomas. On histological investigation, adenomyomas are marked by additional compensatory hypertrophy of the surrounding myometrium [52]. Differentiating this condition from myoma can be challenging, especially when both pathologies are present together. Color Doppler ultrasound may be useful in

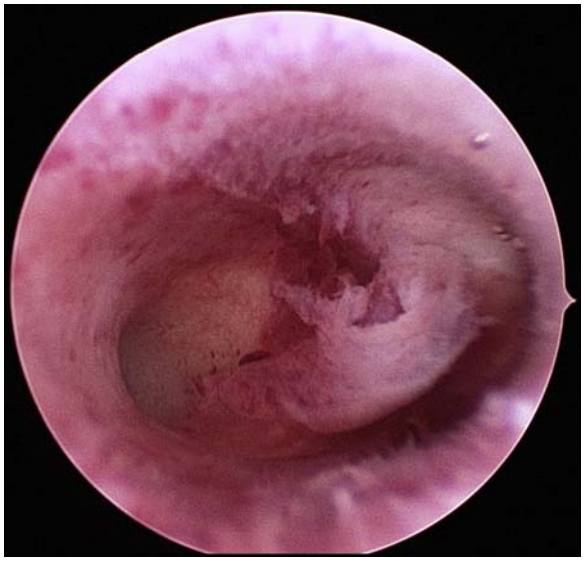

FIGURE 10.3 Hysteroscopic view of an inconspicuous cavum uteri with raised endometrium in the middle.

this setting. Ultrasound findings that indicate the presence of adenomyosis include an asymmetrical thickening of the wall, so-called striae-like vascular patterns, fan-shaped shadowing, myometrial cysts, hyperechoic islands, echogenic buds and strips, and an irregular or interrupted junctional zone [52].

In cases of ambiguous ultrasound findings, MRI provides additional information (specificity 100%, accuracy 97%, and sensitivity 86%–92%) [53].

A hysteroscopy should be performed for an even more detailed investigation or to confirm potential involvement of the uterine cavity. During hysteroscopy the gynecologist may perform an endoscopy of the uterine cavity without anesthesia, usually even without hooking the cervix. The small optical instrument measuring just 3 mm in diameter serves the purpose of inspection. Fig. 10.3 shows the hysteroscopic view of an inconspicuous uterine cavity with a raised endometrium in the center. Polyps, fibroids, adhesions, and septa may all affect implantation; the gold standard for evaluation is hysteroscopy.

Management

Treatment options for fibroids include surgery, medication, and interventional radiology. The treatment improves symptoms by reducing the size of the fibroids, controlling abnormal uterine bleeding, or even curing the fibroids [54].

The key question is when should the clinician treat a fibroid in women with infertility? It primarily depends on the existing clinical symptoms as well as the size and location of the fibroids. The indications for treatment should be established with care because the association between infertility and fibroids may not be evident in some situations. Indications for surgery in intramural fibroids should be evaluated very carefully because surgery involves removal of the fibroid, but also causes scarring of the uterus wall, which may affect subsequent pregnancies. Medication may be used to treat abnormal uterine bleeding, although this approach has no more than a transient effect on fibroids. Available medical treatments include gonadotropin-releasing hormone (GnRH) agonists or antagonists, antiprogestins, progesterone-only treatments, combined hormonal contraceptives, selective progesterone receptor modulators, antifibrinolytic agents, and nonsteroidal antiinflammatory drugs (NSAIDs) [54]. In certain cases, GnRH agonists may be used before surgery to shrink fibroids and restore hemoglobin levels in symptomatic patients. However, due to their side effects, GnRH agonists cannot be used for a long time [54].

A thorough preoperative assessment is essential to determine the surgical strategy according to the size, location, and number of fibroids. A precise preoperative diagnosis will indicate whether a hysteroscopic resection or a laparoscopic myomectomy is feasible, and whether a laparotomy should be performed for numerous or large fibroids [55]. Each approach has its own indications. Currently, hysteroscopic myomectomy is the gold standard for surgical treatment of submucosal fibroids (FIGO 0 and 1 fibroids). FIGO 2 fibroids are more difficult to resect and may require a two-stage treatment, especially if they are larger than 3 cm in size [55].

Complications during the intervention are rare and mainly related to the difficulty of the surgical procedure. The most common problems associated with hysteroscopic myomectomy include uterine perforation, bleeding, infection, and venous intravasation [56,57]. Long-term complications such as intrauterine adhesions were reported in about 10% of cases during second-look hysteroscopy; the risk is higher in cases of multiple apposing fibroids [58]. Prevention strategies include the insertion of a postoperative intrauterine device, intrauterine balloons, hyaluronic acid gel, or postoperative treatment with oral estrogens to stimulate endometrial regeneration [58]. Surgical strategies may also permit prevention of adhesions. Monopolar resectoscopes appear to increase the risk of postoperative intrauterine adhesions compared to bipolar resection of fibroids [59]. However, evidence regarding prevention strategies is very limited. The duration of endometrial wound recovery varies for the different types of hysteroscopic surgery, ranging from 1 month after polypectomy to 3 months after myomectomy. The duration of wound recovery is important for subsequent fertility treatments [60].

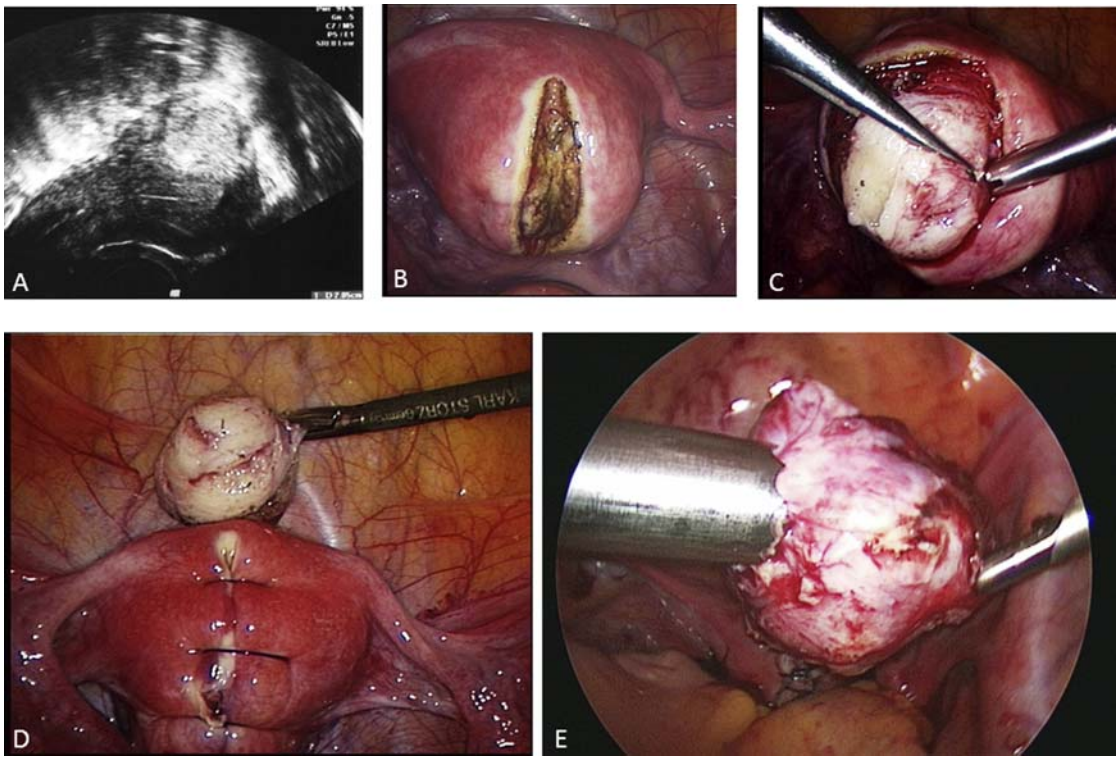

FIGURE 10.4 A: Preoperative presentation of a subserosal fibroid with 2D ultrasound. B-D: Laparoscopic incision, dissection, removal of the fibroid and re-suturing of the uterus. E: Removal of the fibroid by morcellation.

Intramural and subserosal fibroids (FIGO 3 fibroids and above) are best removed by laparoscopy or laparotomy. Laparoscopic surgery is the first choice in the absence of contraindications. Laparoscopic myomectomy is considered more difficult by many gynecological surgeons, but its benefits are noteworthy: less postoperative pain, shorter hospital stays, less blood loss, and faster recovery. No difference was registered between the laparoscopic and abdominal approach in regard of reproductive outcomes [61]. Challenges in surgery include the appropriate use of sutures and the achievement of satisfactory hemostasis. The most frequent intraoperative complications of laparoscopic myomectomy include myometrial hematoma, excessive blood loss, and morcellation accidents [62,63].

Complex conditions would be the presence of concomitant pathologies such as adenomyosis or adenomyoma, or the need for large intramural fibroid extraction [63]. Antiadhesive agents may be useful in reducing postoperative adhesions [62]. Obstetric complications during labor are caused mainly by a weak myometrium after destruction due to extensive coagulation, defective suturing. and poor tissue approximation. The rate of uterine rupture in a subsequent pregnancy is reported to be 1% [63]. During laparoscopic myomectomy, fibroids are usually removed with a morcellator. Although the prevalence of leiomyosarcoma is very rare in fibroids (<0.3%), the risk of uterine fragment dispersion during morcellation remains a highly debated issue and has been addressed by many international societies [54,61].

Contraindications to laparoscopic myomectomy include multiple fibroids (>4) at different sites of the uterus, requiring numerous incisions, and the presence of an intramural fibroid >10−12 cm in size or suspected of being a leiomyosarcoma [61].

Fig. 10.4 shows laparoscopic enucleation of a fibroid with reconstruction of the uterine wall.

In view of the absence of long-term data concerning fibroids and infertility, nonsurgical interventions such as uterine artery embolization, magnetic resonance-guided focused radiofrequency ablation, or transcervical radiofrequency ablation are inconclusive [61].

Recommendation

Clinical pregnancy rates were high after myomectomy in patients with *submucosal* fibroids, but the ongoing pregnancy/live birth rate did not reach statistical significance. No change was registered in abortion rates [4,45].

Subserosal fibroids do not seem to affect fertility outcomes, and removal does not confer benefit [6,22].

In contrast to submucosal fibroids, recommendations concerning *intramural* fibroids that cause no distortion of the uterine cavity are far from clear. There is no consensus as to whether intramural fibroids should be removed in women with infertility. Many clinicians would recommend removal of intramural fibroids if they are ≥5 cm in diameter. A study performed by Hart et al. showed lower implantation/pregnancy and ongoing pregnancy rates in women with large (≥5 cm) intramural fibroids [64]; the authors recommend myomectomy in these cases. The procedure should be discussed individually with each patient, taking other potential conditions such as dysmenorrhea or irregular bleeding into account.

Some authors have registered no clear benefits for surgery and do not recommend the approach. However, the limitation of these studies is that they provide no clear information about the size, number, and location of fibroids. Although intramural fibroids are reported to be associated with poorer pregnancy outcomes, women who underwent myomectomy for intramural fibroids experienced no benefit in regard of pregnancy outcomes compared to controls. Regrettably, studies addressing this specific question and included in the Cochrane analysis are scarce and do not provide precise recommendations [22,65].

Conclusion

Pregnancy and live birth rates appear to be reduced in women with submucosal fibroids. Resection of these fibroids improves pregnancy rates. In contrast, subserosal fibroids do not affect fertility outcomes, and their removal does not confer any benefit. Intramural fibroids appear to reduce fertility, but recommendations concerning their treatment remain ambiguous. Myomectomy should be discussed individually with the patient. In addition to the problem of infertility, potential symptoms such as dysmenorrhea or bleeding disorders should be evaluated and included in the indication for surgery. A conclusive analysis of the value of myomectomy for the treatment of intramural fibroids requires further studies with due attention to the size and number of fibroids, as well as their distance to the endometrium.

a. **Submucosal fibroids**: These should be removed before ART or in cases of habitual abortions.
b. **Subserosal fibroids**: As they do not seem to affect pregnancy rates, myomectomy does not appear to be necessary.
c. **Intramural fibroids**: There is controversial data and lack of homogenous opinion.
   - Intramural fibroids ≥5 cm: Perform surgery before ART or in cases of habitual abortion.
   - Intramural fibroids <5 cm: The reported outcome varies between no difference and significantly reduced cumulative pregnancy rates.

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CHAPTER

11

Endometriosis and infertility

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Endometriosis is an estrogen-dependent chronic gynecological disease characterized by the development of endometrial tissue outside the uterus, especially in the ovaries, pelvic peritoneum, and rectovaginal septum. It affects approximately 150 million women worldwide and 7%–12% of women of reproductive age. The incidence of the disease in infertile patients can increase up to 50%, and 80% of unexplained infertility may be associated with endometriosis [1,2]. Endometriosis is seen in 25% of patients who undergo assisted reproductive treatments (ART), and ovarian endometriosis is found in 20%–40% of these patients [3,4]. Because the development of endometrial implants is dependent on ovarian steroids, endometriosis mostly affects women aged 25–35 [5]. Symptoms range from asymptomatic to infertility, but the most common symptoms are dyspareunia, dysmenorrhea, chronic pelvic pain, and irregular uterine bleeding, which are very prevalent in women in reproductive age [6,7]. Despite endometriosis being a disease that seriously reduces the quality of life of the individual, diagnosis is made approximately 6–7 years after the emergence of descriptive symptoms [8].

Pathophysiology

Although endometriosis was described histologically for the first time in 1860 by Rokitansky, the exact mechanism that led to its development is still not fully elucidated [9]. Current data show that endometriosis develops with the effect of a combination of hormonal, immunological, anatomical, and genetic factors. Many theories have been proposed in the pathogenesis of endometriosis (Fig. 11.1). These theories can be divided into two categories: implants originating from the uterine endometrium (transplantation) or implants originating from extrauterine tissues (transformation). In addition, genetic susceptibility can be added to these theories, although the causal relationship with genetic susceptibility in the development of endometriosis has not been adequately revealed yet [10]. Coelomic metaplasia, one of the theories of nonuterine tissue origin, is the differentiation of normal peritoneal cells to endometrial cells with a hormonal or immunological inducing stimulus factor, proposed by Ferguson in the 1960s [11,12]. It is based on the theory that the peritoneum contains undifferentiated cells that can differentiate into endometrial cells [13]. The theory of embryonic Müllerian rests suggests that residual cells remaining from the migration of Müllerian duct maintain the capacity to form endometriotic cells under the influence of estrogen beginning with puberty [14]. The benign metastasis theory is that endometrial cells turn into endometriotic implants in distant tissues by lymphatic or hematological dissemination [15]. Recently, extrauterine or progenitor stem cells originating from bone marrow have been suggested to have the ability to differentiate into endometriotic tissue [16]. Detection of donor-derived endometrial cells that can be distinguished by human leukocyte antigen (HLA) type in endometrial biopsies of women who underwent bone marrow transplant from a single antigen mismatched donor supports the theory that bone marrow–derived mesenchymal stem cells may also turn into endometrial cells [17]. Stem cell theory may also explain how ectopic endometrial lesions can be found in tissues other than the peritoneal cavity, such as lung and central nervous system. Familial predisposition to endometriosis has been known for many years. Women with familial history of endometriosis are sevenfold more susceptible to developing endometriosis themselves [18]. Although the etiology of the disease

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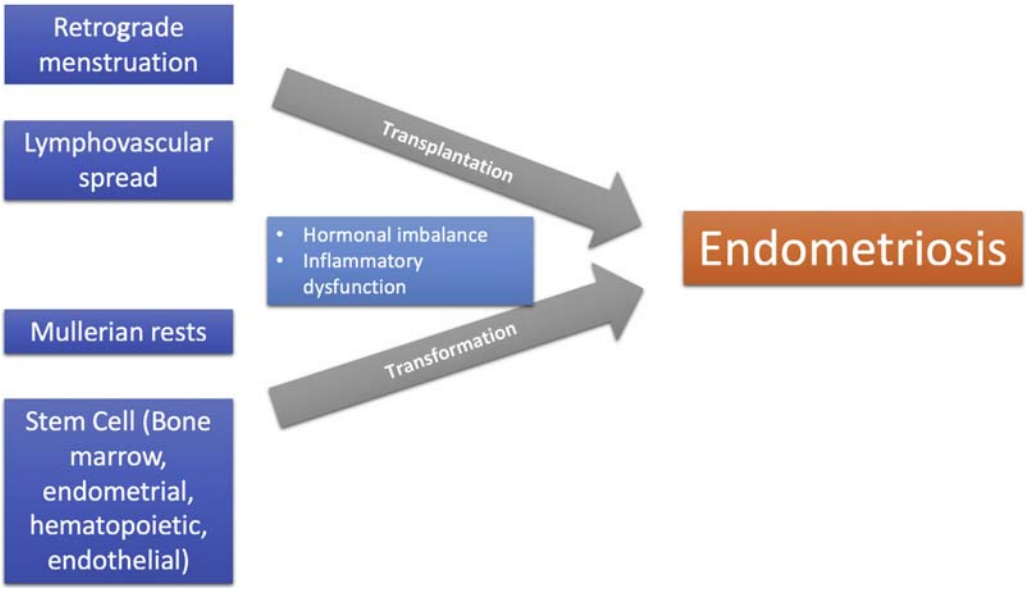

FIGURE 11.1 Theories in the pathogenesis of endometriosis.

has not been fully elucidated, it is thought that genetic polymorphisms may also play a role. Recent meta-analysis suggests that only 5 out of 28 polymorphisms investigated were associated with endometriosis (interferon gamma [IFNG] [CA] repeat, glutathione S-transferase mu 1 [GSTM1] null genotype, glutathione S-transferase pi 1 [GSTP1] rs1695 and wingless-type MMTV integration site family member 4 [WNT4] rs16826658 and rs2235529) [19]. Despite all these theories, the retrograde menstruation theory, first proposed by Sampson in 1927, still continues to be the primary mechanism in the pathogenesis of endometriosis [15]. Endometrial cells that reach the peritoneal cavity by retrograde menstruation attach to peritoneal mesothelial cells and proliferate by blood support. Many subsequent studies support this theory. However, similar rates of retrograde menstruation in women with and without endometriosis suggest that other mechanisms may be effective in its pathogenesis. At this point, with the alterations in the immunity mechanisms, endometriosis arises as a result of insufficient clearance of the endometrial cells from the peritoneal cavity, explaining why some women with retrograde menstruation develop endometriosis while others do not [20].

Potential mechanisms for endometriosis-related infertility

Despite the scientifically supported relationship between endometriosis and infertility, it is difficult to prove a correlation between endometriosis and infertility since endometriosis has impact on fertility status via different mechanism given the heterogeneity of the disease. While the rate of fecundity in couples of normal reproductive age without infertility is 15%–20%, this rate varies between 2% and 10% in women with endometriosis [21,22].

The American Society for Reproductive Medicine (ASRM) scoring system categorizes the disease into four stages; minimal (stage I), mild (II), moderate (III), and severe (IV) [23]. Pregnancy rates after 3 years of unprotected sex are lower in women with mild endometriosis than couples with unexplained infertility (36% vs. 55%) [24], and women with infertility are more likely to have advanced stage endometriosis [25]. Endometriosis disrupts the pelvic anatomy with adhesion and chronic inflammation affects tubal functions adversely. Furthermore, endometriosis has adverse effect on tubal ciliary motility and may lead to irregular myometrial contractions that consequently lead to diminished implantation rates and thus to infertility [26].

Decreased oocyte quality may also be effective in adverse pregnancy outcomes seen in endometriosis patients [27–29]. Since fertility preservation is becoming widespread in endometriosis, the impact of the disease on oocyte quality should be clarified [30,31]. Knowledge in this area is limited, as most of the studies investigated the indirect effects of endometriosis on oocyte quality (i.e., embryo quality, clinical pregnancy rates. and live birth rates, which can also be affected by male partner and many accompanying factors such as implantation and abortion rates) [32].

Increased oxidative stress is a factor in the pathogenesis of endometriosis, and in recent years endometriosis has been thought to modify follicular oxidative stress status [33–35]. Normal spindle structure is essential for adequate cytoplasmic and nuclear maturation and oocyte competence [36], and reactive oxygen species (ROS) reduce oocyte quality by causing meiotic abnormalities and chromosomal instability [37]. Iron-

induced oxidative damage is observed in the follicles surrounding the ovarian endometrioma [38], so increased oxidative stress is considered to provoke spindle deterioration [39]. Zhang et al. reported that ROS-induced stress generates oocyte apoptosis and necrosis in early ovarian follicles [40]. In addition, ROS is a potent stimulator of tissue fibrosis through transforming growth factor-ß (TGF-ß), and chronic fibrosis may lead to progressive decline in ovarian follicle reserve and oocyte quality [41]. However, the results are still controversial because there are studies that did not define any increase in oxidative stress in follicular fluid (FF) of women with endometriosis [42].

In women with moderate/severe endometriosis compared to women with tubal factor infertility, proinflammatory cytokine levels were higher in FF, and follicles having higher concentrations of proinflammatory cytokines were more likely to have immature oocytes. Thus, IL-8 and IL-12 concentrations in mature oocytes were lower, and IL-8 and IL-12 concentrations were found to be significantly higher in FF in endometriosis. These results suggest that endometriosis-induced inflammation in FF may lead to a decrease in oocyte quality [43].

The effects of oocyte morphology on embryo development have not been elucidated yet. However, morphologic defects such as the presence of cytoplasmic granules and/or vacuoles may affect fertilization adversely. Nevertheless, the predictive value of these morphologic changes is limited because of the subjectivity and limitations in evaluation [32]. Furthermore, oocyte morphology may also be affected by other factors such as ovarian stimulation or hormonal milieu [44]. Goud et al. reported that cortical granule loss and zona pellucida hardening causes immature oocyte development in endometriosis [45]. Borges et al. showed that there are extracytoplasmic oocyte defects in endometriosis. However, blastocyst development rate was similar to normal control group. Nevertheless, no information was recorded on blastocyst quality [46].

Disruption in the meiotic spindle apparatus leads to abnormal chromosome segmentation and fertilization. In intracytoplasmic sperm injection (ICSI) cycles, oocytes with normal spindles have higher fertilization and euploidy rates compared to abnormal spindle formation [47]. Results of studies evaluating oocyte spindle morphology in endometriosis are controversial [45,48,49]. It should be kept in mind that the evaluation of spindle morphology in these studies was performed on oocytes in the in vitro maturation protocols and may not reflect the mature oocyte spindle configuration [50].

Through the mechanisms aforementioned, endometriosis leads to ovarian tissue damage and impaired folliculogenesis. Clinical studies demonstrate that endometriosis reduces ovarian reserve. While antral follicle count has traditionally been used in the evaluation of ovarian reserve, serum anti-Müllerian hormone (AMH) measurement has also entered routine practice in recent years [51].When AMH levels of patients were evaluated according to the ASRM classification, there was no difference in AMH levels in women with stage I–II disease compared to healthy control women, and significantly lower AMH levels were found in women with stage III–IV disease [52].When AFC was compared in the ovary with endometrioma and contralateral healthy ovary, a decrease was observed in AFC, which was not observed in other benign cysts [53]. In addition, if left untreated, in women with endometriosis, the reduction in ovarian reserve is progressive and faster than the natural decline [54].

Growing evidence suggests that there is immune system dysregulation in endometriosis, resulting in a chronic inflammatory disease [55]. The number of activated macrophages, MAST, T cells, and natural killer cells in peritoneal fluid increases in women with endometriosis, and there are significant differences in cytokine/chemokine profile [56–58]. A protein that resembles haptoglobulin structurally and that decreases the phagocytic capacity of macrophages by binding and additionally that increases IL-6 production was identified in peritoneal fluid in women with endometriosis [59]. Other cytokines that are found to increase in peritoneal fluid are macrophage migration inhibitory factor, TNF-α, IL-1ß, IL-6, and IL-8 [60–62]. On the other hand, whether this change in cytokine profile is a cause or effect should be clarified.

In natural conception, fertilization occurs in the ampulla, at the distal end of the fallopian tubes. The ampulla is exposed to the peritoneal fluid, and it can be thought that these inflammatory changes in the peritoneal fluid may affect natural conception [63]. Fertilization depends on the physiological processes of spermatozoa that are controlled under the influence of the female reproductive tract such as hyperactivation, capacitation, acrosome reaction, and attachment to the zona pellucida [64]. One can assume the interactions between spermatozoa and peritoneal fluid can last for days until ovulation occurs [65]. Although contradictory results have been reported, increased macrophage activity in peritoneal fluid causes immobilization of sperm in endometriosis [66]. In addition, endometriosis may impair acrosome reaction, lead to DNA damage, and decrease oocyte binding capacity [67–69]. Increased levels of TNF α [70], IL-1 [71], migration inhibitory factor [72], and the RANTES (regulated upon activation, normal T cell expressed and secreted) in the peritoneal fluid may adversely affect sperm function [73]. Furthermore, in vitro studies have reported that cytokines affect fertilization capacity by stimulating lipid peroxidation in the sperm plasma membrane [74,75].

There are higher miscarriage rates in endometriosis [76]. Chronic inflammation impairs endometrial receptivity without causing morphologic changes [77–81].

The SART (the Society of Assisted Reproductive Technology) study, in which 347,185 fresh and frozen ART cycles were retrospectively evaluated, indicates reduced implantation rates in endometriosis [82]. In previous studies, it was shown that there is an interaction between eutopic endometrium and endometriotic implants and molecular, biochemical, and cellular differences were found in the endometrium of women with endometriosis [83–85]. These alterations may reflect the state of the disease and the causal relationship between endometriosis and infertility.

Endometrial stromal cells (ESCs) may detect embryo quality [83] and progesterone withdrawal in the inflammatory cascade associated with menstruation [86]. In a recent study, it was reported that ESCs in severe endometriosis release inflammatory cytokines and act as a biological sensor of the *hyperactive inflammatory niche* during the implantation window [87]. These cytokines have detrimental effects on ovarian functions, preimplantation embryo development, and blastocyst implantation [32,88–91]. Additionally, Anupa et al. determined higher concentrations of IL-18 in the control group ESCs compared to the ESCs with ovarian endometriosis, correlated with previous findings [87,92,93]. IL-18 is a cytokine belonging to IL-1 cytokine family, and its dysregulation is associated with inflammatory diseases [94]. A certain level of IL-18 release from uterine cells is important for a successful pregnancy. IL-18 provides conversion of immune system balance from Th-1 to Th-2 depending on its concentration and ratio to other regulators and fine-tunes endometrial status and functions [95–99]. The immune phenotype of this secretory phase endometrium seen in endometriosis may be one of the mechanisms leading to primary infertility. A recent study reported that chronic endometritis is 2.7 times more common in women with endometriosis [78]. Chronic endometritis impairs normal uterine contractility, and this may facilitate the development of endometriosis by causing retrograde reflux [100].

There are studies reporting that aberrant gene expression in the eutopic and ectopic endometrium may lead to infertility in endometriosis. Guo and Taylor have reported changes in *HomeoboxA10/HOXA10* gene expression in the eutopic endometrium in women with endometriosis [101]. *HOXA10* controls embryonic development and functional differentiation in uterine organogenesis in adults [102]. *HOXA10* gene expression in healthy women is cycle dependent [103]. *HOXA10* mRNA levels increase dramatically in the mid-secretory phase, which corresponds to embryo implantation, histological peak differentiation, and systemic high estrogen and progesterone time [104]. High level of *HOXA10* expression in the endometrium is required for the decidual transformation of endometrial cells. However, this increase in *HOXA10* gene expression is not seen in women with endometriosis [105], and defects in *HOXA10* expression and regulation lead to inadequate implantation and decidualization, resulting in recurrent miscarriages and infertility [106].

Expression of endometrial biomarkers in endometriosis differs from normal women [107,108]. Previously, a decrease in the expression of endometrial proteins involved in embryo attachment and invasion has been reported in endometriosis [109,110]. Endometrial integrins are cell surface receptors for the extracellular matrix, and specific key integrins including the $\alpha v\beta 3$ integrin are involved in implantation [111–113]. However, this integrin is reduced in women with endometriosis and infertility and unexplained infertility [114].

Aromatase converts androstenedione and testosterone to estrone and estradiol. Abnormal levels of aromatase are present in both endometriotic implants and eutopic endometrium, and this causes increased estradiol production [115]. Alterations in estrogen-progesterone balance may impair implantation and lead to disease progression [116]. Progesterone resistance and dysregulation of progesterone receptors also play a role in implantation failure. Progesterone has an important role in the development of normal pregnancy as it induces endometrial decidualization in luteal phase, and progesterone receptor alterations have been noted in both eutopic and ectopic endometrium in endometriosis [117]. While receptor downregulation occurs in normal endometrium before implantation, it is delayed in endometriosis [118]. Eventually, there is an estrogen dominant environment that is not appropriate for implantation as a result of progesterone resistance [119,120].

Management of endometriosis-associated infertility

Spontaneous conception medical treatments in endometriosis (oral contraceptives, progestins, gonadotropin releasing hormone agonists) act by blocking ovarian functions and are used to reduce pain and the risk of recurrence after surgery [121,122]. Contrary to previous beliefs, fecundity does not return after treatment is discontinued. Therefore, medical treatments are not recommended in the treatment of infertility in endometriosis [123,124]. The efficacy of medical therapy as an adjuvant or neoadjuvant to surgical treatment in the treatment of infertility has not yet been revealed [122,125].

Effect of surgical treatment of endometrial lesions on conception is conflicting. The contradiction in this regard stems from different forms of endometriosis (superficial endometriosis, endometriomas, and deep infiltrating endometriosis), different surgical techniques, and differences in fertility evaluations [123].

Ovarian damage during the surgery is the main concern in the surgical treatment, and many attempts have been made to minimize detrimental effects of surgery [126,127]. Cystectomy instead of drainage and coagulation is the preferred method in endometrioma surgery because of the lower risk of recurrence and higher postoperative spontaneous pregnancy rates, especially if the endometrioma is 3 cm or larger in diameter [128]. However, stripping technique for endometrioma excision may damage normal healthy ovarian tissue [129,130], and excision of the ovarian tissue along with the wall of the cyst can lead to follicle loss and a decrease in ovarian reserve [131]. In addition, electrocoagulation may cause thermal damage, resulting in a sudden decrease in AMH levels after surgery [132]. ESHRE recommends clinicians who will perform endometrioma surgery to give consultancy to women before the surgery on the possibility of postoperative decline in ovarian functions and oophorectomy [125].

When evaluating the damage caused by endometriotic implants to the surrounding tissue in women with mild endometriosis (ASRM Stage I–II), operative laparoscopy (excision of endometrial lesions, ablation, and adhesiolysis) is superior to diagnostic laparoscopy to increase spontaneous pregnancy rates [133]. Operative laparoscopy can increase spontaneous pregnancy rates and live birth rates compared to expectant treatment in minimal and mild endometriosis (ASRM Stage I–II) [134,135]. In moderate to severe endometriosis (ASRM Stage III–IV), surgery may be useful in the treatment of pelvic adhesions that interfere with reproductive mechanisms. However, since there are no randomized controlled studies comparing postoperative pregnancy rates in these patients, a strong consensus on this issue has not yet been reached [41]. In a recent meta-analysis, Hodgson et al. reported that surgery alone or gonadotropin hormone-releasing hormone (GnRH) agonist therapy alone can improve fertility outcomes in women with endometriosis and infertility [136]. This finding obtained as a result of the meta-analysis is consistent with the evidence in the literature regarding the beneficial effects of surgery, but the effectiveness of GnRH agonist therapy is not compatible with published systematic reviews [137] or clinical guidelines [125,138]. This different result may be due to the use of indirect evidence. Nevertheless, the evidence from the comparison of GnRH agonist alone with placebo therapy is limited. Reproductive outcomes were similar in a medium-quality randomised controlled trial (RCT) that included 450 women with endometriosis and compared GnRH agonist therapy alone, laparoscopic surgery alone, and the combination of the two [139]. GnRH agonists generate a hypogonadal state in endometriosis and reduce estrogen support and disease progression [140]. GnRH agonists also improve pregnancy rates by causing a temporary reduction in the burden of the disease and by ameliorating adhesions and distorted anatomy that affects oocyte release and transport. GnRH analog therapy increases endometrial integrin levels that are inadequate in the eutopic endometrium [141]. Clinicians should choose the treatment modality according to many factors such as medical comorbidities, surgical risk, and expected anatomic spread of the disease.

Conception by ART: Intrauterine insemination (IUI) is a simpler treatment method compared to in-vitro fertilization (IVF) treatment. IUI combined with controlled ovarian stimulation (COS) can be used instead of IVF, IUI alone, or an advanced surgical therapy in women with surgically diagnosed and treated ASRM stage I–II endometriosis [142,143]. Comparing COS-IUI cycles in women with unexplained infertility and women with minimal-mild endometriosis, lower pregnancy rates were found in women with endometriosis [144]. IUI is rarely tried in moderate to severe endometriosis due to pelvic adhesions and decreased tubal functions, and IVF should be considered the first option in these cases.

It is not clear which protocol should be used for ovarian stimulation in endometriosis. ESHRE guidelines recommend ultra-long protocol to improve clinical pregnancy rates [125]. However, this recommendation is based on a meta-analysis of three randomized controlled trials, and it cannot be determined whether the better pregnancy outcomes are due to better oocyte quality or better endometrial receptivity [145]. In a recent meta-analysis, ultra-long protocol was found to enhance the clinical pregnancy and implantation rates compared to GnRH-a long protocol [146]. In that meta-analysis, when subgroup analysis was performed according to the endometriosis stage in randomized controlled trials, while ultra-long protocol compared with GnRH-a long protocol showed statistically significantly better clinical pregnancy rates in stage III–IV endometriosis patients, no difference was found in stage I–II endometriosis patients. In addition, when ultra-long protocol and long protocol were compared in non-RCT studies, the pregnancy outcomes were found to be similar [146]. Finally, the Cochrane review also reported that the impact of long-term GnRH-a treatment on live birth rates in women with stage I–II or stage III–IV endometriosis compared with conventional IVF/ICSI therapy is uncertain [147].

Current literature indicates inconsistent results on the impact of endometriosis on ART outcomes. In the line with the aforementioned mechanisms, primordial follicular reserve is found to be significantly lower in endometriosis. Patients with endometriosis tend to have fewer oocytes and higher cancellation rates for inadequate response to ovarian stimulation than age-matched patients without endometriosis. Although lower mean number of oocytes and embryos are

obtained in ART cycles in endometriosis, live birth rates are similar to other causes of infertility [148]. Besides, there is a lack of evidence on the fetal and obstetric complications of endometriosis patients after IVF treatment. In a recent meta-analysis, endometriosis was associated with preterm delivery (50% higher risk than controls), caesarean section delivery (73% higher risk), placenta previa (>3 fold risk), and neonatal intensive care unit admission after delivery (twofold increased risk) [149]. These findings were attributed to differential modulation of endometrium, as described above, in implantation and placentation.

The effect of endometrioma on ovarian response is also conflicting in controlled studies, and ovarian responses were similar in women with unilateral endometrioma compared with the contralateral ovary [150,151]. But the size of the endometrioma was small in most of the studies included in these reviews. However, two recent studies in larger endometriomas suggest that the size of the endometrioma may affect the ovarian response above a certain threshold [152,153]. Somigliana et al. retrospectively compared ovarian responses in 67 women with unilateral endometrioma in the affected and the contralateral unaffected gonads, and indicated a statistically significant difference in ovarian response only in women with endometrioma size 40–49 mm [154]. However, with these findings, surgical resection of the endometrioma before IVF can not be affirmed to be effective in overcoming these adverse effects on ovarian response in women with endometriomas of $\geq$ 4 cm [154].

Although there is insufficient evidence to demonstrate the beneficial effect of surgical treatment of endometrioma on pregnancy outcomes before IVF/ICSI cycles, conservative management of women with endometrioma and scheduled for IVF treatment is questioned not only due to a decrease in ovarian response and oocyte competence, but also due to technical difficulties during oocyte retrieval, risk of pelvic organ injury because of the distorted anatomy, risk of infection, abscess formation, contamination of FF with endometrioma content, and missing an occult malignancy. However, according to the limited data, the risk of technical difficulties during oocyte pick-up is low, and there is no data that endometrioma surgery will prevent adhesion formation and facilitate the oocyte pick-up effectively. Currently available evidence indicates that there is no endometriosis and endometrioma progression with IVF/ICSI treatments. The risk of contamination of the FF with the endometrioma content is 2.8%–6.1% [155,156] and the risk of endometrioma infection is 1.9% [157], and prophylactic surgery is not recommended before IVF/ICSI. However, women with endometrioma should be informed about the risk of infection before oocyte retrieval, should use wider spectrum antibiotics, and should be monitored more closely after the procedure [150]. Considering that the risk of baseline malignancy in endometriomas is 0.8%–0.9% [158,159], during IVF/ICSI treatment cycle, the risk of missing an occult malignancy in the endometrioma is very low, but it should not be forgotten that although it is rare, the lifetime risk of developing ovarian cancer increases from 1% to 2% in the presence of endometrioma [160].

In conclusion, the decision for surgical treatment of the endometrioma before ART should be carefully considered, individualized, and the treatment plan should be based on the detailed factors that may affect the ART outcome, such as woman's age, ovarian reserve, presence of the cyst unilaterally or bilaterally, endometrioma size and number, symptoms, presence of radiological features suggestive of malignancy, and previous history of surgery [161]. IVF/ICSI may be preferred in women who are asymptomatic, advanced in age, who have diminished ovarian reserve, bilateral endometrioma, or a previous history of endometrioma surgery. In these cases, pituitary downregulation treatment may be beneficial before IVF/ICSI [145]. Surgical treatment can be opted in women who are symptomatic, young, with good ovarian reserve, and who have unilateral and large cysts or suspected malignancy.

Fertility preservation in women with endometriosis

Fertility preservation techniques have been developed to secure reproductive potential in women who will be treated for cancer with gonadotoxic treatment. With advancing technology, decreasing costs, increased accessibility and clinical experience, fertility preservation is now performed with broader indications, including "social egg freezing," which is to preserve fertility against decreasing oocyte quality and ovarian reserve with advancing age. The reduction of ovarian reserve is progressive and faster than the natural decline in women with endometriosis, if left untreated [54]. Additionally, considering the destructive effects of endometrioma surgery on ovarian reserve, patients should be informed about fertility preservation before endometrioma surgery. Therefore, women with endometriosis should receive individualized counseling regarding fertility preservation. This counseling should be based on the age of the patient, the severity of the disease, the presence of endometrioma, and the history of previous surgery.

Embryo cryopreservation is an effective method of preserving fertility, but a male partner is needed, and it brings many ethical and legal problems when the couple separates or one of the partners dies. However, since oocyte cryopreservation does not require a male

partner in fertility preservation and is accepted as a validated technique by important associations, it has now entered routine practice as a standard approach in fertility preservation [162]. In addition, following the advancements in vitrification methods, similar results are obtained with cryopreserved oocytes compared to fresh oocytes [163]. On the other hand, provided that oocytes are stored with tightly controlled systems, there are no known biological factors that limit the storage time [164]. Since the ovarian reserve is already low in patients with endometriosis, the number of oocytes obtained can be increased with repetitive stimulations. Since the number of oocytes retrieved in fertility preservation is the main concern, not the implantation, the antagonist protocol may be more effective in terms of time.

Ovarian tissue cryopreservation (OTCP) is widely used in young women receiving chemo-radiotherapy. OTCP is also used in some benign cases with high risk of premature ovarian failure [165]. However, it is not recommended to use this method in fertility preservation in women with endometriosis because the procedure is technically difficult due to pelvic adhesions [31]. Besides taking healthy cortical tissue separate from endometriomas further reduces ovarian reserve in the future. However, in patients scheduled for endometrioma surgery, during resection, healthy parts of the ovarian cortex can be separated and cryopreserved. The healthy fragments of cortex to be frozen may be pieces of the tissue attached to the capsule removed during the resection. Ovarian tissue removal can be done in any center where endometriosis surgery is performed. Since the tissue can be transported safely to the fertility preservation center before cryopreservation, there is no need for the patient to be directed to another center [31].

Women with endometriosis should definitely seek fertility preservation counseling based on prognostic factors, and while techniques can be used separately, combined fertility preservation methods can be opted in patients scheduled for surgery.

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CHAPTER

12

Endocrinological causes of male infertility

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Introduction

Spermatogenesis is a unique form of cell division resulting in the production of sperm. It is initiated and maintained in the seminiferous tubules in the testis under the direct control of follicle-stimulating hormone (FSH) and testosterone. Testosterone is produced by Leydig cells in the interstitium of the testis by the effect of luteinizing hormone (LH). The gonadotropins, FSH and LH, are produced by the pituitary gland under control of gonadotropin-releasing hormone (GnRH) secreted by the hypothalamus [1]. Any defect in this hypothalamo-pituitary-testicular axis results in a decreased level of FSH and testosterone and impaired spermatogenesis. On the other hand, intrinsic testicular insults result in an impaired spermatogenesis with a poor response to the stimulatory effect of FSH and testosterone and a pituitary increased production of gonadotropins.

Any endocrinological disorder affecting the hypothalamo-pituitary-testicular axis will cause an infertility problem. Hypogonadism, either primary (hypergonadotrophic hypogonadism) or secondary (hypogonadotrophic hypogonadism), is a clinical syndrome of low serum testosterone level resulting in systemic features of hypotestosteronemia and impaired production of normal sperm [2]. Other examples of endocrinological causes of male infertility are hyperprolactinemia and thyroid disorders [3,4].

Studying the serum levels of the hormones that play a role in the control of spermatogenesis is required in all cases of azoospermia and oligospermia and in some cases of asthenospermia and teratospermia. After full evaluation, an endocrinological disorder behind the infertility problem might be reached, and accordingly, we can start a specific treatment to correct the serum hormone level and restore the endocrinological functions. In cases with no clear endocrinological etiology and in idiopathic infertility, an empiric hormonal treatment is used with a considerable degree of success improving sperm production [5].

Hormonal actions on spermatogenesis

Spermatogenesis, which reflects the fertility status of men, is a hormone-dependent process. Many details of the action and requirements of these hormones are not very clear. But, genetic and pharmacological studies using cell-specific ablation of androgen receptor have confirmed many clear facts. The primary role of FSH is initiation of spermatogenesis and stimulation of Sertoli cell proliferation and determining the number of germ cells at the time of puberty [6]. In addition, FSH has an important role in maintenance of spermatogenesis. Marked reduction in all spermatogenic cells up to the stage of round spermatids is seen following the reduction of FSH after hypophysectomy or treatment with GnRH antagonist. FSH treatment increases all spermatogenic cells prior to elongated spermatids. FSH has another role in spermatogenesis as it may synergize with testosterone by stimulating the synthesis of the androgen receptor. It is suggested that FSH has a role in facilitating the transport and localization of testosterone within Sertoli cells [7].

The role of testosterone is maintenance of spermatogenesis, and it is responsible for maturation of round spermatids into mature sperm. Testosterone has a role in keeping the adhesion between germ cells and Sertoli cells. Testosterone withdrawal leads to premature release of round spermatids. High intratesticular level of testosterone is essential for normal spermatogenesis. Androgen receptors in the testis are required to be saturated with testosterone more than other androgen-dependent tissues. In conclusion both FSH and testosterone are required for initiation and maintenance of spermatogenesis [7].

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Hormonal regulations of spermatogenesis

Understanding the hypothalamo-pituitary-gonadal axis (Fig. 12.1) and the enzyme system that works on related hormones is required to study endocrinological causes of male infertility and to plan a treatment protocol for it. GnRH, which is secreted in a pulsatile manner, enters the pituitary portal circulation and stimulates the gonadotroph cells in the anterior pituitary gland to release FSH and LH. FSH is responsible for initiation and maintenance of spermatogenesis and stimulates the secretion of inhibin B hormone by the Sertoli cells, which has a negative feedback effect at the level of the pituitary gland, decreasing FSH secretion [8].

LH stimulates the testosterone synthesis in the Leydig cells. Testosterone is required for maintenance of normal spermatogenesis. Testosterone has a negative inhibitory feedback effect at the level of the pituitary gland and the hypothalamus. Testosterone is converted by 5-alpha reductase enzyme into dihydrotestosterone (DHT), which is not essential in the process of spermatogenesis, and is responsible for the growth of the prostates and external genital organs and for the development of secondary sexual characters. DHT has also a negative inhibitory feedback at the level of the pituitary gland and the hypothalamus. Testosterone is converted by aromatase enzyme in fatty tissues into estradiol, which has an inhibitory effect at the level of the pituitary gland, decreasing LH secretion, and at the level of the hypothalamus by decreasing GnRH secretion at the hypothalamus [9]. Any conditions affecting the level of these hormones or enzymes may lead to a decrease in the intratesticular level of FSH and testosterone and subsequently suppression of spermatogenesis.

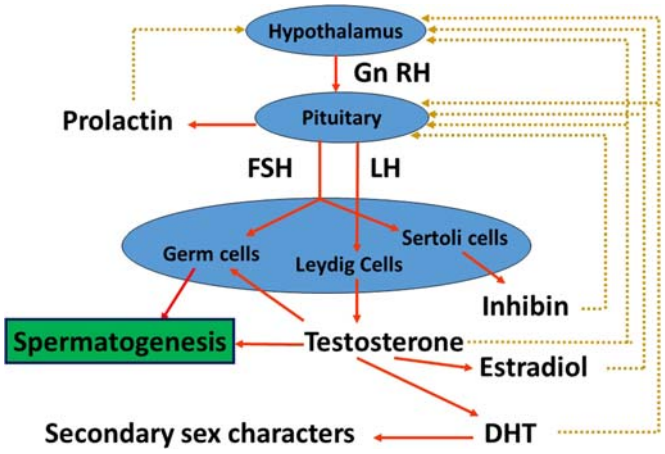

FIGURE 12.1 Hypothalamo-pituitary-gonadal axis.

Endocrine conditions associated with male infertility

Male hypogonadism

Male hypogonadism is a clinical syndrome of lack of testosterone secretion to a normal physiological level resulting in failure of development of masculine body features and/or failure of production of normal testicular sperm. Hypogonadism may be due to congenital or acquired causes, and its clinical presentation depends on the time of presentation and associated hormonal or other system dysfunction. Congenital hypogonadism may be presented with underdeveloped genitalia, small penis and bilateral small testes, and decreased or absent body and facial hair. Late onset hypogonadism may be presented with decreased bone mineral density, decreased muscle strength, visceral obesity, loss of libido, erectile dysfunction, and infertility [2,10].

Hypogonadism may be classified into primary, resulting from testicular failure (hypergonadotrophic hypogonadism), or secondary as a consequence of hypothalamus and/or pituitary dysfunction (hypogonadotrophic hypogonadism). This classification is important from a therapeutic point of view. In men with secondary (hypogonadotropic) hypogonadism, like Kallmann syndrome and isolated hypogonadotropic hypogonadism syndrome, hormonal replacement therapy can successfully induce fertility [2,10]. But, in men with primary (hypergonadotrophic) hypogonadism, like Klinefelter syndrome and androgen insensitivity syndrome, the only treatment for them is microtesticular sperm extraction (TESE) for intracytoplasmic sperm injection [2].

Hyperprolactinemia

Elevated secretion of prolactin has serious effect on sexual activity and fertility. Hyperprolactinemia is often caused by prolactin-secreting pituitary tumors (prolactinomas). It is also seen in chronic renal failure and in primary hypothyroidism and may result from systemic use of some drugs like dopamine antagonists, dopamine synthesis inhibitors, opiates, calcium channel blockers, and H2-blockers. Prolactin has a negative feedback effect at the level of the hypothalamus, and hyperprolactinaemia suppresses the pulsatile secretion of GnRH from the hypothalamus, thus decreasing FSH and LH secretion, with subsequent decrease in testicular secretion of testosterone [11]. Patients with hyperprolactinemia in urology or andrology clinics present with depressed libido and erectile dysfunction and rarely gynecomastia and galactorrhea. Usually, spermatogenesis is affected late after sexual dysfunction [3].

Thyroid disorders

Thyroid disorders, hypo- and hyperthyroidism, affect spermatogenesis by their effect on the hypothalamus and pituitary and on the level of FSH, LH, and GnRH. It is noticed that postpubertal hypothyroidism might decrease semen volume and sperm forward motility and percent of normal sperm morphology [4,12]. Primary hypothyroidism results in a decrease of sex hormone-binding globulin (SHBG) and total testosterone concentrations [12].

In hyperthyroidism, FSH and LH responses to GnRH may be exaggerated, thus increasing the level of FSH and LH. SHBG is elevated and total testosterone is increased. Free testosterone is usually reduced or does not change. The metabolic clearance of testosterone is reduced and the circulating estradiol levels are elevated [4,12]. This altered testosterone-estradiol ratio may explain infertility and any developed gynecomastia in hyperparathyroidism [13].

Obesity

Disturbances in spermatogenesis are seen frequently in men with high body mass index in the form of decrease in sperm concentration and motility and an increase in sperm DNA damage [14]. The strong association between obesity and hypotestosteronemia is a point of study in many series. Large prevalence of hypogonadotropic hypogonadism was proven in men with moderate to severe obesity [15]. Testosterone is converted by aromatase enzyme into estrogen in the peripheral fatty tissues. It is assumed that in obese men, with increased peripheral fat, there is much increase in the peripheral conversion of testosterone and much higher level of estrogen, which via negative feedback decreases the pituitary secretion of gonadotropin and causes acquired hypogonadotropic hypogonadism [16–18].

Exogenous administration of testosterone and anabolic steroid

Exogenous administration of testosterone induces feedback inhibition on the hypothalamo-pituitary-testicular axis leading to reduction of hypothalamus secretion of GnRH, pituitary secretion of FSH and LH, and Leydig cells secretion of testosterone and consequently a decrease of intratesticular testosterone. Low FSH and testosterone may cause azoospermia or oligospermia associated with abnormal sperm motility and morphology [19]. This long-lasting or possibly persistent inhibitory effect of exogenous testosterone and anabolic steroids on spermatogenesis is supported by finding a decreased number of Leydig cells in those patients, and after drug discontinuation, Leydig cells proliferate to below normal counts [20].

Management of spermatogenic suppression following exogenous testosterone administration requires cessation of the exogenous testosterone and administration of human chorionic gonadotropin (hCG) and human menopausal gonadotropin (hMG) [21].

Evaluation of an infertile man from an endocrinological point of view

Endocrinological causes of male infertility are usually suspected during the initial evaluation of infertile men. In history taking, delayed puberty, previous or current use of anabolic steroids, alcohol consumption, decreased sexual desire, and erectile dysfunction may indicate an endocrinological etiology behind the infertility problem. In examination, we should carefully search for signs of endocrine disorders such as abnormal body configuration, decreased masculine features, scanty pubic hair, gynecomastia, and small penis and testes [22].

After semen analysis, hormonal assessment in infertile men is required in all cases with azoospermia and oligospermia and in some cases of asthenospermia and teratospermia. In the initial assessment, we need to have FSH and total testosterone levels. In cases of abnormal FSH and total testosterone levels, it is required to have a full hormonal evaluation measuring LH, estradiol, and prolactin. Thyroid hormone is required in some cases [22,23].

Therapies

Based on hormonal assessment, hormonal treatment of infertile men could be classified into a specific treatment for endocrinological disorder or empiric hormonal treatment for semen abnormalities due to nonendocrinological causes and in idiopathic infertility.

In specific hormonal treatment, our target is to reach a normal level of FSH and testosterone. Endocrinological disorders with low gonadotropins and low testosterone are described as a secondary testicular failure, and the defect is in the hypothalamus or the pituitary and can be treated, with a high level of success, with hormone replacement therapy [24]. Unfortunately, in primary testicular failure with a high level of gonadotropins and low testosterone, it is meaningless to administer exogenous gonadotropins, and the only treatment option in these cases is testicular sperm extraction with a hope of finding enough sperm for intracytoplasmic sperm injection [25].

Empiric hormonal treatment is used in nonendocrinological causes of semen abnormalities as in patients

with a history of testicular insult and in idiopathic male infertility. The goal of the empiric hormonal treatment is to reach a required level of FSH and total testosterone to stimulate spermatogenesis. This required level is expected to be much higher than its normal levels if there is any degree of testicular damage causing its hypofunction [25].

Both FSH and testosterone are essential for stimulation of spermatogenesis, and reaching a high enough level of both of them is the target of any hormonal treatment. Administration of exogenous testosterone, by a negative feedback effect at the level of the hypothalamus and pituitary, inhibits testicular production of testosterone and decreases intratesticular level of testosterone. So, the treatment of choice is a course of GnRH or gonadotropins or any of the drugs that stimulate pituitary production of FSH and LH and subsequently testicular production of testosterone. Drugs that induce endogenous production of gonadotropins and testosterone include antiestrogens and aromatase inhibitors [24–26].

GnRH

GnRH can be administrated in a pulsatile form via a special mini-pump with a subcutaneous needle. It starts with a dose of 4 L(mu)g per pulse [27]. It is effective only when the pituitary is intact and the hypogonadotropic hypogonadism is caused by a hypothalamus hypofunction and GnRH deficiency. It is not commonly used because of its difficult application and dosing [26].

Gonadotropin

Exogenous gonadotropin treatments include the use of hCG and hMG. hCG is analogous to LH, and it stimulates the Leydig cell secretion of testosterone. hMG has both LH and FSH activity. Gonadotropin administration is effective in the treatment of hypogonadotropic hypogonadism. It is also used for treating normogonadotropic oligospermia and azoospermia [28,29].

Antiestrogens

Clomiphene and tamoxifen are the most commonly used drugs as hormonal stimulants for spermatogenesis in idiopathic oligospermia [30,31]. The antiestrogens indirectly stimulate the secretion of FSH and LH by blocking estrogen receptors in the hypothalamus, which increases the release of GnRH. Clomiphene citrate is normally prescribed in a 25-mg daily oral dose (12.5–400 mg/day). Higher doses may cause downregulation of the system [32]. Men treated with clomiphene citrate consistently demonstrate an elevation in serum FSH, LH, and testosterone levels. As a result, serum gonadotropins and testosterone must be monitored to ensure that the testosterone level remains within normal limits, because higher levels may negatively influence spermatogenesis. A small number of patients may suffer deterioration in semen quality with antiestrogen therapy. Therefore, frequent semen analysis is essential during follow-up [33]. Side effects of clomiphene therapy are usually mild and occur in less than 5% of patients. They include nausea, headache, dizziness, weight gain, alterations in libido, visual field changes, gynecomastia, and allergic dermatitis [31].

Tamoxifen citrate has less estrogenic activity than clomiphene citrate. Doses range from 10 to 30 mg orally per day. Side effects are similar to those seen with clomiphene citrate but occur with lower frequency because of its weaker estrogenic properties [34].

Aromatase inhibitors

Aromatase inhibitors are widely used to treat oligospermia and azoospermia specifically if estradiol level is above normal or in case of low testosterone-estradiol ratio. Within fat cells, aromatase enzyme converts the circulating testosterone into estrogen. Markedly obese men may have an excessive endogenous conversion of testosterone into estrogen. In theory, an alteration in the ratios of estrogen and testosterone systemically or within the testis could decrease pituitary levels of LH and FSH and impair sperm production [35,36].

Normal fertile men have a T/E 2 ratio of 16 ± 3; men with nonobstructing azoospermia (NOA) have a ratio of 7. Aromatase inhibitors block the conversion of testosterone to estrogen, thereby enhancing spermatogenesis. Raman and Schlegel used anastrazole and testolactone in 140 patients with oligospermia and low testosterone and a low T/E 2 ratio and found a significant increase in sperm count and motility in addition to increases in the level of testosterone [37].

Patry et al. reported a case of a 31-year-old man with NOA and normal FSH. The patient was given the aromatase inhibitor letrozole for 4 months and repeated FSH, and testicular biopsy. Testis biopsy showed normal spermatogenesis following 4 months of letrozole therapy [38]. Cavallini et al. used the aromatase inhibitor letrozole in four men with NOA and normal hormonal profile for 3 months and found that all patients showed spermatozoa in their ejaculate after treatment [39].

The protocol of hormonal treatment of infertile men

It is clear that the only two hormones having a direct action on spermatogenesis are FSH and testosterone.

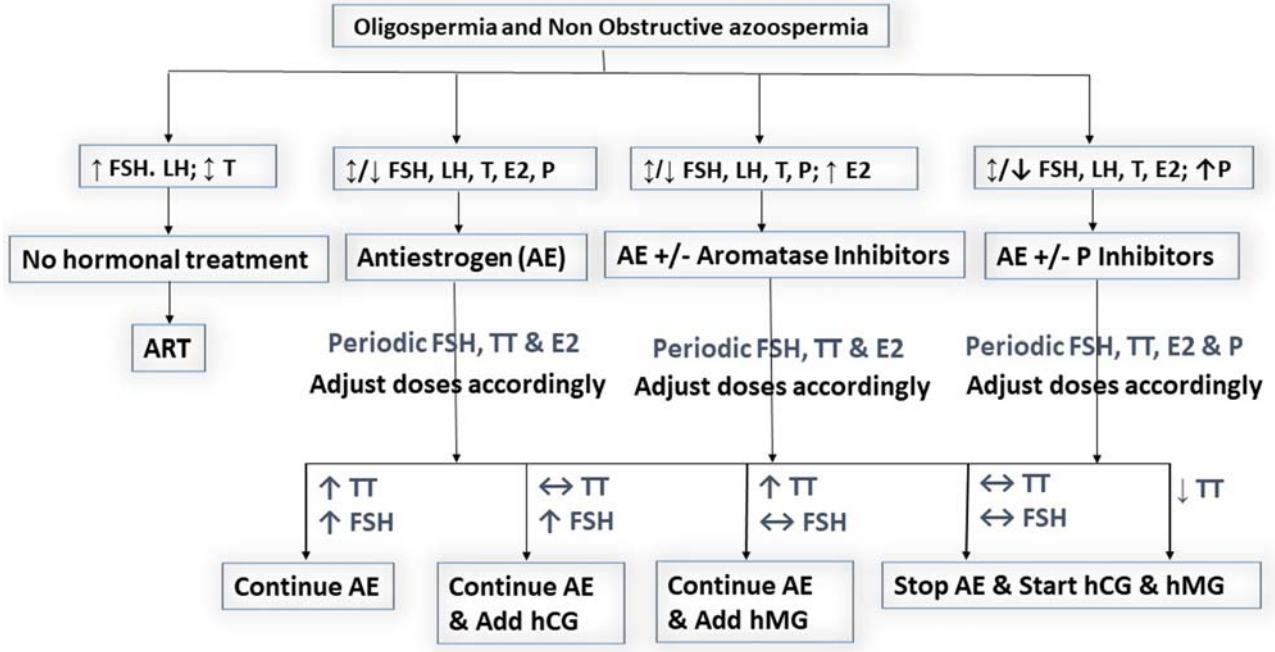

FIGURE 12.2 Algorythm for hormonal treatment for idiopathic oligospermia and nonobstructing azoospermia.

GnRH, LH, estradiol, and prolactin play a role in hormonal regulation of spermatogenesis by stimulatory and negative feedback inhibitory effects on the pituitary gland and testicular Leydig cells to a maintain an optimum level of FSH and testosterone. So, the target of any treatment protocol is to reach an effective level of FSH and testosterone to adequately stimulate spermatogenesis.

It is wise to start with the easily administrated and cheap drugs when effective. That is why the protocol of many centers starts with antiestrogen or aromatase inhibitors. Aromatase inhibitors are specifically the first choice of treatment when estradiol is high or in cases of low testosterone-estradiol ratio [27,40].

Clomiphene is successful in some cases of oligospermia to improve sperm production and might be useful in nonobstructive azoospermia to demonstrate sperm in the ejaculate, potentially improving outcomes of TESE in patients who remain azoospermic [5].

The response to clomiphene is not identical in all patients. Patients differ in the dose and regimen required to achieve the target level of testosterone and FSH. Some patients do not reach the target level of serum testosterone and FSH even if we use the maximum dose of clomiphene. Some patients respond to clomiphene treatment by an obvious increase in FSH without an increase in testosterone. A few patients respond to clomiphene by an unexpected decrease in testosterone that is also manifested with a decrease in sexual desire [25].

Based on these findings, it is necessary to monitor serum level of FSH, testosterone, and estradiol during clomiphene citrate treatment to adjust the dose of clomiphene citrate or to replace it when necessary with aromatase inhibitors or hCG and hMG. Fig. 12.2 demonstrates a simple protocol for treatment of oligospermia and nonobstructive azoospermia prior to TESE [27,40].

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CHAPTER

13

Sperm DNA fragmentation: impact on ART outcome

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Introduction

Infertility is characterized by failure of a couple to achieve clinical pregnancy after 12 months of regular, unprotected sexual intercourse [1]. Male factors strongly influence natural conception and reproductive outcomes to the extent that they are involved in 50% of cases of infertility overall, whether alone or in combination with female factors [2].

Semen analysis (SA) serves as the cornerstone test for male fertility evaluation. While it provides a description of important semen parameters including sperm concentration, motility, and morphology, it alone cannot predict male fertility potential or the success of natural or assisted reproduction, especially considering that 15% of infertile men have SA values classified as normal [3]. The limitations of SA can be attributed to several factors, including inability of conventional SA to analyze functional aspects of spermatozoa such as their ability to fertilize oocytes, variability between laboratories in terms of quality control and standardization of SA, lack of representation of the cut-off values for SA for all men given geographic or ethnic differences, as well as differences in semen characteristics between different ejaculates from the same individual [3,4]. Furthermore, SA does not reflect sperm DNA integrity, nor does it provide an insight into the cellular and molecular processes that lead to successful fertilization.

Sperm DNA fragmentation (SDF) is one of those molecular parameters that can be used to evaluate male infertility and predict the success of natural or assisted reproduction. SDF occurs as a result of endogenous (e.g., defective chromatin maturation, abortive apoptosis, oxidative stress) or exogenous (e.g., environmental exposures and pollutants and testicular hyperthermia) factors. These mechanisms can create single-stranded and double-stranded DNA breaks, which can be evaluated by a variety of methods [1,5,6].

High SDF has been associated with poor reproductive outcomes, and it can adversely affect male fertility potential [1]. For example, a prospective cohort study in couples planning pregnancy for the first time reported that SDF was negatively correlated with pregnancy rate, where SDF $>$ 40% was detrimental to pregnancy success [7]. The generally negative impact of SDF on male fertility has encouraged clinicians to integrate SDF testing in the clinical setting [1].

The aim of this chapter is to evaluate the impact of SDF on artificial reproductive technology (ART) outcomes. First, a description of the various assays used to evaluate SDF will be discussed, before moving to evaluate SDF's influence over the different ART methods used to aid infertile couples.

Measuring SDF within the context of ART

Several methods can be used to measure the extent of SDF in a sample. The following techniques have been studied and recommended for use in evaluating SDF:

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay is the most commonly used assay for measuring SDF [8]. It relies on the addition of fluorescein-labeled dUTP to the 3′-OH ends at the sites of DNA breaks in spermatozoa using the enzyme terminal deoxynucleotidyl transferase. The extent of DNA breakage is then measured by a fluorescent microscope or a flow cytometer [9].
Comet assay is a single-cell gel electrophoresis technique during which fragmented DNA is

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separated by electrophoresis, forming the "comet tail," while intact DNA remains within the nucleus or the "comet head" [10]. This technique is unique as it can distinguish between single-stranded and double-stranded breaks depending on the pH used; alkaline comet detects both types of breaks, neutral comet detects only double-stranded breaks, and the two-tailed comet can distinguish between both types of breaks [9,10].

Sperm chromatin structure assay (SCSA) is an indirect method that evaluates DNA integrity. Sperm DNA is treated with acid that causes denaturation of the DNA strands at the sites of breaks whether single- or double-stranded. The sample is then stained with acridine orange, a small molecule that can intercalate within intact DNA strands and fluoresce green or adhere to denatured strands and fluoresce red. This yields the DNA fragmentation index (DFI), calculated as the percentage of red over total fluorescence, and it reflects the percentage of sperm with fragmented DNA [11].

Sperm chromatin dispersion (SCD) is a simple method to detect SDF whereby sperm DNA is denatured with acid, creating small single-stranded fragments at sites of breaks. After lysis and removal of nuclear proteins, intact DNA disperses away from the nucleus forming a halo, but fragmented DNA does not [12].

Currently there is no universal gold standard and no assay is recommended over the other. In fact, Ribas-Maynou et al. have compared all the assays and have reported significant differences in the levels of SDF between fertile and infertile men when using TUNEL, SCSA, SCD, or alkaline comet. They also reported that these four assays correlate well with each other. However, they did not report such findings when using neutral comet, suggesting its poor ability in determining fertility potential [13]. On the other hand, they highlighted the importance of neutral comet in predicting recurrent pregnancy loss, suggesting that double-stranded DNA breaks could be a male factor related to miscarriage [14].

Many studies have attempted to establish cut-off values for the different assays and have investigated these values in different clinical settings, measuring different reproductive outcomes [15]. For example, Sharma et al. underscored the TUNEL assay's unique potential in diagnosing male infertility. They established a standardized approach for testing and identified a cut-off value of 16.8% that had high specificity (91.6%) and positive predictive value (91.4%) in discriminating between fertile and infertile men [16]. Nicopoullos et al. evaluated different parameters obtained via alkaline comet assay and found them predictive of male infertility and live birth rates after in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) [17]. While variation between the individual studies continues to exist, the meta-analysis conducted by Santi et al. suggested that a cut-off value of 20% could potentially distinguish between fertile and infertile men [18].

The different assays along with their advantages and disadvantages are presented in Table 13.1 [1,9,15].

The impact of SDF on IUI

Intrauterine insemination (IUI) involves placing washed and concentrated sperm into the uterus around the time the ovary releases an oocyte [19]. As a commonly used treatment for infertile couples, IUI is easier, less invasive and less expensive to perform than other ART counterparts [20]. Several factors can contribute to the success of IUI, including the number of mature follicles, hormones used for ovarian stimulation, and the number of motile spermatozoa [21].

SDF presence may have an impact on IUI outcome. A meta-analysis by Chen et al. reported that high rates of SDF corresponded to decreased pregnancy and delivery rates after IUI [22]. Another meta-analysis by Sugihara et al. that analyzed 917 IUI cycles from three studies also revealed an increased pregnancy rate among those with low SDF compared to high SDF ($RR = 3.3$, $P < .05$). However, the significant heterogeneity for its specificity and positive predictive value prompted the authors to conclude that SDF testing has limited power for predicting IUI success [23]. Nonetheless, many individual studies have also looked at the effect of SDF on IUI and whether success can be determined by SDF levels. Duran et al. noted that spermatozoa of infertile couples with SDF $> 12\%$ used for IUI did not achieve clinical pregnancy [24]. Bungum et al. studied the predictive ability of SCSA and reported that DFI $> 30\%$ significantly reduced IUI success, as measured by pregnancy and delivery outcomes. However, they reported no significant differences between low and high DFI groups for couples undergoing IVF or ICSI, further reinforcing the detrimental effect of SDF of IUI [25]. Furthermore, using TUNEL assay to measure SDF and the 8-hydroxy-2′-deoxyguanosine (8-OHdG) biomarker for oxidative DNA damage, Thomson et al. reported a negative effect of increased SDF and 8-OHdG on pregnancy rates with IUI, but not ICSI, though without reaching statistical significance [26]. Generally, presence of high SDF correlates with decreased pregnancy rates following IUI procedures.

The impact of SDF on IVF and ICSI

A large number of studies have investigated and demonstrated the deleterious impact of SDF on

TABLE 13.1 Assays used to measure sperm DNA fragmentation.

Assay	Principle	Advantages	Disadvantages
Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)	Labels DNA at sites of breaks by incorporating dUTP, which is then quantified by microscopy or flow cytometry	- Highly sensitive and reliable, minimal interobserver variability, few sperm needed for test	- Protocols and thresholds still not standardized between labs, needs expensive equipment and trained personnel
Comet	Single-cell electrophoresis during which DNA fragments move away from the nucleus forming a tail	- Sensitive, can discriminate between single- and double-stranded breaks	- Poor repeatability with high interobserver variability, needs appropriate imaging software and experienced observers
Sperm chromatin structure assay (SCSA)	Uses metachromatic acridine orange that fluoresces red with denatured DNA and green with intact DNA, which is measured by flow cytometry	- Reliable and accurate, can simultaneously examine a large number of cells	- Commercial kits not available, needs expensive equipment and trained personnel
Sperm chromatin dispersion (SCD)	Looks at the halo of DNA loops after lysis, where DNA fragments remain in the core and intact DNA disperses	- Simple, easy, and fast to perform, commercial kits available, reproducible and consistent results, no expensive equipment needed	- Interobserver variability

conventional IVF and ICSI. The differences in sperm selection and fertilization conditions between the two procedures can account for the differences in the effect of SDF on various outcomes as described in the subsequent paragraphs.

After sperm preparation and oocyte retrieval and culture, IVF involves addition of spermatozoa onto cultured oocytes and incubation, allowing for fertilization to take place. In ICSI, on the other hand, oocytes are prepared by removing the surrounding cumulus and corona cells, and then a motile spermatozoon with normal morphology is injected directly into the oocyte after piercing the zona pellucida and the cell membrane. Fertilization is then assessed, the zygote is cultured, followed by assessment of embryonic development and finally embryo transfer into the uterus for implantation to take place [27].

Impact of SDF on fertilization

A cohort study by Oleszczuk et al. looked at the effect of DFI on fertilization rate among couples undergoing IVF and ICSI. They analyzed 1117 IVF cycles and reported that mean fertilization rates were lower among groups with higher DFI, as mean fertilization rate was 38.1% for those with DFI $> 30\%$ compared to a mean of 51.4% for those with DFI $\leq 10\%$ ($P = .02$). They also analyzed 516 ICSI cycles but did not report a difference in mean fertilization rates among groups with different DFI [28]. These findings were in line with a meta-analysis that reported fertilization rates for those with high SDF to be lower by 21% compared to those with low SDF among couples undergoing IVF, although no statistical significance was reported. However, the fertilization rates for couples undergoing ICSI were similar for both high and low SDF groups (80% and 78%, respectively) [29]. Furthermore, Tang et al. reported that a DFI cut-off of 31.25% can predict total fertilization failure and low fertilization rates for men with asthenozoospermia undergoing IVF with 72.2% sensitivity, 86.7% specificity, 36.4% positive predictive value (PPV), and 96.8% negative predictive value (NPV) [30].

Impact of SDF on clinical pregnancy

Several meta-analyses have studied the impact of SDF on clinical pregnancy rates for both IVF and ICSI. Deng

et al. analyzed 2130 IVF cycles and reported a significantly lower clinical pregnancy rate among the high DFI group compared with the low DFI group (RR = 0.77, $P = .05$); however no significant difference was reported when they analyzed 278 ICSI cycles (RR = 0.75, $P = .29$) [31]. Zini et al. analyzed 1805 IVF cycles from 11 studies and 1171 ICSI cycles from 14 studies, and they reported a significant association between high SDF and reduced clinical pregnancy rates after IVF (OR = 1.7, $P < .05$); however no such association between SDF and clinical pregnancy was found for ICSI (OR = 1.15, $P = .65$) [32]. Similar findings for the link between high SDF and low clinical pregnancy rates in IVF but not ICSI were reported by other meta-analyses as well [29,33]. An analysis by Simon et al. reported consistent results for IVF (3734 cycles, OR = 1.92, $P = .0005$); however a significant association between SDF and clinical pregnancy was reported for ICSI as well (2282 cycles, OR = 1.49, $P = .0075$). They further assessed the predictive value of SDF testing for pregnancy rates after ART and reported that for IVF (median pregnancy rate of 32%), SDF testing can predict clinical pregnancy rates with a PPV of 79% and NPV of 35%, whereas for ICSI (median pregnancy rate of 36%), PPV and NPV are 64% and 40%, respectively [34].

From the aforementioned studies, it is clear that elevated SDF levels can have a detrimental impact on fertilization and achieving clinical pregnancy in couples undergoing IVF. However, no such effect is observed for ICSI. In couples undergoing IVF, SDF levels were found to be significantly correlated to abnormal sperm morphology and motility [35], so the selection of the morphologically normal and motile spermatozoon for ICSI may result in selection of the sperm with low SDF, and this can account for improved fertilization and clinical pregnancy rates. Differences in the insemination procedure can also explain the better outcomes seen with ICSI, since spermatozoa are directly injected into oocytes, whereas in IVF, sperm are cultured with oocytes for a period of time allowing spermatozoa to be exposed to oxidative stress, adding to SDF and impairing fertilization [25]. Finally, the cumulus and corona cells around the oocyte also contribute to oxidative stress and can increase SDF during IVF, affecting the sperm's ability to fertilize the ovum, and this can directly harm the developing embryo. However, in ICSI, these cells are removed during oocyte preparation, and this can result in improved fertilization and clinical pregnancy rates [28].

Impact of SDF on embryogenesis

SDF can also influence embryonic development and implantation. The meta-analysis by Deng et al. compared 17,879 embryos (8 studies), both from IVF and ICSI, and found that the rate of good-quality embryos was significantly lower among the elevated SDF group compared with the low SDF group (RR = 0.65, $P < .01$) [31]. A retrospective study assessed embryonic quality from IVF/ICSI and its relationship to SDF levels and determined that as SDF increased, the top-quality embryo formation rate decreased, but their results did not reach statistical significance. However, they did report that a 30.7% SDF cut-off could predict top-quality embryo with 80% sensitivity, 54.2% specificity, 13.3% PPV, and 95.7% NPV [36]. Casanovas et al. took this further by investigating the effect of single-strand SDF (ssSDF) versus double-strand SDF (dsSDF) on embryo kinetics and implantation. They found that certain stages of embryonic development took significantly longer with higher dsSDF levels, but such differences were not seen depending on ssSDF levels. They also studied the kinetics of the embryos that were able to achieve implantation and found them to be comparable to those of the low dsSDF group (mean difference 0.4%) but significantly different from the kinetics of the high dsSDF group (mean difference 3.8%, $P = .001$) [37]. Although ssSDF and dsSDF can have different influences on ART outcome, this carries no clinical consequences at this time, as most assays do not differentiate between them, and the management approach does not differ between ssSDF and dsSDF.

Impact of SDF on miscarriage and live birth rate

Once implantation takes place and clinical pregnancy is established, SDF can still influence reproductive outcomes of ART as it is associated with increased risk of miscarriage. A meta-analysis by Zini et al. studied 808 clinical pregnancies from IVF and 741 pregnancies from ICSI and reported significantly higher pregnancy loss among the high SDF groups compared with low SDF groups for both IVF (OR = 2.17, $P < .05$) and ICSI (OR = 2.73, $P < .05$) and found no significant difference in OR between IVF and ICSI [38]. A similar association of increased miscarriage after IVF or ICSI with elevated SDF was reported by other meta-analyses as well [31,39]. Zhao et al., on the other hand, reported somewhat different results. When comparing 301 pregnancies from ICSI, they found a significant difference in miscarriage rate between high SDF and low SDF groups (OR = 2.68, $P = .003$). However no significant difference in miscarriage rate between the two groups was found when they compared 539 pregnancies from IVF (OR = 1.84, $P = .06$) [33]. The process of fertilization is bypassed in ICSI, when the embryologist directly injects a sperm into the oocyte. This would allow SDF to carry on into pregnancy and exert delayed effects, leading to

miscarriage. This delayed effect was demonstrated, as sperm with high SDF did not show reduced fertilization or embryo quality after ICSI, but this was later associated with significant adverse outcomes [40].

Finally, Osman et al. conducted a meta-analysis to address the impact of SDF on live birth rate in IVF and ICSI. They reported a significantly higher live birth rate with low SDF compared with high SDF after both IVF (4 studies, 553 patients, RR = 1.27, $P = .01$) and ICSI (5 studies, 445 patients, RR = 1.11, $P = .04$) [41]. Deng et al. however, did not report any significant differences for either IVF or ICSI [31].

It is worth noting that several of the aforementioned meta-analyses have attributed their inability to make solid conclusions due to the heterogeneity of the studies included. These studies were different in terms of the population of infertile men included, ART conditions, SDF measurement, control of confounding factors in men that can also affect ART outcomes, and control of female factors such as age and ovarian reserve. To further complicate matters, the outcomes of different studies are not in line with each other; for example a recent cohort study found no significant effect of SDF on embryonic development, implantation, clinical pregnancy, or miscarriage rates [42].

Approaches to reduce SDF in ART

Several conditions and risk factors in men have been associated with elevated SDF. Before initiating ART, it is important to recognize these contributors to ART failure due to elevated SDF and address them.

Varicocele treatment

Varicocele is a common condition among men and has been associated with increased oxidative stress as well as increased SDF rates among infertile men [43]. Smit et al. studied the effect of surgical varicocelectomy on 49 infertile men with clinical varicocele and abnormal semen parameters, and they reported that 63% of men were able to achieve more than 50% reduction in DFI after surgery, with a significant decrease in the mean DFI (35.3%−28.6%; $P = .009$). They also compared men who were able to achieve pregnancy with ART and found their mean DFI (21.3%) to be significantly lower than those who failed ART (36.9%; $P = .041$) [44]. Furthermore, a meta-analysis of four studies compared the effect of varicocelectomy on ICSI outcomes. They studied 870 ICSI cycles and reported significant improvement in clinical pregnancy (OR = 1.59; $P = .002$) and live birth rates (OR = 2.17; $P < .000,001$) among men who underwent varicocelectomy prior to ICSI (n = 438) compared to those who underwent ICSI without prior varicocelectomy (n = 432) [45].

Treatment of male genital tract infections

Male genital tract inflammation and infections causing leukocytospermia (>1 million white blood cells in semen) have also been associated with elevated oxidative stress and SDF levels [46]. In fact, significant reduction in SDF was reported in patients who received antibiotics, and 85.7% of couples were able to achieve pregnancy after completion of treatment [47]. However, no significant effect of leukocytospermia on ART outcomes was recently reported [48], suggesting that leukocytospermia may affect particularly natural fertility, and ART can be a final resort after treatment failure for this condition.

Addressing lifestyle and exposure risk factors

Obesity is a condition associated with elevated SDF, which is significantly reduced after weight loss [49]. Furthermore, type 2 diabetes was associated with significantly higher mean SDF percentage among men compared to nondiabetics (37.05 vs. 21.03; $P < .001$) as well as adverse impacts on ICSI outcomes including reduced clinical pregnancy rates (28.57% vs. 46. 34%; $P < .001$) and increased miscarriage rates (50.0% vs. 24.56%; $P < .001$) [50], suggesting that proper management and glycemic control may help improve ART outcomes among men with type 2 diabetes. Exposures to exogenous toxins and other contaminants are also correlated to higher SDF levels; these include smoking, heat, radiation, heavy metals, and chemicals such as bisphenols and phthalate [51]. Therefore, it is important to identify men who are at high risk of elevated SDF levels based on their lifestyle or environmental and occupational exposures. These men should be counseled on the importance of lifestyle modification and exposure limitation that can help reduce their SDF levels and improve ART end results.

Antioxidant therapy

Given the harmful impact of oxidative stress and its contribution toward increasing SDF and alteration of male fertility potential, the use of antioxidants has been investigated. Clinical trials have studied the effect of antioxidant supplementation, such as zinc, docosahexaenoic acid, and vitamins E and C, compared to no treatment or placebo and have reported reductions in SDF levels with antioxidant supplementation [52−54]. Furthermore, the effect of antioxidant supplementation was studied in men with DFI $\geq$ 15% who have failed

initial ICSI. After 2 months of antioxidant treatment, 76.3% of men were able to achieve more than 10% reduction in their DFI, and this was translated into improved outcomes with the second ICSI attempt compared to the first, mainly clinical pregnancy rate (48.2% vs. 6.9%, $P < .05$) and implantation rate (19.6% vs. 2.2%, $P < .01$) [55]. A systematic review by Majzoub and Agarwal on the use of antioxidants for male infertility concluded that antioxidant supplementation resulted in reduction in SDF levels as well as improved ART outcomes, including fertilization rates, pregnancy rates, and live birth rates [56].

Frequent ejaculation

Abstinence time was also found to significantly increase SDF levels, as semen obtained after 1−2 days of ejaculatory abstinence had significantly lower SDF levels compared to longer durations [57]. This was applied to IUI when a study reported significantly improved pregnancy rates (11.3%, $P < .05$) with ≤2 days of abstinence before IUI compared to 3−5 days (6.1%) or >5 days (7.3%) [58]. Also, recurrent ejaculation prior to ICSI was found to significantly reduce SDF by an average of 27% and was subsequently associated with improved clinical pregnancy rates after ICSI compared to 3−4 days of abstinence (56.4% vs. 43.3%, $P = .03$) [59].

Sperm selection techniques and use of ART

SDF levels should be taken into consideration when discussing the options for ART. Given the evidence discussed in the previous section on the improved fertilization and pregnancy rates in ICSI with elevated SDF levels, the couple can be offered ICSI if SDF levels remain high or after failure of IUI or IVF due to elevated SDF levels. Methods to reduce SDF during ICSI, and other methods of ART, have also been described.

Swim-up and diffusion gradient centrifugation (DGC) are commonly used conventional sperm preparation methods for IVF and ICSI [27]. Both methods have been reported to significantly reduce SDF levels compared to fresh or washed semen [60]. More advanced sperm selection techniques can also be used. IMSI (intracytoplasmic morphologically selected sperm injection) selects sperms devoid of nuclear vacuoles and is associated with significantly lower SDF levels [61]. It has also been associated with significantly improved implantation (OR = 2.88, $P < .00,001$), pregnancy (OR = 2.07, $P = .007$), and reduced miscarriage (OR = 0.31, $P = .003$) compared to conventional ICSI [62]. MACS (magnetic activated cell sorting) removes apoptotic spermatozoa and also leads to significantly improved embryo quality, implantation, and pregnancy rates when used with DGC for ICSI compared to DGC alone [63].

Lastly, testicular sperm can also be used for ICSI and was found to contain significantly less SDF compared to ejaculated sperm (8.9% vs. 33.4%, $P < .0001$) and was associated with significantly improved clinical pregnancy rates (OR = 2.42, $P < .001$) and live birth rates (OR = 2.58, $P < .001$) and significantly less miscarriage rates (OR = 0.28, $P = .005$) when used for ICSI [64]. Despite recent publications advocating the use of testicular sperm in nonazoospermic men with repeated failed ICSI cycles and high DNA fragmentation, the majority of studies used for this claim are of poor quality and high heterogeneity, weakening the level of evidence in support of this approach [65−70]. This is further compounded by a recent study demonstrating no benefit of testicular sperm over ejaculated sperm in ICSI [71]. Therefore, the adequate clinical management of patients with high SDF has to be considered a first-line therapy, rather than used as a justification to pursue a potentially harmful surgical sperm retrieval. The control of exogenous factors such medication use, obesity, and smoking combined with an increase of ejaculation frequency and use of appropriate antioxidants can help reduce DNA fragmentation and may decrease the need for invasive procedures. The use of adequate sperm selection methods may also provide sperm with lower SDF levels [72,73].

The different means that can be attempted prior to initiating ART to reduce SDF levels and improve outcomes are summarized in Fig. 13.1.

Future directions

Given the extensive impact of SDF on male infertility and ART outcomes, there is vast room for implementation and improvement. Two recent guidelines regarding SDF have recommended its measurement in patients with ART failure, recurrent pregnancy loss, men with lifestyle risk factors, exposures, and underlying conditions that contribute to sperm DNA damage [1,74]. Measuring SDF can provide possible explanations for ART failure and can also guide reproductive specialists toward management of the couple. In addition, several studies have attempted to assess the value of SDF in predicting ART outcomes, which can be applied to direct management toward a particular ART method. Finally, to reiterate what several studies have conveyed, more controlled and well-designed studies are needed to examine the effect of SDF on ART outcomes as well as to standardize the measurement and practical use of this sperm function test. The authors strongly believe the critical role of basic scientific research into the causes

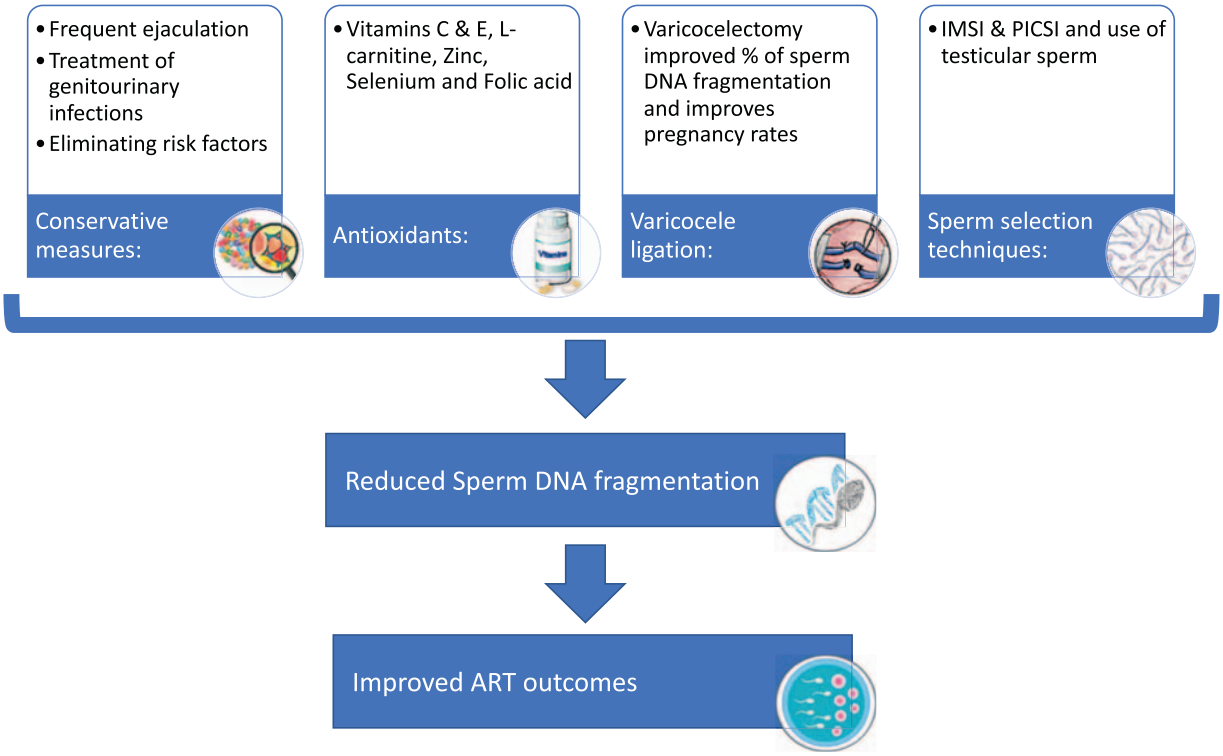

FIGURE 13.1 Methods to reduce SDF to improve ART outcomes. Sperm DNA fragmentation (SDF) can lead to poor outcomes with assisted reproductive techniques (ART). Several practices can be employed to reduce SDF levels to lead to improve ART success. These include controlling the risk factors for SDF prior to initiating ART with use of antioxidants, exposure limitation and lifestyle modification, frequent ejaculation, and treatment of underlying conditions such as varicocele and genitourinary infections. Finally, methods of sperm selection can be utilized with ART for better success, including physiologic intracytoplasmic sperm injection and intracytoplasmic morphologically selected sperm injection (IMSI), as well as the use of testicular sperm.

of SDF and its role in male infertility, as ignoring the value of fundamental research will only delay our understanding and application of this important functional biomarker in the diagnosis of male infertility.

Summary of key points

SDF can lead to adverse male reproductive outcomes and may not be reflected in SA.

The most commonly used assays for SDF include TUNEL, Comet, SCSA, and SCD.

High levels of SDF reduce pregnancy rates with IUI.

SDF reduces fertilization rates and clinical pregnancy rates with IVF but not with ICSI.

SDF increases the risk of miscarriage after ART.

Varicocele treatment, control of male genital tract infections, addressing lifestyle exposures and risk factors, use of antioxidants, and frequent ejaculation can all be employed to reduce SDF prior to attempting ART as a means of improving outcomes.

Advanced sperm selection techniques or even testicular sperm may be used to select spermatozoa with less SDF for use in ART.

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CHAPTER

14

Male accessory gland infection: diagnosis and treatment

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Definition and classification

Male accessory gland infection/inflammation (MAGI) defines a heterogeneous set of inflammatory diseases of the male accessory glands. These include epididymitis, vesiculitis, and prostatitis. They were first recognized by the World Health Organization (WHO) in 1993 [1].

Typically, MAGI is classified based on its etiology and anatomical localization and its extension. According to the etiology, MAGI can be defined as microbial, when microbiological tests (sperm culture, urethral swab culture, and, if deemed necessary, Meares-Stamey test) identify the presence of bacterial, viral, fungal, and/or protozoal (Table 14.1) infection, or amicrobial (inflammatory) when no microorganism is identified [2].

The anatomical localization and extension can only be diagnosed by ultrasound (US) scans of the didymo-epididymal region, seminal vesicles, and prostate. If the US signs of MAGI are confined to the prostate gland alone, MAGI is uncomplicated; if the seminal vesicles and the epididymis are affected, MAGI is defined as complicated. Furthermore, US scan allows distinguishing MAGI into unilateral or bilateral forms. Complicated forms of MAGI associate with worse sperm parameters, compared with uncomplicated ones [3]. Finally, MAGI can be classified into hypertrophic-congestive and fibro-sclerotic forms. These two forms of MAGI, which have different US features, impact differently on sperm parameters. In particular, the hypertrophic-congestive form generally reflects an infection/inflammation of recent onset, whereas fibro-sclerotic MAGI underlies a chronicization of the inflammatory process. The latter negatively impacts the reproductive apparatus more than the hypertrophic-congestive form does. Indeed, hypertrophic-congestive MAGI implies an inflammation in the acute phase, with a high concentration of reactive oxygen species (ROS), while the fibro-sclerotic form involves fibrosis and an irreversible anatomic and functional damage of the efferent seminal ducts [3].

Impact on fertility: explanatory mechanisms

WHO diagnostic criteria of MAGI [1] established the presence of oligo-astheno-teratozoospermia (OAT) as the starting point for further diagnostic examinations. This implies that MAGI negatively impacts sperm parameters, as also the guidelines of the European Association of Urology state [4]. The mechanisms by which this happens can be classified into four main categories [5]:

1. overproduction of reactive oxygen species (ROS) and/or inflammatory cytokines;
2. impaired secretory capacity of the male accessory glands;
3. anatomical obstruction or subobstruction of the seminal tract;
4. direct effect of microorganisms on spermatozoa.

Schematically, the dynamics by which microbial infection can damage spermatozoa start from the presence of leukocytes in the seminal fluid. In fact, the latter increase ROS production, leading to oxidative imbalance, further leukocyte accumulation, and the onset of phagocytosis. These mechanisms trigger specific signal transduction pathways that generate inflammatory cytokines, which,

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TABLE 14.1 Etiology of microbial MAGI [2].

Bacteria	*Escherichia coli, Neisseria gonorrhoeae, Chlamydia trachomatis, Ureaplasma urealyticum, Mycoplasma hominis, Mycoplasma genitalium, Enterobacteriaceae, Klebsiella, Proteus, Enterococci*
Virus	*Papillomavirus*
Fungi	*Candida albicans*
Protozoa	*Trichomonas vaginalis*

in turn, enhance prooxidant systems and hinder antioxidant ones, further increasing ROS responsible for oxidative bursts. This leads to peroxidative damage of sperm proteins, lipids, and DNA, thus impairing conventional and biofunctional sperm parameters. Moreover, remnants of the oxidative stress (OS) response may persist in the seminal fluid for a long time after microbial eradication, contributing to further damage of spermatozoa [5].

Male accessory glands secrete a series of compounds that are necessary for proper sperm function. Epididymis secretes L-carnitine and neutral α-glucosidase, which are involved in sperm maturation; seminal vesicles release fructose, ascorbic acid, ergothioneine, prostaglandin, and bicarbonate, which prevent sperm agglutination; finally, seminal pH, citric and γ-glutaminyl transpeptidase, and zinc seminal concentrations are influenced by prostate function. By affecting the secretory activity of the accessory glands, MAGI can, in turn, alter sperm conventional and biofunctional parameters [6].

Ductal obstruction causes infertility but rarely occurs in patients with MAGI. Subobstruction can ensue in patients with complicated, chronic, fibro-sclerotic, untreated MAGI [5].

Finally, microorganisms can alter sperm function either directly, by the production of soluble factors and/or by adhering to spermatozoa, or indirectly, by stimulating ROS production. More in detail, *Escherichia coli*, mycoplasmas, *Candida albicans*, *Trichomonas vaginalis*, and papillomavirus (HPV) directly adhere to sperm membrane; *Escherichia coli*, *Chlamydia trachomatis*, and *Candida albicans* can also release sperm immobilization factor, lipopolysaccharide, or farnesol, respectively, soluble compounds that reduce sperm motility or induce sperm apoptosis [5].

Diagnosis

WHO first established MAGI diagnostic criteria in 1993 [1], as a disease characterized by OAT associated with specific anamnestic findings and the presence of findings on physical or laboratory examination (Table 14.2).

Symptoms are not always associated with MAGI. Accordingly, asymptomatic or paucisymptomatic forms of MAGI exist, and these result in an underestimation of MAGI diagnosis. Indeed, the lack of symptoms does not lead the patient to andrology counseling, which then starts in the case of infertility. The most common symptoms associated with MAGI, when present, are nocturia, pollakiuria, reduced urinary strength, incomplete bladder emptying, and chronic pelvic pain. The former can manifest as pain in the scrotal, penile, inguinal, suprapubic, and anal region. Moreover, sexual dysfunctions may occur in about 50% of the patients and include erectile dysfunction, premature ejaculation, and decreased libido [5].

Microbiological testing

Among the microbial tests requested in patients with MAGI, sperm culture, nucleic acid amplification tests (NAATs) of the urethral swab, and the Meares-Stamey test are the most widely used. A general agreement has been reached on sperm culture as a diagnostic test for MAGI. In particular, a concentration of urinary tract pathogens $>10^3$ CFU/mL in the seminal fluid is suggestive of significant bacteriospermia [7]. As far as the other microbiological tests, a widely recognized consensus has not been reached so far. Some evidence indicates that the Meares-Stamey test could be used, especially in the case of bacterial chronic prostatitis [8–11]. NAATs of the urethral swab are useful for researching *Chlamydia trachomatis* or mycoplasma search. These microorganisms, in fact, have an in vitro slow growth that precludes culture as a diagnostic method [12]. Other tests have been developed to diagnose *Chlamydia trachomatis*, such as culture, direct immunofluorescence assays, and enzyme-linked immunosorbent assays. However, among these, NAATs show the greatest accuracy, when performed on urethral swabs or urine [13–15], as confirmed by a meta-analysis of cross-sectional studies including 2133 patients [16]. A meatal swab should be avoided since it results in a lower content of cellular material compared to the urethral swab [17]. Accordingly, a prospective multicenter clinical study has recently confirmed the greater accuracy of urethral compared to meatal swabs This study conducted in 1583 patients reported a sensitivity and specificity for *Mycoplasma genitalium* of 98.2% and 99.6% for urethral swabs, 88.4% and 97.8% for self-collected penile meatal swabs, and 90.9% and 99.4% for urine [18], thus confirming the superiority of urethral swabs and urine compared to penile meatal swabs in the diagnosis of mycoplasmas.

TABLE 14.2 Diagnostic criteria of male accessory gland infection [1].

Oligo-astheno-teratozoospermia plus:
- one factor A + one factor B
- one factor A + one factor C
- one factor B + one factor C
- two factors C

Factors	Description
A	• *History:* positive for urinary infection, epididymitis, and/or sexually transmitted disease • *Physical signs:* thickened or tender epididymis, tender vas deferens, and/or abnormal digital rectal examination
B	• *Prostatic fluid:* abnormal prostate fluid expression and/or abnormal urine after prostatic massage
C	• *Ejaculate signs:* leukocyte >1 million/mL, culture with significant growth of pathogenic bacteria, abnormal appearance, increased viscosity, increased pH, and/or abnormal biochemistry of the seminal plasma

Table 14.1 shows the more frequently diagnosed microbes in the clinical practice. However, currently, there is no widely accepted agreement on which pathogen should be investigated. Among mycoplasmas, *Ureaplasma urealyticum* and *Mycoplasma hominis* are significantly associated with male infertility, as reported by a systematic review and meta-analysis on case-control and cohort studies including 611 infertile patients and 506 controls from case-control studies searching for *Ureaplasma urealyticum*, and nine case-control studies on 2410 infertile patients and 1223 controls searching for the presence of *Mycoplasma hominis*. The same study showed that both *Mycoplasma genitalium* and *Ureaplasma parvum* were not associated with male infertility. Indeed, the rate of infection was not similar between the infertile patients and the control group [19]. Recently, a study on 74,376 infertile patients aimed at investigating the effect of semen bacterial infection of sperm parameters reported a significantly lower sperm concentration and motility among patients with a bacterial infection compared with noninfected patients. The bacterial species more frequently identified were *Escherichia coli* with a prevalence of 63.6%; *Klebsiella pneumoniae* subspecies, with a prevalence of 19.8%; and *Proteus mirabilis* with a prevalence of 13.2% [20].

Viruses can also impair male fertility, so their search should be included in the diagnostic work-up of the infertile patients with MAGI. In recent times, HPV infection has been associated with MAGI US features and can be included among the etiological factors of MAGI [21]. HPV is indeed associated with male infertility and its prevalence in infertile patients is ~20%, which is significantly higher than that in control fertile men (~11%) [22]. To understand the impact of HPV infection on sperm parameters, a meta-analysis of observational studies, overall including 5203 patients with and without HPV infection, has reported significantly lower sperm concentration, total sperm count, progressive motility, and spermatozoa with normal morphology in HPV-positive compared to HPV-negative patients. Particularly, asthenozoospermia is significantly more frequent in HPV-positive patients compared with negative ones. No difference in the prevalence of oligozoospermia and teratozoospermia was found between the two groups. A trend toward a lower pregnancy rate was found in HPV-positive patients, although no definitive conclusion can be drawn due to the paucity of data. Moreover, the study reported the presence of a significantly higher miscarriage rate in couples with the male partner positive for HPV compared with the negative controls [23]. Another meta-analysis of observational studies on 616 infertile patients with HPV seminal infection and 2029 infertile controls without seminal HPV infection supports the association between HPV infection and asthenozoospermia in infertile patients [24]. The last published meta-analysis on this issue reported a significantly higher prevalence of HPV infection in infertile patients compared with fertile men, as well as an association between HPV seminal infection and lower sperm motility, normal morphology, and higher sperm DNA fragmentation and miscarriage rate in infertile patients undergoing assisted reproductive technique (ART) [25].

Importantly, in male patients with microbial MAGI, microbiological testing of the female partner is mandatory before establishing a therapeutic approach. In particular, the cultural examination of the cervical-vaginal swab is a useful diagnostic tool.

Ultrasound

US examination is a pivotal diagnostic tool for the management of MAGI with prognostic implications. US diagnostic criteria of MAGI implement the MAGI diagnostic flow chart. Particularly, conventional and additional US diagnostic criteria of MAGI are detailed in Table 14.3 [5]. The accuracy of these criteria has been carefully evaluated in a cohort of 100 patients with MAGI and 100 aged-matched controls [26]. The sensitivity and specificity analysis showed that additional US criteria had a diagnostic accuracy similar to

TABLE 14.3 Ultrasound criteria of MAGI [5].

		Ultrasound criteria
Prostatitis (>2 criteria simultaneously present among the following)	Conventional US criteria	• Asymmetry of the gland volume • Areas of low echogenicity • Areas of high echogenicity • Dilatation of the peri-prostatic venous plexus
	Additional US criteria	• Single or multiple internal similar cystic areas • Area(s) of moderate increase in vascularity (focal or multiple)
Vesiculitis (>2 criteria simultaneously present among the following)	Conventional US criteria	• Increased (>14 mm) anteroposterior diameter, mono- or bilateral • Reduced (<7 mm) anteroposterior diameter, mono- or bilateral • Thickened and/or calcified glandular epithelium • Polycyclic areas separated by hyperechoic septa in one or both vesicles
	Additional US criteria	• Fundus-to-body ratio >2.5 • Fundus-to-body ratio <1 • Anteroposterior diameter unchanged after recent ejaculation
Epididymitis (>2 criteria simultaneously present among the following)	Conventional US criteria	• Increase in size of the head (craniocaudal diameter >12 mm) and/or of the tail (craniocaudal diameter >6 mm) (finding single or bilateral) • Presence of multiple microcystis in the head and/or tail (finding single or bilateral) • Low echogenicity or high echogenicity, mono- or bilateral • Large hydrocele, mono- or bilateral
	Additional US criteria	• Enlargement of the superior part of the cephalic tract and a superior-to-inferior part ratio >1 • Unchanged anteroposterior diameter of tail just after ejaculation

the conventional ones. In addition, the diagnostic sensitivity and specificity of US scans increase with the increase in the number of US signs found. Two or more criteria of prostatitis are in fact associated with a higher predictive value than just one. The same was found for the US signs of epididymitis and vesiculitis [26].

US examination of the male accessory glands can add further insights concerning specific issues. It allows the evaluation of MAGI extension and assessing for the presence of unilateral and bilateral forms. Moreover, US inflammatory signs confined in the prostate allow diagnosing noncomplicated forms of MAGI, with a better prognosis on sperm parameters compared with the complicated forms characterized by the presence of US inflammatory signs present also in the seminal vesicles and/or the epididymis. Accordingly, sperm parameters of 70 patients with prostate-vesiculo-epididymitis had significantly lower sperm parameters than those with prostatitis alone, prostate-vesiculitis, and controls [27].

Finally, based on the specific US features, MAGI can be classified into hypertrophic-congestive and fibrosclerotic forms. Congestive MAGI are characterized by prostate areas of hypoechogenicity, cystic areas, and periprostatic venous plexus dilation; seminal vesicle increased anteroposterior diameter, polycyclic areas, hyperechogenic septa, and increased fundus/body ratio; and epididymal increased tail craniocaudal diameter, head and tail bilateral areas of hypoechogenicity, and unchanged postejaculatory anteroposterior diameters in the epididymis. These features generally reflect a

recent infection/inflammation. In contrast, the fibrosclerotic form is characterized by the presence of areas of hyperechogenicity and asymmetry in the prostate, reduced anteroposterior diameter, thickened and/or calcified glandular epithelium, reduced fundus/body ratio in the seminal vesicles, and areas of hyperechogenicity in the epididymis [5]. The distinction between these two forms is clinically important since the fibrosclerotic variant has a worse sperm output. Accordingly, a case-control study carried out in 100 patients with MAGI and 100 age-matched controls reported that the prevalence of the hypertrophic-congestive form was 56% and the fibro-sclerotic variant was 29%. The same study analyzed sperm conventional parameters and measured seminal ROS between the two groups, reporting significantly higher sperm concentration, motility, and normal forms, but also higher seminal fluid leukocyte concentration and seminal ROS in patients with hypertrophic-congestive MAGI compared with those with fibro-sclerotic MAGI. Expectably, patients with MAGI significantly had worse sperm parameters compared with controls [3].

Taking all this into account, US scan is a useful test in patients with MAGI that provides useful information on MAGI extension and features but also on prognosis, thus allowing better tailoring of the therapeutic approach.

Therapeutic strategies

The therapeutic approach to infertile patients with MAGI is included among the nonhormonal medical treatments available for male infertility. It plays an important role since, in some cases, it can cure infertility and, in others, can improve the microenvironment in which spermatozoa are produced and mature, thus contributing to an increase in the success rate of ART.

Both microbial and inflammatory MAGI deserve a nonempirical medical treatment, which, generally, is the treatment prescribed to the infertile male after the etiology has been identified. Patients with MAGI are at risk for infertility due to the following three main mechanisms: infection, inflammation, and/or increased oxidative stress. Therefore, the therapeutic approach should be aimed at overcoming these specific pathogenetic mechanisms. The main available therapeutic compounds are antibiotics, antiinflammatory drugs, and nutraceutical compounds with fibrinolytic or antioxidant properties.

Antibiotics

The choice of the antibiotic to be prescribed should be guided by the results of microbiologic examinations and antibiotic sensitivity testing since a targeted therapy is recommended. Moreover, the specific antibiotic and its posology have to provide a good penetration into the prostate, since its biofilm has a low permeability. The most effective class of antibiotics include quinolones, trimethoprim, tetracyclines, and macrolides. β-Lactam antibiotics (penicillin derivatives, cephalosporins, monobactams, carbapenems) have limited use in male infertility.

Quinolones (ciprofloxacin, levofloxacin, ofloxacin, norfloxacin, pefloxacin, enoxacin, fleroxacin, lomefloxacin) are considered a first-line therapy. Indeed, these antibiotics show excellent penetration into the prostate tissue and are effective against typical and atypical pathogens. The most used are ciprofloxacin and levofloxacin [28,29]. The dose and duration of treatment should be sufficient to eradicate the infection, e.g., ciprofloxacin 500 mg (once/day), levofloxacin 500 mg (once/day) for 20 to 28 days. The treatment can be divided into two cycles of 10−14 days, separated by an interval of 1 to 2 weeks. However, quinolones are associated with central nervous system adverse events and with tendonitis. In 2018, the European Medicines Agency released a warning on disabling and potentially permanent side effects with quinolone and fluoroquinolone antibiotics [30]. Therefore, they should only be prescribed to patients with MAGI when clearly indicated.

Trimethoprim is second-line therapy. It is active against many relevant pathogens except *Pseudomonas*, some enterococci, and some enterobacteriaceae. Dose and duration should be sufficient to eradicate the infection, e.g., 200 mg once or twice/day for 28 days. The treatment may be divided into two cycles of 10−14 days, separated by an interval of 1 to 2 weeks.

Tetracyclines are second-line therapy. They are active against in vitro slow-growth pathogens, such as *Chlamydia trachomatis* and mycoplasmas. The dose and duration should be sufficient to eradicate the infection. Doxycycline is administered at the dose of 100 mg once or twice/day for 28 days. The treatment may be divided into two cycles of 10−14 days, separated by an interval of 1 to 2 weeks.

Macrolides show a good penetration into the prostate and are active against Gram-positive bacteria and *Chlamydia trachomatis.* The dose and duration should be sufficient to eradicate the infection. Azithromycin is prescribed at the dose of 1 g once/day for 7 to 10 days.

Table 14.4 provides a summary of the microbiological eradication rate of specific antibiotics and suggests a greater efficacy for levofloxacin and azithromycin used either alone, in combination, or sequentially, in patients with chronic prostatitis by *Chlamydia trachomatis.*

Evidence from clinical trials supports the usefulness of the antibiotic treatment to improve conventional sperm parameters in infertile patients with bacterial

TABLE 14.4 Microbiological eradication rate of different antibiotics.

Antibiotic	Eradication rate (%)
Ciprofloxacin	40–77 [29]
Levofloxacin	75 [29]
Azithromycin	80 [31–33]
Doxycycline	77 [32]
Clarithromycin	80 [33]
Azithromycin + ciprofloxacin	62–77 [34]
Azithromycin and/or levofloxacin	>90 [35]

TABLE 14.5 Main antioxidants used for treatment of male infertility.

Antioxidants	Ascorbic acid (vit. C), α-tocopherol (vit. E), ascorbic acid (vit. C), selenium, L-carnitine, L-acetyl-carnitine, glutathione, coenzyme Q10, myoinositol, folic acid, L-arginine, lycopene, picnogenol, N-acetyl-cysteine, pentoxifylline, zinc, astaxanthin, *Lepidium meyenii*, α-linolenic acid, lignans, lycopene, garlic oil, *Morindae officinalis extract*

MAGI [36–38]. Only one study has reported that antibiotic treatment can improve sperm DNA fragmentation [39]. Contrasting data are currently available on the effects of antibiotics on the pregnancy rate [38,40]. Therefore, further studies are needed to cover the impact of antibiotics on the latter two endpoints in infertile patients with MAGI.

Finally, the therapeutic approach cannot be limited to the male partner of an infertile couple. In fact, microbiological testing and targeted antibiotic therapy are also necessary for the female partner of a male patient with microbial MAGI.

Antiinflammatory drugs

Antiinflammatory drugs include nonsteroidal (e.g., salicylates, profens, cox-2 inhibitors) and steroid (glucocorticoids) drugs. Overall, their effectiveness and use for the treatment of MAGI are limited to the inflammatory forms. Nutraceutical compounds with antiinflammatory and/or antioxidant action are more frequently used.

Fibrinolytics

Fibrinolytics include serratiopeptidase, bromelain, and escin. Serratiopeptidase is a metalloprotease of 45,000–60,000 kD molecular weight with a proteolytic activity ensured by the zinc atom [41]. This proteolytic action makes this compound of particular utility in case of increased seminal viscosity due to inflammation. It may also favor the capability of antibiotics (especially quinolones) to penetrate the prostate biofilm [42]. Therefore, fibrinolytics associated with antibiotics may increase their therapeutic efficacy in patients with microbial MAGI.

Similar to serratiopeptidase, bromelain has proteolytic activity mainly exerted on fibrinogen. Therefore, it can be prescribed in association with antibiotics or cases of increased viscosity of the seminal fluid. The dose used ranges from 160 mg/day to 750–1000 mg/day. Finally, escin has mainly antiinflammatory and antiedematous effects.

To summarize, given their proteolytic and antiinflammatory properties, fibrinolytic compounds can be associated with antibiotics for the treatment of microbial MAGI or may be prescribed to patients with inflammatory MAGI.

Antioxidants

Antioxidants represent a wide group of nutraceutical compounds that act supporting enzymatic (superoxide dismutase, SOD; catalase and glutathione peroxidase, GPX) and nonenzymatic (e.g., glutathione; N-acetyl-cysteine, NAC, vitamins A, E, and C; coenzyme Q10, CoQ10; carnitines; myoinositol, MYO; lycopene; astaxanthin; *Serenoa repens*; etc.) antioxidant system to increase the total seminal plasma antioxidant capacity [43]. Table 14.5 shows the main antioxidants used alone or more frequently in association for the treatment of male infertility.

These compounds can be prescribed to infertile patients with microbial or inflammatory MAGI who have already been successfully treated with antibiotics and/or antiinflammatory/fibrinolytics. The rationale for their use in these patients is that the infection triggers nonspecific and specific immune reactions that increase oxidative stress. A chronic nonspecific inflammatory reaction (leukocytospermia, seminal plasma increase of interleukin-1 [IL-1], IL2, IL-8, and tumor necrosis factor α), overproduction of ROS, or specific autoimmune response (production of sperm auto-antibodies) often can continue to be present even after microbial eradication [2]. This leads to an exhaustion of the scavenger systems with consequent oxidative damage of the sperm plasma membranes and DNA fragmentation that impair sperm function. In this context, treatment with antioxidants can be useful to counteract the deleterious effects of oxidative stress on sperm fertilizing ability, as also is

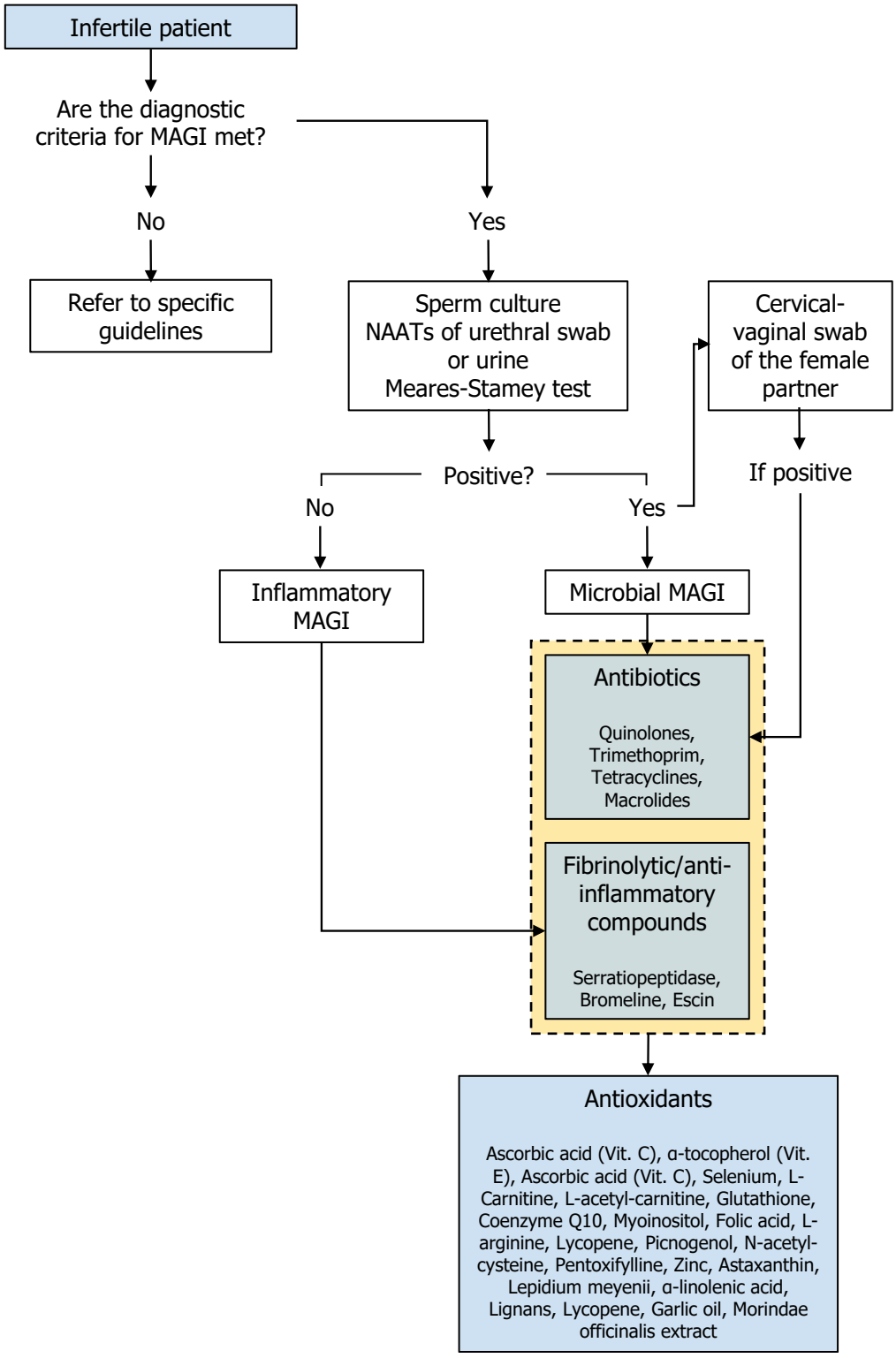

FIGURE 14.1 **Diagnostic and therapeutic flow chart of male accessory gland infection/inflammation in infertile patients.** Patients with male infertility and male accessory gland infection/inflammation (MAGI) should undergo appropriate microbiological tests for the differential diagnosis between the microbial or inflammatory form of MAGI. In the case of microbial MAGI, the female partner should also undergo microbiological tests. Microbial MAGI must be treated with specific antibiotics (based on the type of microorganism and its sensitivity to antibiotics). The antibiotic treatment should also be prescribed to the female partner if the cervicovaginal swab is positive. Fibrinolytic agents or antiinflammatory compounds can be associated with antibiotics to increase their ability of antibiotics to penetrate the prostate biofilm. After microbial eradication, an antioxidant treatment may be considered. Inflammatory MAGI should be treated with antiinflammatory and/or fibrinolytic agent compounds followed by the administration of antioxidant.

suggested by the Italian Society of Andrology and Sexual Medicine [44].

The last published Cochrane review on the use of antioxidants [45] for the treatment of male infertility reported that the use of antioxidants in 6264 infertile patients was associated with increased live birth and clinical pregnancy rates. However, these findings were ranked of low quality [45]. Moreover, several meta-analyses support the positive effects that antioxidants and, in particular, selenium, coenzyme Q10, and ω3 fatty acids have on sperm count, motility, and morphology [46–48].

In summary, the published data on the possible benefits of antioxidants for the treatment of male infertility are contrasting [49]. This is likely due to the different inclusion criteria and nutraceutical compounds administered. Hence, well-designed, randomized, controlled trials on selected cohorts are still needed to clarify this issue.

Conclusion

A correct diagnostic and therapeutic approach of MAGI is important for the proper management of patients with male infertility since both microbial and inflammatory MAGI impact negatively on sperm parameters and function leading to infertility. Fig. 14.1 provides a diagnostic and therapeutic flow chart for patients with MAGI.

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CHAPTER

15

Hormonal and nonhormonal treatment of male infertility

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Etiology of male infertility

Male factor infertility, accounting for 50% of cases of infertility, is caused by abnormal sperm parameters [1]. The extreme of this is azoospermia, which describes the absence of sperm in the ejaculate [2]. The causes of male infertility can be broadly classified by the position of the defect in the hypothalamo-pituitary-gonadal (HPG) axis (Table 15.1). Pre-testicular causes of male infertility are caused by hypothalamo-pituitary disease resulting in hypogonadotrophic hypogonadism (low follicle-stimulating hormone (FSH)/luteinizing hormone (LH) and low testosterone). Testicular causes describe impaired spermatogenesis at the gonadal level and may be associated with hypergonadotrophic hypogonadism (high FSH/LH and low testosterone). At its most severe, testicular dysfunction can result in nonobstructive azoospermia [2]. Post-testicular causes represent anatomic disruption to outflow and thus obstructive azoospermia [3]. However, in 30%–40% of men with abnormal semen parameters the cause of infertility remains elusive and is classed as idiopathic [4]. In these cases it is suspected that genetic factors, environmental pollution, hormonal disruptors, and reactive oxygen species play a causative role [5].

Pathophysiology of spermatogenesis

Spermatogenesis, the stepwise differentiation of germ cells to spermatozoa, is reliant upon an intact HPG axis [6]. Pulsatile release of gonadotrophin-releasing hormone (GnRH) from the hypothalamus stimulates the anterior pituitary to secrete FSH and LH. FSH and LH act upon the testicular Sertoli and Leydig cells respectively. The Sertoli cells lining the seminiferous tubules of the testes are considered to offer structural and functional support to the developing spermatozoa. Furthermore, the Sertoli cells secrete inhibin B under the influence of FSH [7]. The Leydig cells secrete testosterone, maintaining the high intratesticular concentration of testosterone required for normal spermatogenesis. Indeed intratesticular testosterone (ITT) levels are 100-fold higher than serum levels [8]. Aromatase activity in the Leydig cells converts testosterone to estradiol. In turn, estradiol, testosterone, and inhibin B act via negative feedback on the hypothalamus and pituitary. A derangement at any level can disrupt spermatogenesis and cause male infertility [9].

Principles of management

The management of male infertility dependants upon where in the HPG axis the defect is located (Tables 15.2–15.7). Hormone replacement with GnRH or gonadotropins in hypothalamo-pituitary disease has been established as efficacious at inducing spermatogenesis and improving fertility potential [10]. There are no proven techniques to stimulate spermatogenesis in primary testicular dysfunction, and as such the mainstay of treatment for severe male factor infertility has been assisted reproductive technology (ART). Surgical sperm retrieval (SSR) represents a mechanism by which the man's own sperm can be used in *in vitro* fertilization (IVF)/intracytoplasmic sperm injection (ICSI). Hormonal stimulation prior to SSR is used as an off-license treatment in specialist centers with the aim of increasing ITT synthesis to increase sperm retrieval rates. The three drug classes used for this purpose are 1) gonadotropins,

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TABLE 15.1 Typical diagnostic features in the infertile male.

	Hypothalamo-pituitary disease[a]	Testicular dysfunction[a]	Obstructive azoospermia[a]
Testicular volume	↓	↓	Normal
Sperm count	Azoosermia/ oligospermia	Azoosermia/ oligospermia	Azoospermia
FSH	↓/normal	↑	Normal
LH	↓/normal	↑	Normal
Testosterone	↓/normal	↓	Normal

[a]*These are typical features but some patients may deviate from this pattern.*

TABLE 15.2 Hormone replacement in the management of male infertility.

Drug class:	Example:	Rationale:
GnRH analogs:	Gonadorelin Buserelin Leuprolide	Pulsatile GnRH is required for gonadotropin (FSH/LH) release from the anterior pituitary
Gonadotropins[a]:	rFSH rLH hCG HMG	Gonadotropins are responsible for maintaining high levels of intratesticular testosterone and inducing spermatogenesis
Dopamine agonists:	Bromocriptine Cabergoline	Prolactin exerts an inhibitory effect on the pulsatile release of GnRH from the hypothalamus, and dopamine inhibits prolactin production
Aromatase inhibitors (AI)	Anastrazole Letrozole Testolactone	Aromatase activity in the Leydig cells converts testosterone to estradiol Inhibition of aromatase releases the HPG axis from estrogenic negative feedback. As such AIs increase gonadotropin signaling, which in turn increases ITT
Selective estrogen receptor modulators[b] (SERMs)	Clomiphene Enclomiphene Tamoxifen	SERMS inhibit estrogen feedback at the level of the hypothalamus As such, SERMs increase gonadotropin signaling, which in turn increases ITT

[a]rFSH, *recombinant FSH;* rLH, *recombinant LH;* hCG, *human chorionic gonadotropin;* HMG, *human menopausal gonadotropin.*
[b]Clomiphene, *clomiphene citrate (CC);* tamoxifen, *tamoxifen citrate.*

2) selective estrogen receptor modulators (SERMs), and 3) aromatase inhibitors (AIs). The rationale for their use can be found in Box 15.1 and Table 15.2 [11–25]. However, the evidence base for this practice is conflicting. Obstructive azoospermia is managed by surgical correction of the anatomic defect and/or sperm retrieval prior to IVF/ICSI, discussion of which is beyond the scope of this chapter. Empirical hormonal treatment for idiopathic infertility is contentious but lifestyle modification and antioxidants show promise.

Hypothalamo-pituitary disease

Conditions affecting the hypothalamus and pituitary can be congenital or acquired following trauma, hemorrhage, surgery, or radiotherapy [4]. Defective GnRH or gonadotropin synthesis and release results in low levels of downstream androgens and impaired spermatogenesis. The extent of the hypogonadism depends upon the degree of deficiency and whether the insult occurred before or after puberty [4].

BOX 15.1

The rationale for the use of SERMs and AIs in male infertility

Selective estrogen receptor modulators

Mode of action

The proposed mechanism of action is based on SERM blockade of estrogen's negative feedback at the level of the hypothalamus. This results in increased GnRH secretion, followed by increased pituitary secretion of gonadotropins. Gonadotropins stimulate spermatogenesis and testosterone secretion in the testes [11,12].

❖ *Tamoxifen citrate (tamoxifen):*

Tamoxifen is a synthetic nonsteroidal estrogen antagonist that competitively binds to the estrogen receptor in the hypothalamus [11,12].

❖ *Clomiphene citrate (CC)*:

CC is a racemic mixture of two isoforms: enclomiphene, which is a strong estrogen antagonist, and zuclomiphene, which is a weak estrogen agonist [13]. Globally CC inhibits estrogen's negative feedback at the level of the hypothalamus and pituitary and thus upregulates FSH and LH production. There are reports of reversible deceased sperm motility and even azoospermia following treatment [14]. Studies into the use of enclomiphene alone, for pure estrogen antagonism, are undergoing to potentially exclude this complication [13].

Cautions:

Concerns have been raised about the safety profile of prolonged estrogen blockade in men of reproductive age, for example on bone health. However a recent review has found a positive effect of SERMs on bone mineral density [15]. There are also concerns regarding an increased risk of venous thromboembolism (VTE) with hormonal therapy. This is especially important in infertility associated with Klinefelter syndrome as they already have an elevated VTE risk [16]. A recent study demonstrated in a group without additional risks for VTE there is no greater occurrence [17].

Adverse effects: [18,19]

- Gastrointestinal:
  - Constipation
  - Diarrhea
  - Nausea
  - Vomiting
- Cardiovascular:
  - Hypotension
  - Prolonged QT interval on ECG
  - Atrial dysrhythmia
  - Hot flashes
- Neuropsychiatric:
  - Anxiety
  - Insomnia
  - Depression
  - Decreased libido

Aromatase inhibitors

- Letrazole
- Anastrazole
- Testolactone

Mode of action

Aromatase is an enzyme present in the testes, prostate, adipose tissue, brain, and bone of men. It converts testosterone to estradiol, and androstenedione to estrone. Estradiol exerts negative feedback on the hypothalamus and pituitary to reduce the secretion on gonadotropins. AIs reversibly inhibit the action of aromatase and thus release the HPG from the negative feedback effects of estradiol. This results in increased GnRH secretion from the hypothalamus, which stimulates gonadotropin release from the pituitary. Aromatase activity is thought to be of particular importance for infertility in the setting of low total testosterone or low testosterone:estradiol [20]. AIs increase testosterone and may therefore improve spermatogenesis [21].

Cautions

At high doses AIs may induce deleterious negative feedback on the HPG axis and thus ultimately reduce testosterone [22]. Furthermore, increasingly it is understood that estrogen has actions in the male reproductive tract including stimulation of sperm motility, maintenance of sperm morphology, and enhancement of oocyte penetration [23].

Adverse effects: [24,25]

- Decreased libido
- Deranged liver function
- Cutaneous rashes
- Hair loss
- Increased weight

Hypogonadotrophic hypogonadism (HH) secondary to dysfunctional hypothalamic production or release of GnRH can be treated with exogenous pulsatile GnRH. An example regime would be gonadorelin given every 90 min via subcutaneous pump. Doses are subsequently titrated based on resulting FSH, LH, and testosterone levels. This will successfully induce spermatogenesis in 85% of patients, with results seen as early as 4 months from treatment onset [26]. Pregnancy rates are quoted at 60% after 9 months treatment [27]. Response to treatment can also be observed by an increase in testicular volume and maturation of secondary sexual characteristics such as pubic hair growth [22]. Certain pretreatment and intertreatment characteristics can positively predict successful induction of spermatogenesis. These include normal pretreatment inhibin B, normalization of gonadotropin and testosterone levels, testicular size, and secondary sexual characteristics during treatment [22]. There is some evidence that idiopathic HH treatment with pulsatile GnRH can "reset" the hypothalamic-pituitary-adrenal axis so treatment need not be lifelong in about 10% [28]. In the event of treatment failure, testing for anti-GnRH antibodies should be performed [26]. The requirement to dose GnRH in a pulsatile manner limits its acceptability. The alternative 2-h intranasal or continuous intravenous pump administration, though effective, is unrealistic [26].

Due to their position in the downstream pathway, exogenous gonadotropins can be used to treat either pituitary or hypothalamic origins of HH. The options, with comparable efficacy, include recombinant FSH, LH, human chorionic gonadotropin (hCG), human menopausal gonadotropin (HMG), or purified urinary gonadotropins [29]. Structurally similar, LH and hCG both act upon the same receptor on Leydig cells. HMG, historically extracted from the urine of postmenopausal women, has both LH and FSH activity. Classically treatment is initiated with hCG alone, with treatment effect being evidenced by increased testicular volume and appearance of sperm in the ejaculate, and dose titrated by resulting testosterone levels [30]. If spermatogenesis does not occur beyond around 6 months of treatment, recombinant FSH or HMG would be introduced [30]. Gonadotropins successfully induce spermatogenesis in 80% of patients, rising to 94% with combination gonadotropin therapy [31,32]. However clinical pregnancy rates following treatment are quoted at 38%–51% [27,33]. Side effects are uncommon when doses are titrated by testosterone level but include gynecomastia, acne, and weight gain [22].

In the setting of subfertility, a secreting prolactinoma can be managed pharmacologically using a dopamine agonist. Cabergoline is considered first line. In those who fail to respond to cabergoline a trial of bromocriptine is advised. If both cabergoline and bromocriptine have been tried at maximal dose, dopamine agonist resistance is diagnosed and surgery is indicated [34].

Testicular failure

Hypergonadotrophic hypogonadism is usually present in the setting of primary testicular dysfunction [4]. Generally, in the presence of absent or minimal spermatogonia, FSH levels will be found to be high [4]. However individual FSH levels do not predict sperm quality, as in the setting of normal FSH and testes volume, maturation arrest may still have occurred at the spermatocyte or spermatid level, so azoospermia is still found [35].

There is no substantial evidence to suggest that standalone therapy with gonadotropins, SERMs, or AIs improve spermatogenesis if the defect is at the gonadal level [36]. It has been postulated that suppression of high gonadotropin levels by administration of a GnRH analog may overcome desensitization of the Sertoli cells caused by the elevated levels of circulating gonadotropins, though definitive evidence for this is currently lacking. A small study in 1989 treating men with testicular failure (nonobstructive azoospermia and hypergonadotropic hypogonadism) with pulsatile GnRH found that while FSH levels were significantly reduced, there were no improvements in semen parameters [37].

For men with such nonobstructive azoospermia, SSR and ICSI can be offered. Hormone stimulation prior to SSR aims to increase the yield by enhancing spermatogenesis. Although all currently unlicensed, three drug classes are commonly used for hormonal stimulation prior to SSR: (1) gonadotropins, (2) SERMs, and (3) AIs (Box 15.1). They all utilize the same mechanism of action: increased gonadotropin signaling within the testes to increase testosterone. Gonadotropins do this directly, whereas SERMs and AIs indirectly increase gonadotropins by blocking estrogen-driven negative feedback. Pharmacologically increasing gonadotropin levels in men with testicular failure, in whom gonadotropin levels may already be elevated, might seem counterintuitive. However high ITT is a requisite for normal spermatogenesis [8]. Evidence suggests that low ITT is associated with the persistence of immature germ cells [8]. A recent survey of American urologists found 65% of respondents use hormone stimulation therapy prior to SSR [38]. Gonadotropin therapy is generally more costly, and as such is usually reserved for patients intolerant to AIs and SERMs [39]. SERMs such as clomiphene citrate and tamoxifen are the most commonly used drugs as they are low cost and conveniently orally administered [39]. AIs, such as anastrozole, are favored in obese patients due to the action of aromatase in adipose tissue [20].

Although the theoretical rationale for hormonal stimulation prior to SSR is sound, the evidence base remains

incomplete with a paucity of high-quality randomized controlled trials to support this practice (Box 15.2) [40–47] Furthermore the few trials that exist fail to comment on pregnancy outcomes and complication rates. However in the treatment of nonobstructive azoospermia there are no alternative options to optimize spermatogenesis. Given the cost implications and lack of evidence, empirical treatment in all patients is ill-advised. However in select individuals this treatment may be appropriate after thorough evaluation of comorbidities, the couple's age, and the fertility status of the female partner with regard to likelihood of IVF success [48]. This therapy may be of specific value to those for whom the use of donor sperm is unacceptable [39].

Idiopathic male infertility

Empirical hormonal therapy

GnRH analogs

There is no evidence for the empirical use of GnRH analogs in idiopathic male infertility. Two relatively small randomized clinical trials have investigated this and found no significant difference in semen parameters following treatment compared with controls [49,50]. Given this lack of evidence its use cannot be advocated [4].

TABLE 15.3 Summary: GnRH analogues.

Hypothalamo-pituitary dysfunction	Effective
Testicular dysfunction	Definitive evidence lacking
Idiopathic male infertility	No evidence to support

Gonadotropins

Definitive evidence is lacking, but there is evidence to suggest that FSH treatment improves sperm parameters in men with idiopathic infertility [51]. Studies have also found that sperm DNA fragmentation is reduced in the treatment group [41,52]. A 2013 Cochrane review (6 randomized clinical trials with >400 participants) concluded that FSH treatment resulted in higher pregnancy and live birth rates in the setting of natural conception but not with ART [53]. In contrast, a 2015 meta-analysis (15 studies with >1200 participants) found improvements in both spontaneous and assisted conception rates [54].

TABLE 15.4 Summary: Gonadotropins.

Hypothalamo-pituitary dysfunction	Effective
Testicular dysfunction	Definitive evidence lacking
Idiopathic male infertility	Definitive evidence lacking

Androgens

The fundamental importance of testosterone to male reproductive health makes it an attractive candidate for empirical treatment. Low-dose testosterone has been shown to improve epididymal maturation of spermatozoa, and there was a notion that high-dose rapidly withdrawn therapy might induce a rebound gonadotropin surge. However physiology would dictate that regular supplemental testosterone, or its metabolites, will inhibit gonadotropin release from the pituitary. Consequently, ITT, a requirement for spermatogenesis, will be reduced.

Large studies have shown that testosterone supplementation does not improve sperm production or pregnancy rates [55]. In fact, exogenous testosterone administration has been shown to decrease sperm count in a reversible fashion and as such has been investigated as a male contraceptive [55,56]. Upon treatment cessation, 64%–84% of men will recover normal sperm parameters within on average 110 days [56]. Stand-alone androgen therapy is therefore contraindicated in the treatment of male infertility [4].

There has been some interest in co-administration of testosterone and tamoxifen, with evidence suggesting it increases sperm count and motility [47,57]. However, notably neither study reported pregnancy outcome data.

TABLE 15.5 Summary: Androgens

Hypothalamo-pituitary dysfunction	Ineffective
Testicular dysfunction	Ineffective
Idiopathic male infertility	Ineffective

Aromatase inhibitors

The activity of aromatase enzyme has commonly been considered to be associated with male infertility, especially in the setting of testicular dysfunction and a low testosterone:estradiol ratio. Elevated levels of estradiol exert negative feedback on the HPG, resulting in reduced FSH and LH and consequentially impaired spermatogenesis. A 2019 meta-analysis found that AIs lead to a statistically significant improvement in semen parameters and hormonal profile [21]. These results are promising but further larger randomized clinical trials are required to form firm conclusions regarding the clinical applicability of AIs in idiopathic infertility [4].

TABLE 15.6 Summary: Aromatase inhibitors.

Hypothalamo-pituitary dysfunction	N/A
Testicular dysfunction	Definitive evidence lacking
Idiopathic male infertility	Definitive evidence lacking

BOX 15.2

Evidence review: hormonal stimulation prior to surgical sperm retrieval in men with testicular failure [40–47]:

Cocci et al. (2018)	A case-control study of men with idiopathic nonobstructive azoospermia treated with rFSH prior to SSR ($n = 25$) compared with a control ($n = 25$) who did not receive hormonal stimulation prior to SSR - 24% of the intervention arm had positive sperm retrieval compared with 12% in the control group
Shinjo et al. (2013)	A case series of 20 patients with nonobstructive azoospermia + hypergonadotropic hypogonadism who had had a negative SSR were administered gonadotropin therapy (HCG ± FSH) prior to a second SSR attempt - Sperm was successfully retrieved from three patients (15%) - ITT was statistically significantly elevated (measured via testicular fluid obtained during SSR)
Cavallini et al. (2013)	A randomized control trial that included 11 men with nonobstructive azoospermia + normal hormone levels; the intervention group received letrozole for 6 months ($n = 6$) - All participants in the intervention group ($n = 6$) were found to produce sperm in their ejaculate compared with one participant in the control group ($n = 5$) - Serum FSH, LH, and total testosterone were significantly elevated and estradiol decreased in the intervention group, compared with no statistically significant change in the controlled group
Hussein et al. (2013)	A multicenter case-control study of patients with nonobstructive azoospermia treated with a combination of clomiphene, hCG, and HMG ($n = 496$) prior to SSR compared with a control ($n = 116$) who did not receive hormonal stimulation prior to SSR - 57% of the intervention group had positive sperm retrieval compared with 33% in the control group
Reifsynder et al. (2012)	A retrospective cohort study of consecutive men undergoing SSR for nonobstructive azoospermia; those with preoperatively low testosterone (<300 ng/dl) were treated with AIs, CC, or hCG per the units standard operating procedure; 736 men were included in the study, of which 348 had low testosterone warranting hormonal stimulation and 388 had normal pretreatment testosterone - Men with nonobstructive azoospermia + hypogonadism responded to hormonal therapy with an increase in testosterone levels - There was no statistically significant difference in sperm retrieval, pregnancy, and live birth rates between the group with normal and abnormal testosterone levels
Ramasamy et al. (2009)	A cohort study ($n = 68$) of men with Klinefelter syndrome testolactone or anastrozole (for 2–3 months), followed by hCG or CC if hormone parameters did not improve - In patients who responded to hormone stimulation (improved total testosterone levels) SSRs were higher
Foresta et al. (2009)	A randomized control trial of men with hypergonadotrophic hypogonadism + oligospermia treated with a GnRH agonist (once every 30 days for 4 months) followed by recombinant FSH and hCG - Statistically significant improvement in sperm parameters in the intervention group ($n = 57$) compared with controls
Pavlovich et al. (2001)	A case series of 43 men with nonobstructive + hypergonadotropic hypogonadism receiving testolactone (for a mean of 5 months) - Significant increase in total testosterone and reduction in estradiol - None of the participants produced sperm in their ejaculate - The study did not report success rates at SRR

Selective estrogen receptor modulators

SERMs act to release the hypothalamus from estrogen-driven negative feedback. This elevates GnRH and subsequent FSH/LH levels. The rationale for its use lies in the resulting increase in testosterone and thus assumed enhanced spermatogenesis. An initial 1999 meta-analysis (11 randomized clinical trials with 459 participants) found no association between the use of SERMs and increased pregnancy rates in idiopathic male infertility [58]. However subsequent meta-analyses in 2013 and 2019 found SERMs to be associated with significant improvements in semen and hormone parameters, as well as increased pregnancy rates [11,12]. However in all three of the meta-analyses, only a few of the included

studies were placebo controlled. As such, no firm conclusions can be currently drawn regarding the value of SERMs in idiopathic male infertility.

TABLE 15.7 Summary: SERMs.

Hypothalamo-pituitary dysfunction	N/A
Testicular dysfunction	Definitive evidence lacking
Idiopathic male infertility	Definitive evidence lacking

Empirical non-hormonal therapy

Antioxidants

Reactive oxygen species (ROS), considered to be one of the most important contributing factors to idiopathic male infertility, have gained attention in recent years. Described as unstable by-products of cellular metabolism, high levels of ROS have been suggested to induce sperm DNA fragmentation and contribute to impaired sperm function via effects on the acrosome reaction and sperm motility [59,60]. However, the pool of data regarding the value of empirical antioxidant therapy remains conflicting, and the quality of the contributing studies has been criticized. Cochrane reviews in both 2014 and 2019 reported an increased live birth rate, while the 2020 MOXI trial (Males, Antioxidants, and Infertility) found no improvement in sperm DNA fragmentation, semen parameters, or live birth rates [61–63]. Furthermore, concerns have been raised about the safety profile of the empirical, over-the-counter use of antioxidant supplements such as those containing L-carnitine and acety-L-carnitine. The recently coined term male oxidative stress infertility describes men with idiopathic infertility and the finding of raised semen ROS [5]. A 2021 single-center prospective cohort study compared outcomes following antioxidant therapy (acetyl-L-carnitine) in patients with normal pretreatment semen ROS with those with raised pretreatment levels [64]. This found that sperm count and motility was improved only in those with initially abnormally elevated ROS [64].

Antibiotics

It is established that urogenital infection is associated with a pathologic number of leukocytes in the ejaculate (leukocytospermia, $>1 \times 106$ leukocytes/mL) [65,66]. Studies suggest that leukocytospermia is associated with a greater rate of deranged semen parameters and ROS [67]. However, a 2016 systematic review concluded that the data was too limited to definitively confirm an association between established male urogenital infection and infertility [68].

Evidence for empirical antibiotic treatment in the setting of leukocytospermia in the absence of identified infection is lacking. A 2016 meta-analysis found that antibiotic treatment might improve sperm parameters such as motility, morphology, and concentration, but there was no evidence that this improved conception rates [69].

Lifestyle modification

Evidence suggests that improving deleterious lifestyle factors such as smoking, caffeine consumption, and alcohol use may improve semen parameters. However it is not clear if this translates into improved pregnancy and live birth rates. Although the evidence that lifestyle change improves male fertility is incomplete, low semen quality is associated with increased all-cause mortality and reduced life expectancy, so addressing modifiable risk factors has a benefit beyond conception [70].

Obesity and weight loss

Data shows a statistically significant relationship between deranged sperm parameters and obesity. A 2013 systematic review and meta-analysis found obese men are more likely to be oligo- or azoospermic compared with their normal weight counterparts [71]. However, currently, there is insufficient evidence to suggest weight loss is an effective therapy. Studies are conflicting with reports of improvement, no change, and deterioration of semen parameters [72–77]. Indeed during the first 6 months following bariatric surgery, sperm concentration is reduced [77].

Diet and exercise

A 2018 systematic review and meta-analysis found a positive association between certain food types and sperm quality, namely fruit, vegetables, fish, poultry, and low-fat diary [78]. On the corollary, full-fat diary, processed meats, sugary drinks, alcohol, and caffeine are associated with poorer semen quality [79]. A 2017 meta-analysis found selenium, zinc, coenzyme Q10, and carnitines improve semen parameters [79].

A recent meta-analysis found that moderate-intensity exercise is associated with an improvement in semen parameters [80]. Hormonal profiles may also be improved by exercise [81].

Alcohol

Given its prevalence in society it is perhaps surprising that the effect of regular alcohol consumption on male fertility is incompletely understood. Alcohol use has been shown to reduce semen volume, but its effect on semen parameters is less well delineated [82]. A 2017 meta-analysis found heavy alcohol consumption impairs semen volume and morphology but found no

correlation between that and occasional alcohol use [83]. Heavy alcohol use reduces testosterone levels reversibly, which improve upon cessation of alcohol consumption [84].

Smoking

A number of systematic reviews and meta-analyses show an association between smoking and reduced sperm count and motility, and an increase in abnormal morphology [82,85]. This effect is enhanced both by preexisting subfertility and by intensity of smoking habit [86]. Sperm DNA fragmentation is higher and gonadotropin and testosterone levels are lower in smokers [87,88]. Furthermore, animal studies show a similar pattern with the use of vaping, an often considered safer option [89].

Caffeine

Systematic review has found that caffeine may be associated with increased sperm aneuploidy but not with derangement in classical semen parameters [90].

Recreational drugs

There is clear evidence of a causal link between infertility and some recreational drugs. For example in animal models, regular administration of cocaine interrupts spermatogenesis and decreases pregnancy rates [91]. Anabolic steroids, used to enhance athletic performance, are the most common cause of profound male hypogonadism [92]. The resultant increased testosterone level exerts negative feedback on the HPG axis suppressing spermatogenesis, with a recovery time of up to 2 years [93]. Sexual function is also impaired by the hypogonadic state [94].

Stress

Raised corticosteroid levels suppressing testosterone represents a viable explanation for impaired fertility when under psychological stress and depression [95,96]. Indeed a large meta-analysis found an association between stress and lower sperm concentration, progressive motility, and normal forms [82]. However, in this setting, psychotherapeutic techniques may be preferable to pharmacological as antidepressant drugs can impair semen quality and psychosexual function [97,98].

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CHAPTER

16

Surgical management of male infertility and sperm retrieval

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Surgical management of conditions related to male infertility

Varicocele

Introduction

Treatment of varicocele represents a matter of debate in reproduction medicine. The higher incidence of infertility in varicocele-affected men does not correlate with the evident increase of pregnancy rate after treatment [1,2]. International guidelines are not in agreement about the role of varicocele management; e.g., National Institute for Health and Care Excellence guidelines suggest avoiding treatment for male infertility, but European Association of Urology guidelines support a different approach [3,4]. Effectively, this debate mirrors the uncertain pathophysiology of varicocele-related infertility.

For these reasons, overtreatment has been probably faced in the past; in particular, varicocelectomy has been proposed in adolescents with preventive aims [4]. More recently and contrarily, assisted reproduction techniques (ARTs) have been considered directly as they show to be faster in providing results [5], avoiding treatment of varicocele. A real evidence-based medicine and cost-effective evaluation is still missing. On other side, debate is still open about which clinical settings should be suggesting surgical management.

It has been strongly suggested to treat males with generic "altered sperm parameters" [6]. Nonobstructive-azoospermia (NOA) has been proposed [7], and high DNA fragmentation could be considered still an investigational indication [4]. A Cochrane review in 2012 concluded that treatment in unexplained infertility-affected couples may lead to an increase in spontaneous pregnancy rate [8]. Otherwise, we have no real confidence about what "unexplained infertility" was in different Randomised Clinical Trials. In this uncertain set, different procedures have been proposed to treat varicocele, which are generally divided in two groups: radiological and surgical approaches.

Radiological techniques: retrograde sclerotherapy or embolization

Developed during the 1970s, it was the first technique not requiring a surgical approach. Direct injection into the vein of hypotonic solution, after a percutaneous approach to the femoral vein, has been reported (Fig. 16.1).

Later, venographic placement of a balloon or coil in the gonadal vessels was reported by different authors, with a relatively high success (75%–90%) [9–12] (Table 16.1). Criticism of this techniques has been its "time consuming" feature and the (extremely rare) reported complications related to migration of the coil or balloon (in renal vein, pulmonary embolization) or femoral thrombosis or perforation [13]. On other hand, the two-dimensional view afforded does not enable the surgeon to identify the location of collaterals, and a significant number of men undergoing attempted radiographic occlusion will ultimately require a surgical approach [14].

Radiological techniques: anterograde sclerotherapy or embolization

This technique has the aim to treat a sclerosing spermatic vein, in which the access is obtained from the scrotum. This technique was developed in Europe to reduce the time and invasiveness of the retrograde approach [15–17]. Otherwise, the recurrence rate seems similar to retrograde technique, and complications are probably the same, despite different vascular anatomy faced in these approaches [18,19]. Modification of the technique has been proposed to reduce incidence of

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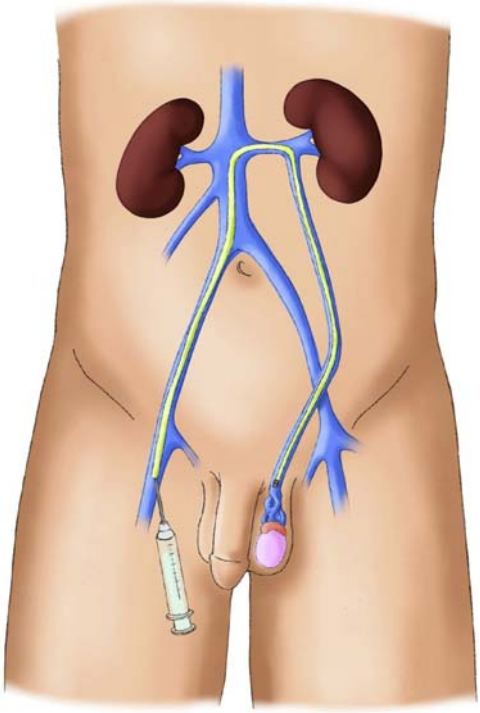

FIGURE 16.1 Anatomical scheme of retrograde embolization of varicocele.

complication, but considering the possible increase in invasiveness as well as time and resources consumed, it strongly conflicts with the initial aim [20].

Surgical techniques: retroperitoneal approach

Retroperitoneal repair involves incision at the level of the internal inguinal ring or higher, splitting internal and external oblique muscle, and exposing gonadal vessel (artery and vein) retroperitoneally. The real advantage is the isolation of the vessel proximally, relatively near the point of drainage to the left of the renal vein. At this level, only one or two veins are present, and the testicular artery has not branched and is distinctly separate from the vein. Didactically, the technique is referred to as "Palomo technique" (where the approach is more cranial and proximal, with a "nearly abdominal approach," used more traditionally in children) or "Ivanissevich technique" (more caudal, at the passage between abdomen and groin, in an adult setting) (Fig. 16.2).

In the first one, the artery preservation is more difficult, due to the very low diameter and position. So especially in children, systematic artery ligation has been described as part of the technique. These approaches are considered to be affected by higher incidence of recurrence or complication (Table 16.1). In particular, the Ivanissevich technique seems to be less affected by hydrocele for the preservation of artery and lymphatic vessels; otherwise, incidence of recurrence could be faced, for persistence of *venae comitantes* (periarterial plexus) (Table 16.1) [21–25]. The procedure is fast and conceptually easy, and if the surgeon will be working in a "deep hole," effectively dissection and ligation is in situ in the retroperitoneum.

Surgical techniques: laparoscopic approach

It could be considered a retroperitoneal approach, with all the advantages and disadvantages of open surgery [26–28]. Magnification of vessels and lymphatic and internal inguinal rings by laparoscope is the major advance provided by this technique, and in time, lymphatics may be visualized and preserved, as for the artery [29] (Fig. 16.3).

Some experiences report a relatively low recurrence rate (2.9%–4.5%) [29,30]. The cost-effectiveness of this approach must be considered, considering general anesthesia, and potential complications related to abdominal access (e.g., bowel injuries) and materials seem to undermine potential use of this technique. Otherwise, in rare occurrence of bilateral varicocele, it seems to be a rational procedure [29,30].

Surgical techniques: inguinal and subinguinal approach

These techniques, proposed more recently than retroperitoneal approaches, have gained popularity for relatively low incidence of recurrence and hydrocele. Traditional inguinal approaches involve an up to 7 cm

TABLE 16.1 Comparison between different techniques.

	Retroperitoneal	Conventional inguinal	Laparoscopic	Radiographic	Microsurgical (sub/inguinal)
Artery preservation	Palomo: no ivanissevich: Yes	No	Yes	Yes	Yes
Hydrocele (%)	7	3–30	12	0	0
Recurrence (%)	15–25	5–15	3–15	15–25	1–5
Risk of serious morbidity	No	No	Yes	Yes	No

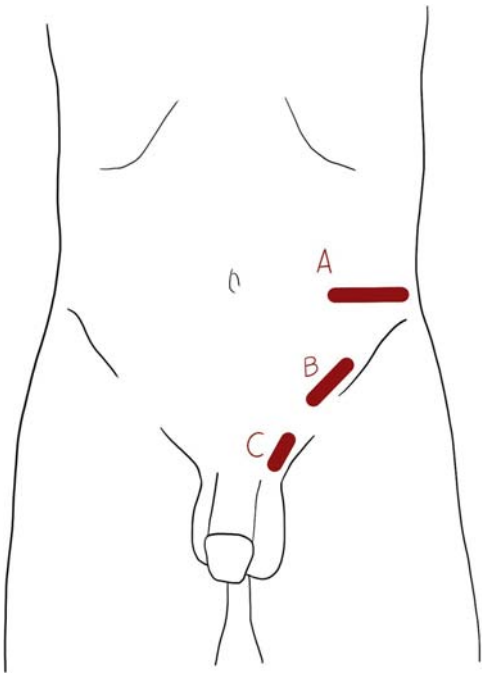

FIGURE 16.2 Surgical access in different techniques: (A) Palomo approach, (B) Ivanissevich approach, (C) inguinal approach.

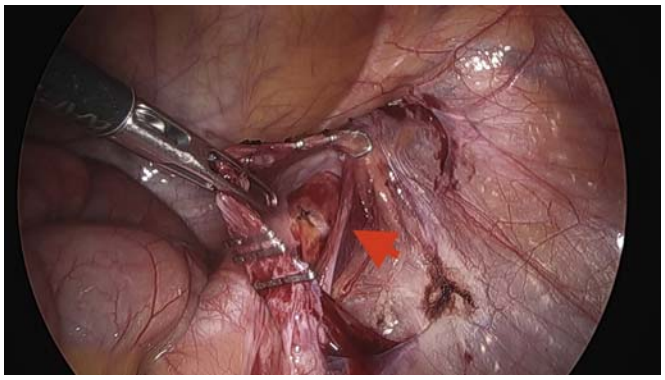

FIGURE 16.3 Laparoscopic view of gonadal vessels during laparoscopic treatment (the right vessel shown here in a bilateral setting). *Red arrow* indicates artery that has been preserved.

incision (Fig. 16.2) made over the inguinal canal, where the external oblique aponeurosis is to be opened; then the cord is isolated and spermatic veins ligated [31]. First, authors reported an incidence of hydrocele varying from 3% to 15% [32]. Later, microsurgical techniques were proposed with the aim to reduce (successfully) complications: use of magnification allows one more easily to identify lymphatics, analogously to the artery, to enable preservation [33,34].

The subinguinal approach, just below the external inguinal ring, described by Marmar in 1985 [35], obviates the need for fascial opening and so enables rapid recovery, and easier pain management has been reported. Contrastingly, in this site, the artery is surrounded by tiny veins (to be ligated) and is divided in different branches. So, this technique needs surgical skill and a longer training period [36]. For this reason, it has been suggested that subinguinal surgery should be used by surgeons with a high level of experience [37]. Another criticism of the technique is that it is quite time consuming. Therefore, the use of an automatic clip applier has been proposed [37]. On other hand, it would increase disposable materials and consequently costs. Terminally, the right procedure will bring a complete dissection with preservation of cremaster muscle fibers, testicular arteries, cremasteric arteries, lymphatic, nerve, and vas deferens.

Subinguinal and inguinal approaches also allow testicular delivery, with the aim to clamp the gubernacular veins, so all venous drainages are managed (described to account for 10% of recurrences). Improvements are reported to be due to the access of the external spermatic vein and even gubernaculum veins (that potentially bypass the spermatic cord and justify recurrences) [38]. Negatively, management of testis sometimes suggests use of drainage (e.g., Penrose), in particular if a little amount of hydrocele fluid is removed [37]. This aspect could extend the hospital stay.

Summary about varicocele treatment

Surgical treatment in varicocele has a long tradition in urological and andrological units. In the last 20 years, indications have been a topic of debate, and effectively, evidence-based medicine in this field is far away. On other hand, a large number of techniques are available. Some of them fare better in complications rate or preventing recurrence, but all the aforementioned ones are still more or less employed. Reasons could be the lack of real cost analysis, a need for less "wasted time," and, finally, the need for a long (or, contrarily, really shorter) learning curve.

Retractile testis in adulthood

Indication of treatment of retractile testes in adults is rare, but it must be considered that some evidences reported that a subset of infertile men has retractile testes [39]: semen parameters seem to be altered, as in varicocele. It is possible that a higher temperature accounts for the alterations.

Two techniques are possible in these cases:

a. Dartos pouch is performed by incision of the skin and then creation of adequate space between *derma* and Dartos. By incision of Dartos and *vaginalis*, the testis is isolated and placed in the pouch. Cremasteric fibers could be ligated, and then the opening in Dartos is closed around the cord. No suture involves the

albuginea, with the aim of protecting the testicular artery [40].

b. More easily (but sometimes considered less effective), as in prevention of testicular torsion, a little transverse incision of skin, derma, Dartos, and *vaginalis* is performed. Finally a suture (generally two stiches: upper and lower testicular pole) between albuginea and inner face of Dartos could be performed [37].

Ejaculatory ducts obstruction

Transurethral resection

Ejaculatory duct obstruction is a congenital or acquired condition, accounting for a large number of causes and clinical development. In case of aplasia of terminal tract or compression by seminal vesicle or prostate cyst, transurethral resection of ejaculatory duct (TURED) could have a role [41]. Also, if it seems to reduce the need of ART in couples affected by azoospermia or severe oligoasthenospermia [42], this procedure should be proposed only in select cases, and when the couple prefers to avoid the Intracytoplasmatic sperm injection/*In-vitro* fertilization techniques.

Considering a correct and complete workflow to diagnose the disease, performing TURED is technically easy for a urological surgeon with minimum endoscopic expertise. Resectoscope, by a 24-Fr loop is engaged with manual (a finger in rectum) control of posterior lobe of prostate. By that, the space between bladder neck and *verumontanum* should be treated to open the ducts, which effectively course in this zone. Sometimes, a cyst cavity or the enlarged duct could be shown. Otherwise, anatomical respect is of paramount importance: bladder neck fiber should not be engaged, to avoid retroejaculation. Distally, the striated sphincter must be preserved. Finally, excessive coagulation should be avoided, to not further new stricture development. The delivery rates of up to 38.5% per attempt (in best series, but must considered that all reported are quite little ones) should be balanced with possible serious complications: chronic epididymitis, reflux of urine in ejaculatory ducts (with new impairment of semen quality), and retrograde ejaculation.

Vasovasostomy and vasoepididymostomy

Treatment of Ejuculatory Ducts obstruction by anastomosis is conceptually very easy, but surgically quite challenging. Technique could be different, and it changes also by grade, causes, and level of obstruction. This surgery should be reserved to experienced centres, at best in strict collaboration with an ART Unit [3], to permit a clear balance between cost and benefit of surgery. The success of operation is strictly dependent on the primary causes of obstruction, duration, and surgical technique. In multiple vasal obstruction unsuccesful operation are a matter of facts: anastomosis in two different sites is affected by high rate of devascularization and fibrosis [37].

By some authors, re-anastomosis has a better long-term pregnancy rate than ART technique and a benefit in costs [43,44]. Couples should be informed also that the patency could be (re-)established, but data series are large only in vasectomy reversal (that is a specific indication), otherwise fertility could be also affected by sperm antibodies (subsequent to surgery), finally that secondary obstruction may occur [45].

Before these procedures are proposed, a correct analysis of spermatogenesis should be documented. Some conditions discourage anastomosis: *e.g.* elevated FSH or evidence of small or soft testis. Strong suspicion of impaired spermatogenesis is a relatively strong contraindication.

Vasovasostomy is generally performed for vasectomy reversal. It is estimated that 2%–6% of men will require reversal [37]. Other indications are occlusion secondary to orchiopexy or herniorrhaphy. In the first case, a scrotal approach is preferred, differently infrapubic or inguinal incision should be performed.

Preparation of vasa is an important part of surgery, considering that length of deferens gap is sometimes a problem, and vas should not be stripped of its sheath, preventing the vasal vessel from damages. Additional length could be achieved by dissecting the entire convoluted vas free from its attachment to the epididymal tunica. Injury to the testicular artery is a complication to avoid, considering the high risk of testicular atrophy resulting.

These aspects, which explain how surgery could be time consuming and not easy to project, associated with a need for absolute absence of movement during microsurgical approaches, justify the preference for general anesthesia.

Accordingly to Goldstein and Hagan [37,44], six surgical aspects are of paramount importance:

a. accurate mucosa to mucosa approximation
b. leakproof anastomosis
c. tension-free anastomosis
d. good blood supply
e. healthy mucosa and *muscolaris*
f. good atraumatic anastomotic technique

Anastomosis in the convoluted vas could be needed but is more challenging, considering the higher risk of fibrosis, due to the lower blood supply in the testicular end. When approaching the vasectomy site, evidence of copious thick and toothpaste-like fluid is present or/and no sperm and no granulomas have been found at the surgery site, vasoepididymostomy is preferred. In this case, different techniques are possible: end-to

side technique (classical), end-to-side intussusception, or variations of the latter.

In all cases, also considering the high report of sperm appearance in the ejaculate [46,47], a contextual semen cryopreservation is mandatory, to permit in vitro fertilization also in men with low quality, low count, or that remain azoospermic after anstomosis [48].

Sperm retrieval in azoospermic men

Azoospermic men should undergo accurate diagnostics according to a flow-chart, aimed to understand the cause of absence of spermatozoa in the ejaculate. These findings have a huge role in choice of technique employed for sperm retrieval. Effectively, some techniques are suggested only in obstructive azoospermia (OA). Therefore, others are effective in both cases, but overtreatment could become a matter of fact.

Sperm retrieval in obstructive azoospermia

Obstruction site influences technique.

In intratesticular obstruction, only surgical testicular sperm extraction (TeSE) is suggested [4]. The technique is similar to the one used in nonobstructive azoospermia (NOA) and so is in specific chapter more extensively presented. The only difference is that a single-site specimen in OA is generally adequate.

In epididymal obstruction more options are available: TeSE has a role, but also microsurgical epidydimal sperm aspiration (MESA), percutaneous epididymal sperm aspiration (PESA), or testicular sperm aspiration (TeSA) could be effective. The first one seems to be more effective to retrieve materials for repeated ICSI procedure, but real RCTs are lacking [49,50]. In some studies, it has been proposed that epididymal sperms have a better outcome in pregnancy rate than testicular ones [51,52]. Otherwise, no conclusive results are really available.

Microsurgery is generally carried out under general anesthesia and that increases cost and length of procedure [53,54]. So, in experienced hands, percutaneous sampling has been developed: by different experiences, it seems really effective. Technically, percutaneous procedures (TeSA/MESA) are quite easy, so they are largely used. Consequently, in a large amount of ART centers, they have become the standard, even if sometimes not really correctly indicated (e.g., in NOA). Briefly, a 19-gauge butterfly needle is placed in the anterior testicular midpole, and suction is obtained by a 30-mL syringe by pulling back by the plunger (Fig. 16.4).

It has been proposed contestual ultrasonography to locate vessels and to avoid hematomas; otherwise, the low incidence of this complications in conventional (not US-guided) TeSA and MESA has affected its widespread acceptance [53,55,56]. Another option, described as an evolution of TeSA, could be PercBiopsy [57], where the testicle should be fired by biopsy needle. The pros are the significant amount of tissue retrieved (making it a mediation between TeSE and TeSA) and the ease of procedure. The cons are its invasiveness (VAS scale reported is higher) and higher complication rate (in particular hematomas).

Sperm retrieval in nonobstructive azoospermia

Different from OA in the clinical setting, in which success rate is higher and different techniques are available, NOA sperm recovery is slightly ineffective. Technique, experience, and correct indication are of paramount importance for results.

First, it should be clear that testicular biopsy, in past proposed in the diagnostic flow-chart in azoospermia, actually is absolutely to be avoided and histology diagnosis only inserted as a part in a TeSE (or microsurgical TeSE) scheme (so called *therapeutic biopsy*) [58]. Prior to proceeding with surgical sperm retrieval, a series of fine-needle aspirations (TeFNA) has been proposed to guide TeSE/m-TeSE and to provide a preliminary insight of retrieval chance by some authors. Actually, this preliminary procedure should be considered only in RCTs and for investigational aim [4]. Different from that stated earlier, TeSA in NOA should not be recommended, though it sometimes is performed for ease and low cost [4,59].

TeSE is a technique that permits retrieval of spermatozoa by low invasiveness for patients. It could be proposed with local anesthesia, sometimes associated with mild sedation. The aim is to provide sperm for cryopreservation and delayed use in ICSI. In some cases, "fresh use" has been proposed, but it requires a experienced center with significant coordination between teams to guarantee effective synchronous procedures [53,60].

In the last years, two different techniques have been proposed. "Trifocal manner" has a tradition, first described by Giessen group [53]. Upper, middle, and lower surfaces of the albuginea are incised over 0.5 to 1.0 cm. That permits in general to avoid significant bleeding, considering that vascular structures are not closed over. Under gentle pressure, small protruding pieces of testicular tissue are exposed, and so by using fine surgical scissors, they can be removed [61,62].

An alternative technique could be to perform an equatorial (or two little) scrototomy with multiple biopsies collected in an equatorial manner around the testis [63]. Considering the vascular architecture of the testis, the transverse approach is preferred to longitudinal incision of the tunica albuginea for avoiding subtunical blood vessel damage (Fig. 16.5) [53].

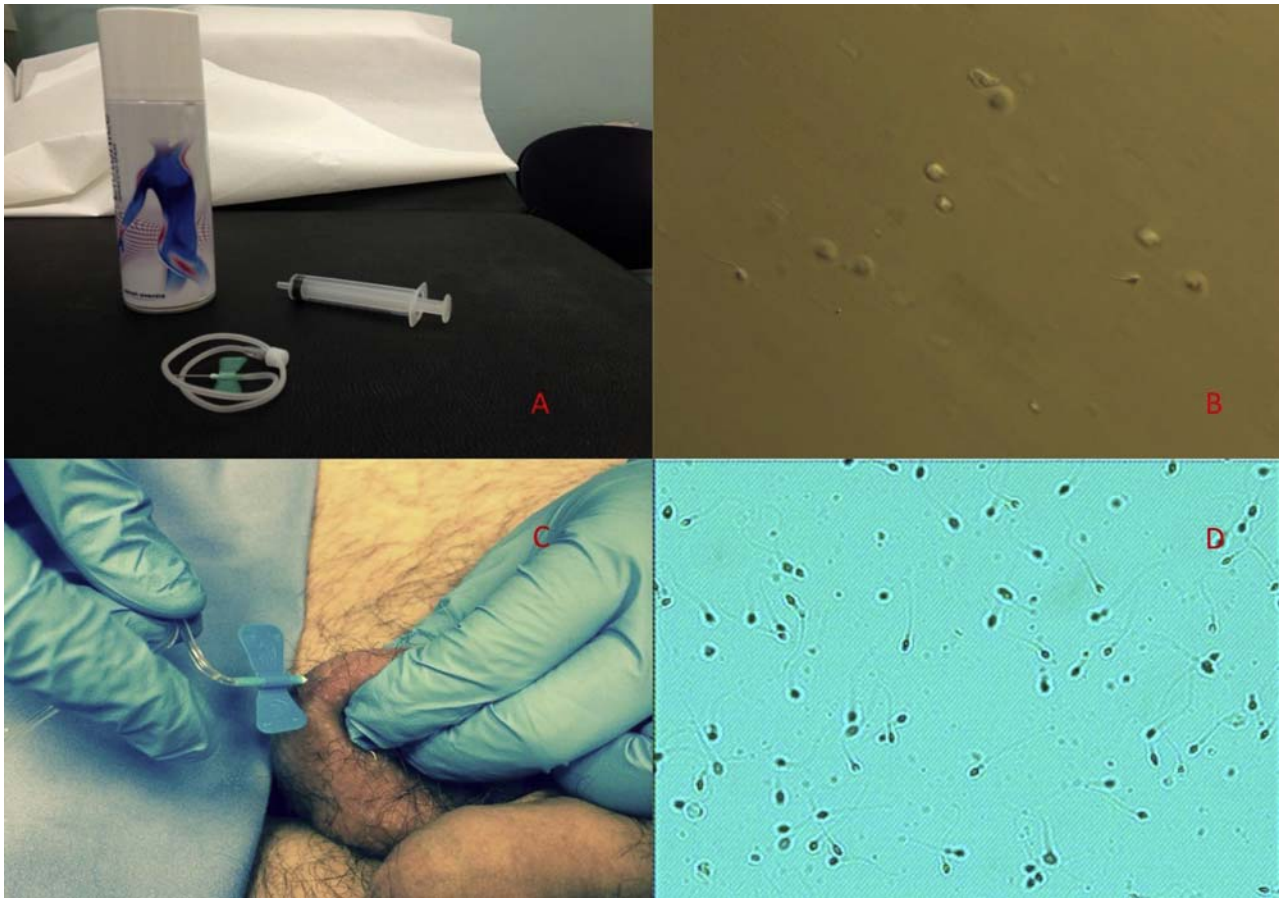

FIGURE 16.4 Material (A), technique (C), and direct observation of specimen obtained by TeSA (upper, B) and PESA (lower, D).

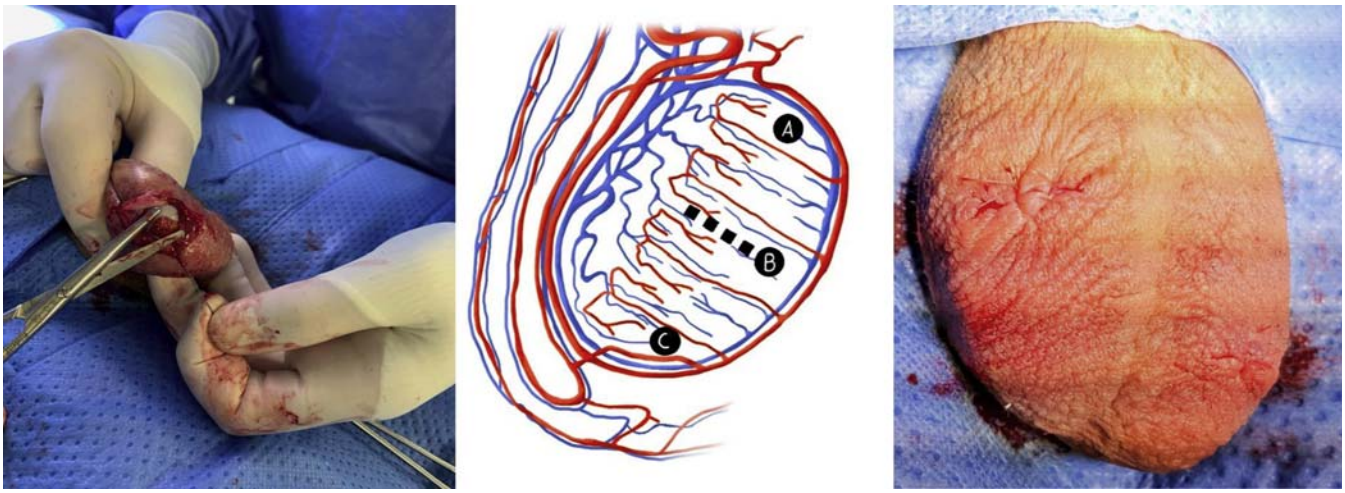

FIGURE 16.5 Technique of trifocal TeSE: generally micro-assisted TeSE is performed in the B area.

In the past, three incisions, at least in NOA, have been strongly suggested [64]. Otherwise, debate has been focused on evidence that the number of incisions does not affect success rate, but the total amount of tissue harvested. Sometimes, cryopreserved sperm cells are few, reducing ICSI attempts. In these cases, experience in repetitive TeSE (so called re-TeSE) is available [60]. This approach has been focused on the risk of a large amount of testicular tissue being removed, thus the relatively high incidence of delayed hypogonadism. About that,

the results are not conclusive: a relatively short follow-up period (2 years) has been proposed to detect a hypogonadal state [65].

In the last years, different innovations have been proposed to increase success rate and lower incidence of immediate complications (e.g., intratesticular bleeding). Ultrasonography seemed to provide interesting findings, although never reaching clinical acceptance [53,66].

So, the only real innovation in TeSE technique has been the increasing use of a microscopic approach, thus the wider use of m-TeSE. Use of x25 magnification permits identification of individual seminiferous tubules. Considering that size of tubules correlates directly to the possibility to detect the full range of spermatogenic cells, the correct identification of larger ones seems to correlate with higher success rate of m-TeSE. In particular, patients affected by hypospermatogenesis or other forms of mixed pathology could benefit from that [62,67].

For this reason, in difficult cases (in particular, with low testicular volume and high FSH blood level), a double approach has been proposed: conventional trifocal TeSE associated with a middle microsurgical approach, where the upper pole specimen should also be providing materials for molecular diagnosis and conventional TeSE and cryopreservation [54]. Authors have reported a success (retrieval rate) up to 66%. It should be considered that the microsurgical approach needs a longer surgical time, sometimes 120 min, considering that conventional TeSE could in experienced hands consume 20 or 25 min by sampling three sites. Length of procedure suggests general anesthesia (or spinal with deep sedation). By that, also if a lower intratesticular bleeding for better vessel identification has been reported [68,69], hospital stay could be longer. This last aspect, the increase in technological need and surgical time, should be considered when proposing m-TeSE as a gold standard. Cost-effectiveness of m-TeSE has not really been made clear, in comparison with conventional TeSE.

A higher success rate has not been confirmed in recent meta-analysis [70], and a very similar success rate (46%) between the two techniques has been reported. In addition, it should be remembered that sperm retrieval is an intermediate result: pregnancy rate and birth rate should be considered in a clinical setting. In the same meta-analysis the success rate in ICSI has been reported up to 28%.

In addition, in some studies, "sperm competence" could be strongly related to total amount collected [71]. In other words, we are lacking data about pregnancy rate in cases in which TeSE resulted uneffective, but mTeSE reported some amount (likely low number). Effectively, this aspect has to still be corroborated by evidence. So, several variables should be considered before proposal of one specific technique, including surgical skills, testicular histology, costs of the procedure, and risk of complications [4].

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CHAPTER

17

How to choose the appropriate ART technique and counseling about reproductive outcomes

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With one in six patients struggling with infertility, a growing number of couples will seek medical assistance in achieving a much desired pregnancy. Selection of what treatment is appropriate for each patient will depend on a number of factors. The patient's age, Anti Müllerian Hormone (AMH), BMI, cause of infertility, previous treatments, as well as the patients own wishes, aspirations for family size, and religious and cultural beliefs must all be factored into the decision-making.

In this chapter, we will consider the treatment options available at a modern-day fertility clinic and the rationale you may wish to consider when choosing the correct treatment for your patient.

Too often, in vitro fertilization (IVF) is seen as the only way forward for the management of infertility. Patients frequently believe this, and many clinicians promote this view based upon the concept that it is the fastest way to achieve a pregnancy. While not denying this view is factually correct, many less invasive approaches can produce the desired pregnancy. For instance, there is an increased fertility in the months after tubal flushing [1]. Ovulation induction works in those with ovulation disorders with up to 60% success after six ovulatory cycles [2].

One of the most simple, noninvasive treatments available to the subfertile couple is intrauterine insemination (IUI). This involves the introduction of a prepared male sperm sample into the uterus with a fine catheter, around the time of ovulation, following tracking of the patient's cycle. This may be done using the patient's unstimulated natural cycle, or more commonly, after ovulation induction or supra ovulation with oral agents or injected gonadotrophins (OI-IUI).

A number of considerations must be made before recommending IUI as a reasonable treatment option for your patient. First to consider is the cause of infertility in the couple.

Patients with ovulation disorders in the absence of any other contributing factors have the highest success rates when considering OI-IUI, with cumulative pregnancy rates quoted between 46% and 84% [3,4] after four cycles. IUI as first-line treatment or following failure of ovulation induction with timed intercourse can be confidently recommended in this group.

It is key to have assessed tubal patency with either Hysterosalpingogram (HSG), Hystero Contrast Sonography (HycoSy), or laparoscopy prior to recommending IUI. While bilateral tubal occlusion is undoubtedly a contraindication to IUI, the finding of unilateral occlusion does not preclude it as a treatment option. As we know, many women will go on to conceive spontaneously following removal of a tube following, for example, an ectopic pregnancy. Studies have suggested comparable pregnancy rates with stimulated IUI in patients with an identified unilateral occlusion when compared to patients with unexplained infertility (17.3% versus 16.5% per cycle respectively) [5], with higher pregnancy rates observed in those with proximal occlusion compared with distal [5–7]. Therefore, in the absence of any other contributing factors, IUI remains a very reasonable recommendation for first-line treatment.

With regards to endometriosis, the role of IUI is less straightforward. Studies have shown significantly lower pregnancy rates in patients with endometriosis undergoing IUI compared with those with unexplained infertility, with figures quoted as low as 6% per cycle [8], and a study suggesting a diagnosis of endometriosis halves the success rate of IUI [9,10]. While more optimistic figures have been put forward for patients with stage I/II

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disease [11], there is overwhelming evidence to suggest those with stage III/IV do not do well with IUI, with cycle success rates rarely quoted above 5% [8,11]. A study comparing IUI and IVF as a primary treatment for patients with endometriosis found significantly higher cumulative pregnancy rates in the IVF group regardless of disease stage or patient age, with the biggest benefit in the stage IV group and those aged over the age of 38 [12]. Taking all this into consideration, in an infertile patient with a history of endometriosis, moving straight to IVF is a reasonable choice.

Age-related oocyte aneuploidy, as well as diminishing ovarian reserve sees a steady decline in fecundity, most marked after the age of 40. Consequently, maternal age is undoubtedly the single biggest predictor of outcome in fertility treatment, and IUI is no exception. In a female over the age of 40, success rates per cycle of IUI are around 4%–7% [8]. This infers no real benefit when compared to the chance of natural conception. The 40 and Over Treatment Trial (FORT-T) [13], which investigated pregnancy rates and time to pregnancy in patients aged 38–42 undergoing IUI versus IVF, demonstrated significantly higher clinical pregnancy rates per cycle (24.7% versus 7.3%) and live birth rates per cycle (15.3% versus 5.1%) in the IVF group. Therefore, in the case of a patient presenting with subfertility aged over 40, most would recommend IVF as the first-line treatment. However there are patients who do not wish to embark on IVF, who will elect to try the less invasive IUI in the first instance.

Male factor infertility accounts for 30% of couples presenting with a delay in conception. While more severe abnormalities of semen analysis may necessitate a move straight to assisted reproductive technologies (ART) with intracytoplasmic sperm injection (ICSI) for fertilization, IUI may still be appropriate with a borderline low result. In this instance, it is pertinent to perform a trial wash, to assess if an adequate concentration of healthy, motile sperm can be achieved. This involves removing any immotile or misshapen sperm from the sample and preparing it into a highly motile clean pellet [14]. Figures for a suitable lower limit for IUI have been debated with no definite consensus, with suggestions of a postwash total motile count anywhere from 1 to 10 million put forward [8]. However we recommend a postwash count of two million as a reasonable threshold.

Duration of infertility is the final factor we will consider with regard to suitability for IUI. It stands to reason that patients with a longer duration of infertility are more likely to be afflicted with more severe fertility issues, be they identifiable or not, so this is an important prognostic consideration when offering treatment [9,15,16]. Again, while studies have suggested various recommendations on the cutoff, a duration of infertility >3 years as a poor prognostic indicator for OI-IUI can be deemed a reasonable threshold, and strong consideration of IVF as first line in these patients is recommended.

Having discussed the merits and shortcomings of IUI, the next step is the decision to move on to ART, defined by the Society for Assisted Reproduction as therapies requiring manipulation of eggs, sperm, or embryos outside the womb, i.e., IVF or ICSI. Since the birth of Louise Brown, the first baby born via IVF in 1978, ART has evolved from basic IVF to many more complex clinical and laboratory approaches to improve pregnancy rates and patient tolerability. With the increasing technologies available comes the need for measured decision-making on part of the clinician in the selection of the most appropriate treatment protocol. ART should be the last line of treatment of infertility after all other options have been exhausted or deemed inappropriate.

Having decided to recommend ART to a patient, one of the first decisions to be made is regarding the regimen for controlled ovarian hyperstimulation, or COH. This differs from COH-IUI, where the primary aim is to stimulate one to two dominant follicles, instead aiming to stimulate around 10–15. Thus most common protocols used in modern-day practice center around much higher doses of follicle-stimulating hormone (FSH) in combination with either gonadotrophin-releasing hormone agonists or gonadotrophin-releasing hormone antagonists. The higher FSH dosage should result in higher oocyte yields, while the GnRH analogs provide suppression of endogenous luteinizing hormone. This prevents premature ovulation and allows planned timing of oocyte collection procedures.

For many years the standard method for COH was that of the agonist protocol, also known as a “long protocol.” In this regimen, GnRH agonists, such as nafarelin (inhaled), lucrin, or decapeptyl (injected), suppress the release of endogenous pituitary FSH and luteinizing hormone (LH) by desensitizing the pituitary receptors. The agonist medication is started in the cycle before stimulation, usually in the mid-follicular phase, about 1 week following ovulation. Baseline bloods are then taken on day 1 of the next menses to ensure adequate suppression has been achieved. Gonadotrophin injections then start on day 2–3 of menses at the time of normal recruitment of follicles. The agonist and gonadotrophins are continued daily throughout stimulation. When follicles have reached an adequate number and size, these medications are stopped. Various criteria for this decision are used on a clinic-by-clinic basis based on follicle diameters from 16 to 18 mm. The “trigger injection” is then administered 36 h before scheduled oocyte retrieval. The standard trigger is human chorionic gonadotropin (hCG) (either urinary derived or genetically engineered). This mimics the normal LH surge that results in final maturation of the oocyte by

stimulating the second meiotic division and ultimately ovulation at between 40 and 44 h.

In the last decade, an alternative "short protocol" using GnRH antagonists has become the favored approach. Antagonists directly and rapidly inhibit gonadotrophin release within hours through competitive binding to pituitary GnRH receptors. This means that treatment can be restricted to the days during which a premature LH is likely to occur [17]. The antagonist, for example ganirilex or cetrotide, is commenced in the mid-follicular phase, starting day 5 or 6 of FSH stimulation, known as a fixed regimen. An alternative is to start using a more flexible model awaiting a proven rise in estrogen levels. Some data has shown trends suggesting better pregnancy rates when a fixed approach is adopted, potentially attributed to better LH control [17,18].

This "short" protocol has the advantage of avoiding the hypoestrogenic side effects of the longer downregulation, such as hot flushes, bleeding, and mood dysfunction, while the shorter duration of medications and less monitoring is more patient friendly and potentially more cost effective. Perhaps the most marked advantage is the reduction in moderate and severe ovarian hyperstimulation syndrome (OHSS), which has been achieved with the introduction of antagonist protocol. This is attributable to the more rapid suppression of gonadotrophins, and the fact it allows for an agonist trigger to be used rather than HCG. A 2016 Cochrane review comparing agonist and antagonist protocols showed a substantially higher rate of OHSS in the agonist group, with no difference in ongoing pregnancy or live birth rates between the two [17]. The improved safety profile and comparable efficacy means that the antagonist cycle has become the protocol of choice for many clinicians. It should certainly be considered first line for those at higher risk of OHSS, including patients with Polycystic Ovarian Synndrome (PCOS), a high AMH, and patients <35.

Certain groups of patients may benefit from a longer downregulation, for example those with significant endometriosis. Some studies have suggested better outcomes in these patients after long downregulation (LDR), particularly ultra-LDR, that is where downregulation is adopted for three or more months [19]. However other studies have failed to demonstrate any such benefit [20,21], and potential benefit should be weighed against potential side effects and long cycle duration. Another possible subgroup who may benefit from an LDR protocol are those who have demonstrated asynchronous follicular development or premature ovulation during a short cycle.

When it comes to choosing the correct dose of FSH for the patient, no clear consensus exists, and a variety of dosing regimens are advocated. A Cochrane review of dosing described a desirable response to stimulation as the collection of 5–15 eggs [22]. Both poor response and hyper-response are associated with an increased chance of cycle cancellation, which can be both costly and distressing for the patient. Key factors to consider include patients age, BMI, and their ovarian reserve, measured by AMH or antral follicle count. The below table summarizes the author's strategy for gonadotrophin dosing in the first cycle.

Age	Dose	Weight >90 kg	AMH <10	AMH>20
<30	100 units	+50 units	+25 units	−25 units
30–35	150 units	+50 units	+25 units	−25 units
35–40	200 units	+50 units	+50 units	−25 units
40+	300 units	+50 units	+50 units	−25 units

Caution should also be used when considering the patient with a high AMH or low BMI, and a lower dose prescribed to reduce hyperstimulation.

When it comes to choosing the dose for subsequent cycles, clinicians often depend on the previous response. When considering a patient who has failed to produce an adequate number of eggs, the obvious response is to increase the dose of gonadotropin. Large cross-sectional studies have suggested "the more oocytes the better" [23,24], demonstrating an increased cumulative live birth rate with increasing oocyte yield. However Randomised Control Trials (RCTs) and meta-analyses have failed to support this, and while they have suggested increased FSH doses may lead to fewer cycle cancellations and a better oocyte yield, this does not necessarily translate into a better live birth rate (LBR) [25,26]. Regardless, this better oocyte yield and less chance of cycle cancellation may still be of importance to the patient, and an improvement of these intermediate outcomes may still improve satisfaction with the cycle [27,28] and thus reduce patient stress and treatment discontinuation even though the LBR may not be affected. It is important to discuss this strategy with the patient balanced against the cost of increased medications and side effect burden.

On the opposite end of the spectrum are those who overstimulate on their original dose. It is pertinent to advocate dropping the dose in future cycles for this patient to avoid cancellation and/or hyperstimulation.

The next decision to be made is the appropriate fertilization method. The standard IVF method involves combining the harvested eggs with prepared sperm in a Petri dish and allowing fertilization to occur spontaneously. The alternative is ICSI, which involves the injection of a single sperm into a mature egg. Since its introduction over 20 years ago, ICSI has revolutionized the treatment of male factor infertility where low sperm quantity and quality result in poor fertilization rates

with standard IVF. However its use has continued to increase dramatically in recent years, even in the context of nonmale factor infertility. In some countries, ICSI rates have reached over 90%. This change in practice is not evidence based. The primary justification is to avoid failed fertilization. However, there is a strong body of evidence suggesting that ICSI does not increase the LBR when compared to IVF in those with normal sperm parameters [29,30]. The increased lab work, along with potential for more oocyte degradation during the stripping of the oocyte cumulus required for ICSI [29] means that ICSI should not be the first choice fertilization method in those with nonmale factor infertility. The belief that those with advanced maternal age may benefit from ICSI given their thicker zone pellucida has failed to be proven, with studies showing no improvement in outcomes when compared to IVF [32,33].

However, couples with nonmale factor infertility who have experienced failed or low fertilization rates with IVF in previous cycles despite seemingly normal sperm parameters may benefit from ICSI, with one study suggesting improved fertilization rates of up to 60% in these patients [34]. Those males with borderline low sperm parameters also need consideration. Evidence suggests when morphology is not severely impaired and in the context of otherwise normal parameters, IVF may be preferable [31]. Indeed with those a borderline low motility but an adequate concentration, IVF remains a very reasonable treatment option, as sufficient good quality sperm should be available. Of note, it is always good practice to counsel the patient that the final decision regarding the best fertilization method may change on the day of treatment, as it is dependent on the quality of the sample available.

Once fertilization has occurred, embryos are cultured in an incubator. These have become more sophisticated over the last decade to allow minimal handling of the embryo. Introduction of time-lapse photography of the embryo has allowed monitoring of their development without the need to take them out of the incubator. It seems that stable environment improves success [35].

A decision must then be made regarding at which stage of embryo development to perform the transfer. In the first 25 years of IVF, transfer took place on day 2 or 3 of development when the embryo is in six to eight cell stage. Success rates per embryo transfer were, at best, less than 30% in women under 38. However many embryos were frozen for further cycles.

With improved culture conditions growing the embryo further until day 5, those embryos reaching blastocyst stage have a significantly higher success rate per embryo transferred. By this stage blastocyst formation has occurred, and the transfer at this point mimics nature since it is at this stage in a spontaneous pregnancy that the embryo travels from the fallopian tube into the uterus to implant. The extended culture also imparts a selection process whereby those day 2 or 3 embryos that would not develop if transferred fall by the wayside [36]. Thus the number of embryos reaching day 5 is reduced, but each has a higher pregnancy potential. Data from units with excellent laboratories suggest that the cumulative pregnancy rate per egg collection (the number of babies born per egg collection after transfer of all fresh and frozen embryos) is similar whether the strategy is early or later stage of development. However time to pregnancy is shorter with blastocysts and cost is less since there are fewer frozen cycles [37].

The next decision relates to the number of embryos to transfer. Worldwide, double embryo transfer has been the norm. This habit arose when day 2 or 3 embryo transfer was standard with their lower individual success rates. This increased pregnancy rates. However this also resulted in higher twinning rates demonstrated as upward of 30% [38,39]. The downstream impact of multiple pregnancy is significant due to preterm delivery and pregnancy complications. Admissions to neonatal intensive care are significantly higher than singleton pregnancies with the associated massive costs [40]. Perinatal mortality doubles and the incidence of cerebral palsy increases fourfold [41]. Thus, single embryo transfer has been recommended across the globe. Scandinavia and Australia led the way with either legislation or regulation. Today in Australia, 90% of transfers are single, and multiple pregnancy occurs in less than 4% of pregnancies [42]. This has been supported by developments such as 85% of embryos being transferred at the blastocyst stage (with their higher success rate), as well as the development of vitrification of frozen blastocysts which now carry an equal chance of pregnancy to freshly transferred embryos.

In terms of embryo transfer, the procedure is straightforward, little more than a Pap smear. The American Society for Reproductive Medicine (ASRM) guidelines for the conductance of embryo transfer include careful cleaning of the cervix and the use of ultrasound to ensure the accurate placement of the embryo at the border of the upper third and lower two-thirds of the uterine cavity [43].

The final decision to make is the use of luteal phase support. Early evidence showed clearly that if no hormonal support is given in the luteal phase of an IVF cycle with fresh transfer, pregnancy rates are lower [44]. The explanation probably relates to the abnormal hormonal environment on the endometrium. It has been stimulated by supraphysiological levels of estrogen in the lead up to oocyte collection, then exposed to high but rapidly falling progesterone levels postprocedure. Early menstruation is common. Support involves supplementation of progesterone to the uterus. Oral progesterone may be helpful, but concerns of poor absorption

and metabolism have led to the primary use of vaginal progesterone preparations in the form of gels, tablets, or capsules [45]. Intramuscular progesterone in some countries has been popular but is painful and has documented side effects. An alternative is the stimulation of progesterone production by the corpora luteal by using HCG subcutaneously or GnRH agonists through the effect on pituitary LH release. The risk with HCG is an increased incidence of OHSS [46].

After all these decisions are made and an embryo has been transferred, what follows is the harrowing wait for the pregnancy test result. Given that the majority of transfers will not be successful, the next discussion is what to do next.

Should there be embryos frozen from the fresh cycle, a further transfer should follow. A decision on the cycle type is then required. For a woman with a regular ovulatory cycle, a natural cycle is appropriate. Monitoring with hormonal levels of estrogen, LH, and progesterone in association with ultrasound assessment of endometrial thickness and the presence of a developing follicle allows the prediction of the day of ovulation. Depending on the stage of embryo development, transfer will be undertaken 3 or 5 days later. In regularly ovulating women, luteal phase support is probably unnecessary, but many clinicians add this in. For anovulatory women, e.g., PCOS, ovulation induction with low-dose gonadotrophins or oral letrazole or clomiphene can result in good ovulation and subsequent embryo transfer. An alternative approach is to use hormone replacement to control endometrial development. This is obviously the only way forward in egg donation cycles in postmenopausal women. However some units favor this approach generally. The advantage is the ability to control the embryo transfer day. There, evidence suggests little, if any, difference in pregnancy outcomes with this approach. Women start an oral estrogen medication e.g., estradiol valerate, 6 mg daily, from day 1 of menses and continue this up to 10 weeks gestation should they fall pregnant. After 10 days an ultrasound is performed to confirm an endometrial thickness of at least 7 mm. A thickness less than this has poorer success rates. From that point, progesterone supplementation can begin. This is usually vaginal application of one of the various forms, i.e., gel, tablet, or pessary. Optimal timing of the transfer is thought to be around 120 h after the first dose. Obviously the commencement of the progesterone is timed to allow the transfer to occur at a time convenient to the clinician. The disadvantage to the patient is the need to continue the vaginal progesterone and oral estrogen for 10–12 weeks when pregnancy occurs to replace the lack of endogenous ovarian function.

Should the supply of embryos be exhausted after fresh and frozen transfer, a full review of the case is required to determine if there could be changes in protocol to possibly improve the next cycle. An alternative outcome of that review may be cessation of attempts with ART. These are difficult discussions and require experience to be handled well.

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CHAPTER

18

Preparing the couple for ART: necessary and unnecessary diagnostic tests

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Introduction

To reduce worldwide variability in clinical practice, several national or international guidelines have been developed. The National Institute of Health and Care Excellence (NICE) guidelines make evidence-based recommendations on a wide range of topics including infertility.

The NICE guidelines aim at proposing an exhaustive diagnosis work-up that could be applied internationally [1], but some local conditions related to specific social and health contexts raise critical issues, making NICE guidelines difficult to apply everywhere.

Furthermore, most countries of the world do not include ART treatments in the minimum levels of care provided by the public health system, and performing ART in a private setting inevitably affects the diagnostic path and the possibility of accessing specific tests. It is therefore possible that certain tests are not carried out not because they are useless, but because they are expensive, orienting diagnostic choices more toward the cost-benefit ratio, than to the real need of a given exam.

In this context, it is difficult to build an algorithm including the essential diagnostic tests that a couple should undergo prior to ART treatment. This algorithm, in fact, should consider several factors: (a) the prevalence of some pathologies in specific ethnic groups and populations, (b) the possibility of the public health system to offer some tests and, last but not least, (c) the national legal rules context in which the treatment is performed.

In this chapter, the tests that should and should not be performed before an ART treatment will be discussed with a worldwide application perspective, suitable for most international areas in which ART is carried out. Both the World Health Organization (WHO), the Center for Disease Control and Prevention (CDC), and the European Society of Human Reproduction and Embryology (ESHRE) define ART as "all profertility treatments in which both eggs and sperm are handled. In general, ART procedures involve surgically removing eggs from a woman's ovaries, combining them with sperm in the laboratory, and returning them to the woman's body or donating them to another woman" [2–4].

The diagnostic tests presented in this chapter will be those of a homologous in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI) treatment and will be grouped into three categories: tests that are common to both partners, tests for the woman, and tests for the man.

Tests to be performed by both partners

Blood group with Rh factor

People who carry the Rh D antigen are identified as Rh D-positive, whereas those who do not carry it are identified as Rh D-negative. The frequency of the Rh D-negative phenotype is higher among individuals of European and North American descent (15%–17%), less common in Africa and India (3%–8%), and rare in

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Asia (0.1%−0.3%) [5,6]. Alloimmunization refers to the maternal formation of antibodies against fetal Rh D-positive cells, occurring as a consequence of events such as miscarriage, ectopic pregnancy, antenatal bleeding, and delivery, as well as of procedures such as chorionic villus sampling, amniocentesis, pregnancy-related uterine curettage, and surgical treatment of ectopic pregnancy [7].

Knowing the blood group and Rh status of both partners before ART allows to safely plan a pregnancy. This is particularly relevant considering that ART pregnancies are characterized by an increased risk of miscarriage, threatened abortion, and ectopic pregnancy, all situations that can cause alloimmunization [8,9]. Women with a recent history of such events may also be tested with indirect Coombs test, able to identify women already sensitized to Rh D antigen.

Besides alloimmunization prevention, some evidence demonstrated an association between patient's blood type and IVF cycle outcome. In particular, women with blood type B were observed to have significantly higher odds for live birth (LB) compared to other blood types, even after adjusting for factors recognized to impact IVF cycle outcome [10]. These findings have not been confirmed by subsequent studies, so further studies are warranted to clarify whether non-O blood group holds any prognostic value in women undergoing IVF [11].

Conclusions:

Blood group typing of both partners is advisable before ART, and indirect Coombs test is useful as screening of preexisting maternal alloimmunization.

Blood glucose

The blood glucose test may be useful for the early identification of women with polycystic ovary syndrome (PCOS) affected by insulin resistance [12] that are known to be at high risk of developing ovarian hyperstimulation syndrome (OHSS) [13,14]. For these subjects, lifestyle changes (diet, body weight reduction, etc.) before ART are essential to optimize reproductive and obstetric outcomes and to eventually plan a therapy with oral hypoglycemic agents both during ART and the following pregnancy [15]. Indeed, metformin was found to be effective in controlling the levels of circulating vasoactive factors implicated in the pathogenesis of OHSS [16].

The presence of a metabolic syndrome (MS) should systematically be checked at the beginning of medical care, also in infertile males [17]. Indeed, hyperglycemia, hyperinsulinemia, and insulin resistance in the context of type 1 diabetes or MS was significantly associated with the impairment of sperm motility and with higher levels of sperm DNA damage [18,19]. The following pathogenetic mechanisms may explain this association: endocrine disorders, neuropathy, increased oxidative stress, and epigenetic modifications during spermatogenesis that could be transmitted by the male germline to the offspring [20−22]. The identification of men with altered glycemic levels, as in women, can allow one to plan lifestyle or pharmacological interventions (i.e., metformin), potentially able to improve spermatogenesis [23].

Conclusions:

Blood glucose measurement is recommended to identify women with insulin resistance, who are prone to complications during both ART and pregnancy. Furthermore, it allows identifying a possible cause of male infertility with potential consequences on ART outcome.

Kidney and liver function

Infertility and sexual dysfunction are common clinical findings in men and women affected by chronic kidney disease (CKD), which can be screened by creatinine assay, even if false positive results have been reported.

Gonadal dysfunction is estimated to affect one-quarter to one-half of men with CKD overall [24]. The hormone profile in men with CKD is characterized by elevated luteinizing hormone (LH), elevated prolactin, decreased anti-Müllerian hormone (AMH), and markedly reduced testosterone [25−28]. This results in impaired stimulation of Sertoli cells and in a severe reduction in spermatogenesis [25]. Higher stages of CKD have been associated with a reduced volume of ejaculate, a decreased sperm count and concentration, and a progressive decline of motile sperm [29].

In women, CKD is associated with an increased risk of adverse pregnancy outcomes; it is therefore important to identify patients with CKD in the preconception period, defining the disease stage to predict kidney function during pregnancy [30].

There are no studies that have specifically addressed the effect of IVF in women with CKD or end stage kidney disease. In women with CKD, attention to kidney-related complications during IVF is warranted. OHSS can increase thromboembolism and acute kidney injury resultant from ischemia or obstructive nephropathy [31−33]. Reducing the risk of OHSS by using gonadotropin-releasing hormone (GnRH) antagonist protocol should be considered in all patients with CKD, and single embryo transfer should be recommended to reduce the risk of multiple pregnancies [31,34].

Liver tests performed to detect, evaluate, and monitor liver disease or damage are bilirubin (total and direct), alkaline phosphatase (ALP), aspartate aminotransferase

(AST), and alanine aminotransferase (ALT) assays. Fertility problems are common in patients with liver disease, chronic or not, due to a complex interaction of genetic, environmental, lifestyle and hormonal factors. In males, clinical studies indicate that men with liver disease have significantly lower levels of serum testosterone and sex hormone-binding globulin compared with healthy individuals, resulting in impaired spermatogenesis [35]. In addition to hormonal impairment, a further cause of infertility is the increased fat deposit in the groin and scrotum, with consequent higher local temperature and spermatogenesis deterioration [36].

In women with advanced liver damage, fertility is reduced and pregnancy is rare due to metabolic and endocrine dysfunction [37]. Disruption of the hypothalamic-pituitary axis in conjunction with disturbed estrogen metabolism leads to anovulation, amenorrhea, and infertility. When pregnancy occurs, there is an increased rate of adverse obstetric and perinatal outcomes [38].

Furthermore, the majority of patients with liver disease are insulin resistant, and elevated ALT is common in women with PCOS [39], although increased liver enzyme levels may not be present until late liver damage [40]. The identification of women at risk of liver damage and with concomitant MS or PCOS is essential to reduce the risks of IVF (primarily OHSS). For this reason, it is important to identify women with liver damage during preconceptional counseling and ART access.

Conclusions:

Kidney and liver function should be tested prior to accessing ART to identify women at risk for adverse pregnancy outcomes and men with potentially severe impaired semen quality.

Viral screening: HIV, HBV, and HCV

The viral serological assessment of the couple is crucial to minimize the risk of transmission in case of sero-discordant couples, for IVF lab safety, and for the correct interpretation of subfertility caused by active viral infections or antiviral therapies. The panel for viral diseases includes several tests: nucleic acid test (NAT), usually performed from 10 to 33 days after exposure to the virus, or antibody tests, which can take 23–90 days to detect the viral infection after exposure. In general, subjects positive for HIV, hepatitis B, or hepatitis C should be offered counseling and appropriate clinical management [1].

It has been reported that there is a 25%–40% reduction of fertility in HIV-infected subjects compared with sero-negative controls, possibly due to a direct effect of HIV at the gonadal level of both male and female partners [41,42]. The antiviral treatment seems unable to fully restore fertility in case of HIV-positive women. Also a role of HIV in reducing ovarian reserve and causing premature ovarian failure has been suggested [43,44].

In HIV-positive men, semen alterations such as low volume, reduced sperm motility, concentration, and morphology, with values directly correlated with the CD4 count, suggest that HIV patients are less fertile than unaffected males [45,46]. As a result, there is an increasing number of HIV-infected people accessing ART, which is a safe option once the semen has been processed to get it free of HIV [47].

After HIV-1 infection, HIV-specific markers appear in the blood in the following chronologic order: HIV RNA, p24 antigen, HIV IgM and IgG antibodies. The standard-of-care test for diagnosing HIV is the serum immunoassay test (EIA), known as the HIV fourth-generation test, which is a combination antibody (Ab) and antigen (Ag) tests. If there is a strong suspicion of a very early HIV infection (less than 14 days), or an inconclusive test at EIA, a NAT can be performed to detect HIV RNA (as early as 5–10 days after the putative transmission, depending on the sensitivity of the assay). Rapid tests have been developed in the last years and mostly used in low-income countries: they are ELISA tests and provide the result in 20–30 min [48]. The advantage of rapid tests is that they can be performed outside of a clinical setting; however, their use is not recommended before access to ART.

Regarding HBV viral infection and its impact on fertility, the presence of the virus has been demonstrated in the ovary of affected women both within oocytes and granulosa cells, suggesting a possible viral transmission to progeny via infected gametes [49,50]. In addition, it remains to be clarified whether virus-infected ovaries may show a different response to controlled ovarian stimulation (COS) during ICSI/IVF, and lead to lower fertilization and implantation rates, or whether hormonal stimulation itself can induce viral replication [51].

Conflicting evidence have been reported about ICSI outcome in HBV-affected men [52,53]: however, a recent study reported comparable clinical pregnancy rate, implantation, miscarriage, and LB rate between the HBV-positive group and the control group [54]. For the diagnosis of chronic HBV infection a serological assay (either rapid diagnostic test or laboratory-based immunoassay format: enzyme immunoassay [EIA] and chemo-luminescence immunoassay [CIA]) is recommended to detect hepatitis B surface antigen (HBsAg). Serological tests for the detection of hepatitis B (HB) e-antigen and antiHBe antibody may help in the management of the patient and are widely available. Directly following a positive HBsAg serological test, the use of quantitative or qualitative NAT for detection of HBV DNA is recommended [55]. These assays detect the

presence of viral DNA through targeting a specific segment of the virus, which is amplified to detect minimal levels.

Women with HCV are at greater risk of reduced ovarian reserve and impaired fertility [56]. Meanwhile, several studies have shown that HCV infection alters seminal parameters, inducing higher sperm diploidy, mitochondrial membrane potential impairment, chromatin compaction, and DNA fragmentation [57,58]. Virological markers of HCV infection are anti-HCV antibodies, HCV core antigen, HCV RNA, and HCV genotype [59].

Screening for HCV is based on detection of total HCV antibodies (IgM and IgG). EIA and CIA are the most used techniques. Confirmatory antibody testing can be done with recombinant immuno-blotting assays for individuals who have tested positive at EIA. Confirmation of HCV infection and circulating viral genome is based on detection of HCV RNA. NAT, in particular real-time polymerase chain reaction (PCR) [60], is the gold standard and most commonly used confirmation test [61,62]. Antigen can be used as an indirect marker of HCV replication, and assays have the potential to replace NAT, with the advantages of reducing costs and being performed on the same diagnostic platforms as some EIA assays [63].

Conclusions:

Screening for infectious diseases is recommended before ART because infectious diseases impact fertility, cause a biological risk in the lab, and potentially cause infection transmission during ART.

Human papillomavirus (HPV) screening

HPV is recognized as responsible for fertility impairment in affected subjects. In particular, the presence of the virus affects up to 35% of the sperm population in infertile subjects [64,65]. The most frequent semen alteration is a significant reduction of mean sperm motility. At present it has not yet been defined whether HPV-infected spermatozoa are able to adequately fertilize and then transfer viral DNA to the egg [66]. Some experimental studies have demonstrated the role of HPV in causing pregnancy loss by transmission of viral genes to oocytes and enhancement of DNA fragmentation and apoptosis in embryonic cells [67–69]. Furthermore, recent studies have shown a significant reduction in pregnancy rate and a higher rate of abortion also in women undergoing IVF with HPV cervical infection [70]. This finding could be explained by the fact that HPV-infected trophoblast cells show higher rates of apoptosis and reduced placental invasion capability when compared with healthy controls [71]. The actual gold standard for HPV detection is NAT, which also allows the genotyping of the virus. NAT currently uses PCR techniques, as well as blotting tests (line blot assay, linear array, and dot-blot hybridization). The assays to detect HPV antibody response in the blood can be distinguished into neutralization assay, competitive immunoassay, and enzyme immunoassay. The test to be used should be chosen based on the experience of the center, though a novel droplet digital PCR (ddPCR) method to simultaneously detect and quantify HPV DNA from different HPV types seems to be the most promising [72].

Conclusions:

HPV testing of couples pursuing ART treatment is still not included in the European Tissue and Cells directory; however, this is a rapidly developing area and HPV impact needs to be considered in the field of medically assisted reproduction.

Hemoglobin electrophoresis

In recent years, the screening of thalassemia and abnormal hemoglobin-linked diseases resulted in a slight decrease of infants with major hemoglobinopathies [73]. International guidelines recommend screening for hemoglobinopathies by molecular and biochemical investigations aimed at the identification of healthy carriers [74].

In populations where hemoglobinopathies are endemic, about 20 different mutations should be searched by molecular DNA analysis both for alpha- and beta-thalassemia, in association with one or two significant globin variants. The molecular characterization of carrier genotypes requires a wide range of methods, most of which are based on PCR. The early identification of carriers in ART favors both preimplantation genetic testing (PGT) of *in vitro*-derived embryos and prenatal diagnosis, avoiding the risk of generating an affected offspring [75].

Conclusions:

Hemoglobinopathies represent one of the main indications for PGT, and the status of healthy carriers should be investigated in the couple before accessing ART.

Karyotype

Chromosome analysis is usually performed on circulating white blood cells. Chromosomal abnormalities (e.g., translocations or complex chromosomal rearrangements) represent one of the causes of infertility in animal models and in humans. In humans, aneuploidy is found in approximately 0.3% of newborns, 30%–60% of embryos, 30%–70% of oocytes, and 35% of spontaneous abortions [76]. The detection of karyotype abnormalities

in couples undergoing ART should be part of an accurate genetic counseling, including correct information regarding the specific type of abnormality, its clinical relevance, the rate of transmission to the offspring, and the possibility of PGT. The prevalence of chromosomal abnormalities is increased in infertile men, and it is inversely proportional to sperm count: less than 1% in men with normal sperm concentration, 10%–15% in azoospermic men, and approximately 5% in men with severe oligozoospermia (<5 million/mL) [77,78].

In infertile men, sex chromosome aneuploidy (Klinefelter syndrome; 47,XXY) accounts for about 60% of all chromosomal abnormalities [79]. Inversions and balanced translocations are also more frequent in infertile men than in the general population [80]. In the absence of evidence showing a benefit of karyotype evaluation in all males undergoing ART, at least men with nonobstructive azoospermia or severe oligozoospermia (<5 million/mL) should be evaluated with a high-resolution karyotype before using their sperm to perform ICSI [81]. Among women with indication to ART, the frequency of chromosomal aberrations was reported to be seven times higher than in the general population (4%), especially in subjects with repeated implantation failure (RIF) or lack of fertilization. The prevalence of karyotype abnormalities in a female population affected by RIF was reported to be about 2% [82]. Therefore, a karyotype analysis is indicated in all women with RIF [83]. Furthermore, nulliparous women with a history of miscarriage are at greater risk of chromosome abnormality and should be advised to undergo karyotyping [84], although an individual assessment of risk should be carried out rather than a routine screening of all couples affected by recurrent pregnancy loss [85].

Conclusions:

In the absence of a complete cost-effective study investigating the routine use of karyotyping in both partners accessing ART, chromosome analysis should be limited to couples with a family history of genetic disease, severe male factor, or previous RIF/miscarriage.

Cystic fibrosis mutations

Cystic fibrosis (CF) is the most prevalent autosomal recessive disease in the Caucasian population; more than 300 mutations have been identified, some of them with an uncertain clinical consequence [86]. The prevalence of infertility among patients with CF is around 35%, compared with 14% in the general population [87]. Infertile women affected by CF show hypothalamic dysregulation, anovulation, abnormal cervical mucus, and abnormal uterine secretions that may impair spermatozoa motility and capacitation. In addition, women with CF may have a reduced ovarian reserve compared with age-matched controls [88]. In the male, congenital absence of the vas deferens (CBAVD) is one of the most frequent clinical presentations related to cystic fibrosis transmembrane conductance regulator (*CFTR*) mutations, while forms of non-obstructive azoospermia or severe oligozoospermia seem to be often associated with less common mutations such as IVS8-5T [89].

In couples at higher risk of CF, the search for *CFTR* mutations in both partners is recommended, considering the risk of transmission to the offspring and the possibility to use preimplantation genetic testing for monogenic diseases (PGT-M). However, no consensus document has been formulated regarding CF-related screening programs in cases of couple infertility. If CBAVD is present in the couple, it is indicated to proceed to the investigation sequence of all exons, intronic regions adjacent to exons, promoter regions, 3′ untranslated regions, and fully intronic regions, whose rearrangements could be sites of pathogenic variants of the *CFTR* gene.

With the aim of reducing the incidence of the disease in the population, it has been proposed to extend the search for carriers even in the absence of familiarity; the American College of Medical Genetics and the American College of Obstetricians and Gynecologists recommend to offer the research of carrier testing to all couples with reproductive projects [90,91].

Conclusions:

In the absence of a conclusive cost-effective study investigating the routine assay of *CFTR* mutations in both partners accessing ART, genetic analysis should be recommended to couples with a family history of respiratory diseases, azoospermia, or CBAVD. Carrier screening could be offered also in absence of these conditions.

Tests to be performed by the woman

Preconception counseling

Before accessing ART, all women should receive adequate preconception counseling to identify relevant comorbidities and potential complications of the future pregnancy. Further, modifications of risk factors should be advised, such as increasing folic acid intake to reduce the risk of neural tube defects, stopping smoking, reducing alcohol intake, and avoiding medications that may compromise fetal development [92].

Thrombophilia screening

Investigation and treatment of congenital or acquired thrombophilic status have become common practice in

management of RIF [93], although its impact on ART outcome is still a matter of debate among reproductive specialists [94]. Recent meta-analyses show contrasting results regarding IVF outcome in women affected by thrombophilia, some highlighting an increased risk of failure in the presence of antiphospholipid antibodies (APS) and Leiden factor V (FVL) mutation [95], and others showing no correlation [96].

The predominant thrombophilic mutations include FVL mutation, methylene tetrahydrofolate reductase C667T mutation (*MTHFR*), prothrombin gene mutation *G20210A*, and deficiencies of the natural anticoagulant proteins C and S, and antithrombin III (ATIII). Almost all these congenital thrombophilic conditions (CoT) are inherited in an autosomal dominant fashion [94]. APS, heparin-induced thrombocytopenia, paroxysmal nocturnal hemoglobinuria, myeloproliferative disorders (polycythemia vera and essential thrombocytosis), and paraneoplastic syndromes are common causes of acquired thrombophilia.

APS is generally recognized as a cause of recurrent pregnancy loss (RPL), even if most studies used arbitrary cut-off values for APS positivity, and only a few used the standardized Sapporo criteria for diagnosis [97]. Preconception testing for APS antibodies and treatment is currently recommended after two pregnancy losses [85]. Conversely, routinely testing for APS women undergoing ART does not seem to be justified because a clear association between APS positivity and ART failure is lacking [94].

Conclusions:

Given the lack of evidence of a strong correlation between CoT and ART outcome, to date it is not indicated to test patients undergoing ART for thrombophilia, in the absence of a personal or family history of venous thromboembolism. Testing for APS antibodies is recommended after two pregnancy losses.

Cervico-vaginal swab

The microbiota of the female reproductive tract has long been studied using culture methods to identify the microorganisms that can be isolated and to assess their impact on reproductive physiology. Highly sensitive molecular techniques have recently been introduced, being able to identify microorganisms that cannot be grown in culture [98,99].

The traditional culture of cervico-vaginal swabs, combined with the use of Amsel criteria or Nugent scores, allow the diagnosis of bacterial vaginosis, which is associated with tubal factor infertility and early spontaneous abortion [100,101]. Vaginal swab and Nugent's score processing is a simple, reproducible procedure with limited costs. However, more complex vaginal dysbiosis can be identified only by a broader characterization of the bacterial communities of the vagina [102].

The impact of the vaginal microbiota, characterized by studying the length of the 16–23S rRNA gene interspace regions during an IVF cycle, was recently explored, showing that microbiome profiling enables stratification of the chance of becoming pregnant prior to the start of an IVF treatment [103]. Also the endometrial microbiota appears to affect embryo implantation rate [104]. Analyzing microbiota at the species-level resolution may be necessary for identifying the true pathogenic bacteria of the endometrium and avoiding overtreatment against harmless non-*Lactobacillus* microbiota [105]. However, the application of such an innovative and expensive method is still experimental and worth planning larger studies before a transversal clinical application is recommended [106,107].

Conclusions:

Traditional culture swabs associated with Nugent score can be used prior to IVF treatment to identify women with bacterial vaginosis, at risk of adverse outcome. Advanced molecular analyses could be employed in experimental trials but is not yet applied in the clinical practice.

FSH, LH, and estradiol

Serum levels of follicle-stimulating hormone (FSH), LH, and estradiol (E2) can vary greatly between cycles for the same woman, as well as between different women.

A serum FSH and E2 level obtained on cycle day 3 is commonly used to define ovarian reserve. FSH greater than 20 IU/mL has been associated with a low ovarian reserve and therefore with a reduced ability to conceive [108,109]. Baseline serum E2 alone is not used to assess ovarian reserve, but it can provide additional information to better assess the significance of FSH levels; in fact, when serum E2 levels are elevated in the early follicular phase (>80 pg/mL), there is an advanced follicular maturation and an early selection of the dominant follicle, as observed in women with advanced age [110,111]. In this case, FSH levels may be falsely low due to the negative feedback of E2, not reflecting accurately the extent of ovarian reserve.

Basal FSH is rather variable from cycle to cycle and also from laboratory to laboratory due to differences in the assay kit [108,112,113]. For this reason, the predictive value of FSH has been questioned, and its use has been reduced in favor of other ovarian reserve markers, such as AMH and antral follicle count (AFC).

The plasma LH measurement is indicated in the differential diagnosis of amenorrhea and more generally

of hypogonadism. LH secretion occurs in a pulsatile manner, presenting daily fluctuations of 30% as well as physiological variations in the different phases of the cycle. High LH values are indicative of primary ovarian failure, while low LH values associated with low FSH and E2 levels are indicative of hypothalamic-pituitary hypofunction [114]. On the other hand, a high LH/FSH ratio is indicative of the presence of polycystic ovaries, although this is no longer considered a diagnostic criterion according to ESHRE guidelines [115].

Conclusions:

FSH, LH, and E2 are essential tests in the diagnostic process of the infertile woman. However, before ART treatment, other more reliable indicators of ovarian reserve can be used.

Clomiphene citrate challenge test

The clomiphene citrate challenge test (CCCT) is the daily administration of 100 mg of clomiphene citrate from day 5 to day 9 of the cycle. An elevated FSH concentration after clomiphene stimulation suggests a poor ovarian reserve. A systematic review of studies comparing basal FSH and the full CCCT showed that the CCCT has probably no additional value. Since newer tests such as serum AMH and AFC are simpler and highly predictive of ovarian response, CCCT should not be used as a screening test for reduced ovarian reserve before ART [116,117].

Conclusions:

CCCT should not be performed before ART.

AMH

AMH is a hormone produced by granulosa cells of preantral follicles. Its secretion is gonadotropin and estrogen independent, so it can be measured at any time during the menstrual cycle. AMH level represents a marker of ovarian function and seems to be a good predictor of ovarian responsiveness to exogenous gonadotropins [118,119]. However, AMH values can be modified by some factors: the use of exogenous hormones (e.g., oral contraceptive pills), obesity, and hypogonadotropic hypogonadism are associated with a reduction of AMH levels [120,121]. On the other hand, the presence of large ovarian endometriomas seems to be associated with an increase in AMH concentrations, although conflicting results have been reported [122–124].

Multiple commercial kits for AMH assay are available: AMH levels measured by different assays can be combined for research or interpreted in the context of established clinical cut-offs. Therefore, clinicians should be aware of their own laboratory's reference ranges [125,126]. The introduction of new and recent automated assays has made it possible to solve the problem of the low comparability of AMH values measured in different laboratories. Automated assays have been shown to be efficient particularly in identifying women with reduced ovarian reserve [127].

Conclusions:

AMH measurement should be performed prior to ART treatment to assess the extent of ovarian reserve and predict the responsiveness to COS.

Progesterone

Serum progesterone determination provides a reliable and objective measure of ovulatory function. It should be obtained approximately 1 week before menses, rather than in a fixed day of the cycle (e.g., day 21).

Serum progesterone levels are a poorly reliable diagnostic tool to assess the adequacy of the luteal phase, as no minimum serum progesterone concentration defines the "fertile" luteal function [128]. Moreover, the secretion of progesterone from the corpus luteum occurs in a pulsatile manner, and the serum concentration of the hormone can vary up to seven times within an interval of a few hours [129].

Conclusions:

Progesterone assay may be useful in diagnosing anovulation but is not required before ART.

TSH and TPOAb

The relationship between ART outcome and thyroid function has been a hot topic in recent years [130]. An increased prevalence of thyroid autoimmunity (mainly antithyroperoxidase antibody [TPOAb]) is reported in women with RPL and subfertility and is associated with lower AMH levels. Meanwhile, subfertile women with hyperthyroidism should be informed of the increased risk of maternal and fetal complications [131], and euthyroidism should be restored and maintained prior to an ART treatment. Furthermore, COS generates a rapid increase in E2 levels that enhances the hepatic synthesis of thyroid-binding globulin and finally leads to a reduction in free T4 [132–135].

A recent metanalysis showed no difference in ART outcome when a thyroid-stimulating hormone (TSH) cut-off value of 2.5 mIU/L was used. However, using a broader cut-off value of TSH, a higher miscarriage rate was noticed. It is likely also that subclinical hypothyroidism can lead to adverse obstetric and neurodevelopmental outcomes [136,137], suggesting that a thyroid

function test should be routinely performed in women seeking ART [138,139].

Conclusions:

Regardless of the cause of infertility, all women seeking ART should be screened for TSH and TPOAb.

Pelvic ultrasound examination

Ultrasound (US) examination of the pelvis is the first level of investigation for evaluating the uterus and the ovaries. In particular, transvaginal US allows to evaluate AFC (a reliable marker of ovarian reserve), uterine pathologies, endometrial characteristics, and adnexal anomalies.

To overcome the 2D limit of US, 3D US has been introduced in the obstetric field with interesting applications in the diagnosis of infertility. 3D US is more sensitive and specific than 2D US in defining and mapping uterine lesions such as fibroids, adenomyosis, and intrauterine synechiae. Recent evidence shows that AFC can be better estimated using 3D US compared to 2D technology [140–142] and that 3D US allows a better evaluation of endometrial junctional zone anatomy, suitable as a predictor of ICSI outcome [143]. Further, the 3D imaging of uterine pathology and identification of intratubal and intrauterine devices consistently reported higher rates of diagnostic accuracy when compared to the standard 2D US.

Studies regarding the value of assessing the endometrial volume and vascularization prior to embryo transfer have reached conflicting and inconsistent conclusions, discouraging a routine use of Doppler velocimetry prior to ART.

Conclusions:

Offering a TV-US examination is mandatory for all women accessing ART. 3D US can better support the diagnosis of utero-adnexal diseases and should be considered in the presence of a suspect disease.

Hysterosalpingography and hysterosonosalpingography

Hysterosalpingography (HSG) is the fluoroscopic examination of the uterus and the fallopian tubes.

The use of HSG involves exposure to X-rays and exposes the patient to a risk, albeit minimal, of tubal and pelvic infection (1% of cases) [144]. HSG defines the size and shape of the uterine cavity and can reveal developmental anomalies (unicornuate, bicornuate, septate uterus) or other acquired abnormalities with potential negative reproductive consequences (polyps, myomas, synechiae). However, HSG has relatively low sensitivity (50%) and positive predictive value (PPV: 30%) for the diagnosis of endometrial polyps and submucous myomas in asymptomatic infertile women [145].

Hysterosonosalpingography (HSSG) better defines the size and shape of the uterine cavity and has higher PPV and negative predictive value for detection of intrauterine pathology (endometrial polyps, submucous myomas, synechiae) [145–147]. HSSG is an X-ray-free, well-tolerated US test to investigate tubal patency in infertile women. However, the absence of filling of one or both fallopian tubes has a relatively low PPV for tubal occlusion: this finding may represent proximal tubal spasm rather than a real occlusion and may require additional imaging tests or laparoscopy with methylene blue injection to get a definitive diagnosis [148].

Conclusions:

HSSG may be recommended for patients with a suspicion of utero-adnexal pathology, but there is no evidence that its application before all ART treatments would be of benefit.

Chlamydia antibody test (CAT)

Chlamydia trachomatis infection causes a sexually transmitted disease responsible for damage of the fallopian tubes with demonstrated consequences for fertility. The test for IgG antibodies against chlamydia (CAT) has different estimates of accuracy due to the use of different assays and cut-off values. The accuracy of CAT in diagnosis of tubal disease was assessed for three different CAT assays (microimmunofluorescence, MIF; immunofluorescence, IF test; or enzyme-linked immunosorbent test, ELISA) and revealed that MIF is the most accurate in evaluating tubal disease and should therefore be the test of choice [149].

Conclusions:

CAT is of pivotal importance before tubal investigation, but it can be omitted in patients who are candidates for IVF, after cervico-vaginal infection has been ruled out.

Hysteroscopy

Hysteroscopy represents the gold standard for the study of uterine cavity, as it allows direct visualization of the intrauterine pathology and offers the opportunity for performing treatment at the time of diagnosis.

Although the use of hysteroscopy before ART is part of the clinical routine of several centers, its impact on IVF outcome is still discussed. Several studies were performed to evaluate the efficacy and safety of hysteroscopic screening in subfertile women undergoing evaluation for infertility and in those undergoing ART.

In the general population with a normal US or HSG, there is no high-quality evidence to support the routine use of hysteroscopy as a tool for improving reproductive success rates. This uncertainty is also present for women with previous failed IVF attempts [150]. Regarding operative hysteroscopy, a recent Cochrane systematic review concluded that uncertainty remains concerning the benefit of hysteroscopic removal of submucous fibroids for improving the clinical pregnancy rates in women with otherwise unexplained subfertility [151]. It remains unclear whether endometrial scratching improves IVF/ICSI outcomes: if a true effect exists, it may be smaller than previously anticipated or may be limited to specific groups of women undergoing IVF/ICSI [152]. At present, endometrial scratching should not be performed outside of clinical trials [153].

Conclusions:

More research is still needed to measure the effectiveness and safety of routine hysteroscopy before ART; by now, its use should remain limited to patients with clinical or US suspicion of endometrial pathology.

Laparoscopy

The role of laparoscopy in the evaluation of infertility is controversial. Laparoscopy offers the possibility to perform both diagnosis and therapy at the same time, and the opportunity to add hysteroscopic exploration of the uterine cavity with endometrial biopsy. There is not enough evidence to assess whether laparoscopy should be part of pre-ART testing. According to data published in retrospective studies, diagnostic laparoscopy could be useful for the detection and treatment of pelvic pathology [154].

Currently, the NICE guidelines recommend a less invasive procedure, such as HSG, as the first option for testing tubal patency over laparoscopy in women without comorbidities such as pelvic inflammatory disease, previous ectopic, or endometriosis. However, laparoscopy could be recommended for women with pelvic comorbidities [1].

In women with bilateral ultrasonically visible hydrosalpinx, moderate-quality evidence shows that salpingectomy prior to ART probably increases the pregnancy rate compared to no surgery [155]. In women with endometriosis-related infertility, although randomized controlled trials are lacking, the benefit of laparoscopic surgery in moderate or severe endometriosis has generally been accepted [156].

Conclusions:

In some specific clinical settings, the use of diagnostic laparoscopy in current fertility practice should be recommended. There is however a need for more RCTs to answer remaining questions regarding its value in the diagnosis and treatment of otherwise unexplained infertility.

Tests to be performed by the man

Semen analysis

Semen analysis is the cornerstone of the infertile male diagnostic pathway, and it helps to define the impact of the male factor on ART [157]. Standardized instructions for semen collection and transport should be provided, including a defined pretest abstinence interval of 2–5 days. Semen should be evaluated according to the WHO manual [158], and preferably performed in laboratories that have expertise in reproductive medicine.

The semen analysis provides information on semen volume, sperm concentration, motility, and morphology [158]. Clinical reference ranges help to classify men as fertile or subfertile [159]. Nevertheless, even some men with abnormal semen parameters may sometimes be fertile. When semen contains no sperm, the diagnosis of azoospermia can be established only after the specimen is centrifuged. As spermatogenesis is a long process, lasting about 80 days, a pathologic semen analysis deserves reevaluation after some weeks, preferably after a spontaneous cycle of spermatogenesis.

Conclusions:

Semen analysis should be offered as a first level investigation in the infertile couple, before accessing ART, and in case of pathologic values, it should be repeated after 3 months.

Microbiological assessment

Patients with increased numbers of white blood cells (WBCs) in the semen should be evaluated for the presence of male accessory gland infection/inflammation, such as orchitis, epididymitis, vesiculitis, prostatitis, and urethritis [160]. These are potentially reversible causes of male infertility and can be easily treated with anti-inflammatory and/or antibiotic therapy [161].

Pyospermia is defined as the presence of $>10^6$ (1 million) leukocytes per milliliter of ejaculate; it occurs more frequently in infertile patients compared with fertile men, and it has been associated with sperm motility abnormalities [157,162]. When pyospermia is present, semen culture is indicated, and if negative, a second-level test (urethral swab, urine culture) may be useful to detect intracellular microorganisms such as mycoplasmas (in particular *Mycoplasma hominis* and *Ureaplasma urealyticum*) [161,163].

Conclusions:

Men with pyospermia (WBCs $> 10^6$/mL) should be evaluated to exclude genital tract infection or inflammation. In this case, semen culture tests should be performed.

FSH, LH, testosterone, prolactin

Hormonal abnormalities of the hypothalamus-pituitary-testicle axis represent uncommon causes of male infertility and are extremely rare in men with normal semen analysis [157].

Serum FSH levels negatively correlate with the number of spermatogonia. A markedly elevated FSH level, or even an FSH value in the upper normal range (above 7.6 mIU/mL), is indicative of an abnormal spermatogenesis or is associated with nonobstructive azoospermia due to severely impaired sperm production [162,164]. Conversely, low FSH and LH levels are suggestive of hypogonadotropic hypogonadism. Normal hormonal parameters are found in cases of obstructive azoospermia. A single measurement of serum FSH and LH is considered adequate despite the oscillation due to pulsatile secretion.

Testosterone levels should be measured in a blood sample collected in the morning, and if it results low the assay should be repeated, in addition to serum free or bioavailable testosterone, LH, and prolactin [157]. There is no consensus on the lower cut-off value for total testosterone concentration: the diagnostic value for hypogonadism is 300 ng/dL according to American Society for Reproductive Medicine (ASRM) and 230 ng/dL (8 nmol/L) according to European Association of Urology (EAU) [162,165]. In couples with unexplained infertility, low total testosterone in the male partner has been associated with abnormal sperm morphology and lower LB rates, but it is not clear if its pharmacological correction could be clinically relevant [166,167].

An often missed endocrine etiologic factor of male infertility is the disorder of the prolactin hormone [168]. It plays an antagonistic action on the male gonadal functions decreasing the pulsatile release of GnRH, thereby depressing the secretion of FSH, LH, and finally of serum total testosterone. A recent study reports a 16% prevalence of prolactin disorders in males with altered seminal parameters and FSH levels, suggesting that diagnostic and treatment protocols should include the prolactin measurement and management of its disorders during infertility evaluation in males [169].

Conclusions:

The determination of serum FSH, LH, and total testosterone concentration should not be routinely performed; it is recommended in men with sperm concentration below 10 million/mL, sexual dysfunction (impaired libido, erectile dysfunction), or suspected endocrinopathy (such as prolactin disorders).

Scrotal ultrasonography

Scrotal ultrasonography is a noninvasive, safe, and economic exam that allows the study of anatomy, size, and echogenicity of the testes and epididymis, plus the color-Doppler evaluation of blood flow in spermatic cord veins. A scrotal US examination can be helpful in case of abnormal findings at scrotal clinical examination, and it should also be considered for men presenting with infertility and risk factors for testicular cancer, such as cryptorchidism [157]. Scrotal US may detect signs of testicular dysgenesia (nonhomogeneous testicular architecture and micro-calcifications), often related to an impaired spermatogenesis, or testicular lesions suggestive of malignancy [170]. Scrotal color-Doppler US can confirm the clinical diagnosis of varicocele.

Conclusions:

Scrotal US should not be routinely used in all men seeking ART. A scrotal US can be helpful in case of abnormal findings at scrotal clinical examination/sperm analysis, and it should also be considered for those presenting with risk factors for testicular cancer.

Transrectal ultrasonography

Transrectal ultrasonography (TRUS) can be useful to identify enlarged seminal vesicles or ejaculatory ducts, midline cystic prostatic structures, and diagnose complete or partial ejaculatory duct obstruction [157]. Men with distal obstruction may exhibit similar clinical findings to those with CBAVD, including a low-volume, acidic ejaculate containing no sperm, and no fructose. Men with partial ejaculatory duct obstruction often exhibit low semen volume and oligo-asthenospermia.

Some experts recommend routine TRUS for oligozoospermic men having low-volume ejaculates, palpable vasa, and normal testicular size with normal serum testosterone [157]. However, currently TRUS should be recommended only in infertile patients with a suspected distal obstruction [162].

Conclusions:

TRUS is recommended only for infertile patients with a suspected distal obstruction (severe oligozoospermia or azoospermia, seminal volume <1.5 mL, semen pH < 7.0, and absent fructose), normal testosterone, and palpable vas deferens.

Y chromosome microdeletion analysis

Y chromosome microdeletions are the second most common genetic cause of infertility in the male, after karyotype anomalies. Such microdeletions are too small to be detected by standard karyotyping but can be identified by PCR techniques. Most deletions causing azoospermia or oligozoospermia occur at three sites of the long arm of the Y chromosome (Yq11), known as the azoospermia factor regions: *AZFa* (proximal), *AZFb* (central), and *AZFc* (distal). Men with *AZFc* deletions may have severe oligozoospermia or azoospermia, but in about half of the cases, spermatozoa can be retrieved in the ejaculate or via testicular sperm extraction [162,171]. In contrast, deletions involving the entire *AZFa* and/or *AZFb* region predict a very poor prognosis for sperm retrieval [171,172], being associated with Sertoli cell only syndrome or with spermatogenic arrest [165].

An appropriate counseling should be offered to the infertile male with Y chromosome microdeletions. The couple should be informed that all male offspring will inherit the microdeletion as well as the risk of being infertile, but no other health problems seem to be associated with this condition [173].

Conclusions:

Y chromosome microdeletion analysis could be offered to men with nonobstructive azoospermia or severe oligozoospermia (<5 million/mL) before performing ICSI. It is highly advisable for sperm counts <1 million/mL.

Sperm DNA fragmentation tests

Sperm DNA fragmentation (SDF) is the accumulation of single- and double-strand DNA breaks. Impaired sperm DNA integrity is detrimental for normal fertilization, embryo development, successful implantation, and pregnancy following ART treatments [174]. SDF may increase miscarriage rate and negatively affect the likelihood of natural conception as well as the outcome of ART [175].

Various tests have been developed to assess SDF. The definition of the pathologic SDF threshold is still not standardized, and there is high variability in reported cut-off values, representing the main limiting factor of SDF tests. However, a recent meta-analysis compared the most commonly used SDF assays and suggested that a threshold of 20% may differentiate between fertile and infertile men [176].

Although emerging evidence supports the role of sperm DNA fragmentation in affecting ART outcome [157], routine use of SDF testing is not recommended by current guidelines and can be considered only in couples with RPL or in men with unexplained infertility [157,165].

Conclusions:

SDF testing should be considered in couples with unexplained RPL or in men with unexplained infertility.

Tests for antisperm antibodies

Antisperm antibodies (ASAs) can be generated when there is a disruption in the blood-testis barrier and sperm antigens are exposed to the immune system. ASAs are a rare cause of male subfertility and do not require routine testing. ASAs can decrease sperm motility and impair spermatozoa penetration into the oocyte, negatively affecting the conception rate [177]. However, the lack of penetrating ability of spermatozoa is successfully overcome by ICSI. Therefore, ASA testing is unnecessary if ICSI is planned [157].

ASAs may be detected in serum or seminal plasma through indirect antibody agglutination assay, while direct test is used to detect ASAs bound to the sperm head or tail. Although ASA testing has been suggested for couples with unexplained infertility, its clinical utility in such couples is uncertain.

Conclusions:

Testing for ASAs has been proposed in cases of isolated asthenozoospermia (with normal sperm concentration) at semen analysis or when sperm agglutination is observed. As ICSI overcomes the problems caused by ASAs, their assay is not a recommended test before ART.

Diagnostic testicular biopsy

Diagnostic testicular biopsy can be helpful to determine the etiology of azoospermia, but it is recommended only when clinical and laboratory parameters are inconclusive. In most cases semen volume, clinical exam, and FSH levels can easily distinguish obstructive from nonobstructive azoospermia. Men with FSH = 7.6 mIU/mL or greater and/or with testicular long axis of 4.6 cm or less may be considered to have nonobstructive azoospermia [164]. In these patients, therapeutic testicular biopsy and sperm extraction can be useful to harvest sperm for cryopreservation and ICSI.

Testicular biopsy may be also performed in the subgroup of infertile men at increased risk for testicular malignancy [178].

Conclusions:

Diagnostic testicular biopsy is rarely indicated and should not routinely be performed to differentiate obstructive from nonobstructive azoospermia.

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CHAPTER

19

Ovulation induction protocols

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Introduction

Intrauterine insemination (IUI) also known as artificial insemination is one of the earliest and simplest assisted reproductive technologies (ARTs).

With this technique, sperm from either a partner or donor (such as from a sperm bank) is inserted with a syringe into the woman's uterus during ovulation to increase the probability that fertilization occurs and leads to a pregnancy.

IUI has a long history, first in animals and later in humans.

The use of this technique in animals dates back the fourteenth century when Arabs used it to breed stallions.

Lazarro Spalianzani is known as the first to use the technique to breed dogs in late 1784, where this insemination resulted in the birth of three puppies 62 days later. In London in 1793, John Hunter was the first person reported to achieve a successful human pregnancy using IUI. Although Hunter received credit for the first human pregnancy using the procedure, it is likely there were earlier successful attempts.

The rationale behind artificial insemination is increasing the gamete density at the site of fertilization. The primary reason for using this technique in farm animals was to speed up the rate of genetic improvement by increasing the productivity of food-producing animals. This was accomplished by improving the selection differential wherein one highly selected male is mated with thousands of females. The AID industry was born.

For humans the situation is different: artificial insemination was originally developed to help couples to conceive in case of severe male factor subfertility of a physical or psychological nature. Nowadays artificial insemination with homologous semen is most commonly used for unexplained and mild male factor subfertility. In the previous century, donor insemination was mainly used for male infertility due to azoospermia or very low sperm count and for inherited genetic diseases linked to the Y-chromosome. Nowadays donor insemination is more commonly used in women with no male partner (lesbians or single women).

Indications

The indications for IUI with a partner's sperm remain varied and poorly defined.

The indications currently described for IUI are cervical infertility, disorders (physical or mental) preventing sexual intercourse (vaginismus, ejaculation disorders, malformations, or neurological disorders and other sexual disorders), unexplained infertility, and moderate endometriosis.

In some cases IUI can be proposed as a conversion of *in vitro* fertilization (IVF) in case of insufficient response to OS.

Cervical indications

It is the most logical indication for IUI and is characterized by repeated negative Huhner tests (postcoital tests), even though the benefit of this test is seriously questioned. In fact, IUI leads to "bypass" the uterine cervix, where in these cases, sperm ascension is not satisfactory. These negative Huhner tests may be the consequence of insufficient cervical mucus (e.g., history of conization), impenetrability (e.g., patient with a CFTR gene mutation), or of qualitative sperm insufficiency. In all cases, it is recommended to perform simple induction before switching to IUI, which may sometimes be sufficient to optimize the mucus.

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Sperm etiology

IUI is indicated for "moderate sperm insufficiency" or moderate oligoasthenospermia. The sperm criteria conditioning the prognosis of IUI are still discussed to this day. However, certain parameters such as the number of inseminated motile spermatozoa (NSMI) and the percentage of morphologic disorders of these spermatozoa seem to influence the chances of pregnancy.

It is therefore essential to have a sperm survival test carried out, before considering the IUI and knowing the decision thresholds for these parameters for the laboratory in question.

Unexplained etiology

Although this indication is still the subject of controversy, the vast majority of studies published to date conclude that the pregnancy rates are statistically significant. After ovulation induction + IUI compared with scheduled intercourse with or without ovulation induction pregnancy, rates are significantly higher after 2–3 years of conception attempts.

Ejaculation disorders

Insufficient sperm volume, abnormalities in the mouth of the urethral meatus, and even certain sexual disorders, may represent rare indications for IUI.

Immunological etiology

The presence of antisperm antibodies of male origin, or more exceptionally and controversially of female origin, will impede sperm mobility and the progression of sperm through the cervical canal. IUI by sperm preparation and rapid contact of gametes can promote the onset of pregnancy; however IVF with micro-injection obtains more consistent favorable results in this context. Other indications are still poorly assessed to date: endometriosis with healthy tubes, single patent tubes, and failure to induce ovulation.

Pre-IUI tests

Before beginning IUI treatment, women must undergo an X-ray test, called a hysterosalpingogram, to document that they have at least one open fallopian tube.

Male partners providing a semen specimen for IUI must be tested for infectious diseases. The specific tests required are HIV, RPR (a test for syphilis), hepatitis B surface antigen, and hepatitis C antibody. Before an IUI can be performed, the tests must be complete and there must be no exceptions to this policy.

Performing intrauterine insemination

Ovarian stimulation

Spontaneous cycle

The IUI can theoretically be performed in a spontaneous cycle or most often stimulated by antiestrogens or gonadotropins.

The OS protocol should be set after a positive and etiological diagnosis of the ovulation disorder.

Antiestrogens

Clomiphene citrate The only indication for clomiphene citrate is polycystic ovary syndrome (PCOS) and it has no relevance in unexplained infertility.

The usual dose of clomiphene citrate is 50 mg/day for 5 days from the second or third day for 5 days of the spontaneous cycle or induced by progestins in case of spaniomenorrhea or amenorrhea (most often 20 mg/day for 10 days of didrogesterone).

In the absence of an echographic and or hormonal response, the treatment will be modified to 100 mg/day and then 150 mg/day. In the absence of a response to this dosage, most teams propose to consider the patient as resistant to clomiphene because of the possible antiestrogenic effects on the endometrium at higher dosages.

Aromatase inhibitors Actually many teams prefer antiaromatase, such as with antiestrogen activity without any negative impact on the endometrium.

The protocol of letrozole is 25 mg/day from day 2 or 3 of the cycle for 5 days.

If there is no response, we can increase the dose to 50 then to 75 mg/day.

In the absence of a response to this dosage, we propose to consider the patient resistant to letrozole and start another protocol such as the gonadotropins.

Gonadotropins

The protocol of choice for gonadotropins is the step-up low dose.

The protocol is defined by a low initial dosage, possibly increased in slowly progressive steps. The starting dose is 50–75 IU per a day with increments of 25–37.5 IU per day. The increment dose is proposed for each level in the absence of an ultrasound response after 10–14 days of treatment.

Gonadotropins are administered subcutaneously for an average of 7–12 days depending on the ovarian response.

In all cases, including for clomiphene citrate or letrozole, regular monitoring by ultrasound and incidentally laboratory depending on teams (dosage of estradiol luteinizing hormone [LH] and progesterone) is required to assess the ovarian response.

The hormonal monitoring is still questioned and several datas did not show any benefit with a high cost effectiveness.

When one or two follicles maximum have reached the size of 17−18 mm in diameter with an estradiol level of 150−250 pg/mL per mature follicle, absence of an LH surge, and premature rise in progesterone, an injection of hCG (human chorionic gonadotropin) is performed to trigger ovulation.

There seems no interest of adding antagonists of GnRH.

Results concerning the higher chances of pregnancy are debated since it is not recommended to have more than one or two mature follicles because of the higher risks of multiple pregnancies.

When we compare gonadotropins to antiestrogens and no treatment, a recent meta-analysis demonstrated a higher pregnancy rate with gonadotropins than no treatment or antiestrogen.

Though, IUI with gonadotropins should be the gold standard protocol to increase chances of pregnancies. Progesterone support for the luteal phase is debated and is not based on consensus or on definite bibliographic data. When prescribed, the usual strategy is 600 mg/day of micronized progesterone orally or intravaginally. There is no more consensus or certainty on whether or not support is maintained for up to 10 or 12 weeks or on its discontinuation during the positive pregnancy test considering that the hCG secreted by the trophoblast then becomes sufficient to support the corpus luteum. The orientation of care in ART now favors the search for single-fetal pregnancies. In IVF, this objective is ensured by the development of selective transfers of an embryo. In simple stimulation or in IUI, the risk is assessed by analyzing the ultrasound and hormonal results. The usual criteria for discontinuation of cycle and for abstinence or safe sex counseling are the presence of three or more mature follicles associated with estradiol levels greater than 800 pg/mL. The triggering of ovulation proposal should be discussed on a case-by-case basis if two or three follicles are present. Estradiolemia is therefore not an absolute criteria and multiple pregnancies are observed during stimulation leading to a dominant mature follicle and one or more follicles of intermediate size (12−14 mm). The follicular growth from one ultrasound to another, the age of the patient, the duration of infecundity, the rank of the attempt, and the analysis of any previous cycles will be the elements to be evaluated to make the best decision. Depending on the clinical situation, the objective may be strictly monofollicular or bi- or even trifollicular in older women (more than 40 years old) with a poor prognosis.

Semen preparation

A step prior to insemination, the purpose of sperm preparation, is to eliminate the seminal plasma, which inhibits fertilization, as well as any debris, round cells, and bacteria, and select the most motile and normal sperm. This preparation aims to reproduce the capacitation step *in vivo* during passage through cervical mucus and uterotubal secretions. Several techniques exist such as simple washing, passage through discontinuous gradients of colloidal silica particles with centrifugation, migration, but none has shown its superiority with regard to normal sperms. Centrifugation on discontinuous gradients (two or three different layers) of silica particles makes it possible to select the spermatozoa according to their morphology and their mobility. On the day of insemination, the partner goes to the ART laboratory to collect sperm by masturbation. He will have taken care beforehand to respect an abstinence from 2 to 5 days and to drink at least 1.5 L of water the day before to avoid bacterial contamination of the sample. After observing a liquefaction time of 30 min at room temperature, the sample can be processed. Several milliliters of sperm, however, with a maximum volume of 4 mL, are deposited on the surface of the gradient layers. A centrifugation step of 15 min at 1800 revolutions is carried out, followed by a step of washing the pellet obtained in a culture medium. Depending on the size of the base, a resuspension or a swim-up can be carried out. Once these selections, migration steps, have been carried out, the sample is stored at 37°C for at least 1 h.The minimum quantity of motile sperm deposited in the uterine cavity varies according to the authors from one to two with a maximum of 10 million. In case of IUI with donor or in special cases of IUI with sperm of the spouse or IUI (collection failure, gonadotoxic treatment, work absences, etc.), the sperm selection is carried out at from frozen semen. In these cases, the number of straws required to obtain a satisfactory insemination fraction is decided on the basis of the results of the thawing test. The quantities and concentrations of the silica gradients used will be lower than for the preparation of fresh semen. In some cases, simple washing will be preferable to discontinuous silica gradients.

IUI procedure

After a simple cleaning of the cervix, without the use of antiseptic, insemination is performed using a semiflexible catheter mounted on a 1 mL syringe. The

injected volume is approximately 0.3 mL. Insemination takes place slowly and the catheter is not withdrawn immediately, thus reducing possible reflux. The patient remains lying down for about 10 minutes, then she can resume normal activity. A pregnancy test is carried out 14 days after the sowing. In the absence of a pregnancy, a new insemination can be carried out. As the IUI technique is not very restrictive for the couple, four IUIs can be completed in one semester. The technique of fallopian tube sperm perfusion (FSP) was first described in 1992 by J. A. Kahn. It consists of injecting 4 mL of a sperm preparation under pressure into the uterus using a special probe, while trying to avoid cervical reflux. The latest meta-analysis comparing the efficacy of FSP and IUI does not show any significant difference in terms of pregnancy rate. The additional cost and the more difficult technique of FSP leads to a preference for IUI.

How many IUIs do we need to achieve?

Although the literature data on this subject is debatable, it appears that most IUI pregnancies occur within the first three or four cycles of treatment.

A still satisfactory pregnancy rate (>10%) is obtained in the fifth cycle of IUI, beyond which the chances of pregnancy diminish; however some authors suggest performing up to nine cycles of IUI. The number of cycles performed must take into account the existence of a previous pregnancy, the patient's age and ovarian reserve, the indication for IUI, and the number of motile spermatozoa inseminated.

Prognostic factors of IUI

Etiologies and prognostic factors

The cervical etiology characterized by several negative Huhner tests, not improved by estrogen therapy or simple induction, obtains the best scores with a pregnancy rate per cycle of about 20%. However, it can be noted that a recent review of the literature concluded that, despite the large number of publications on this subject, the insufficiency of the methodologies used did not allow a conclusion to be drawn on the effectiveness of IUI in case of cervical etiology. Finally, a controversy exists for these patients on the usefulness or not of performing a controlled ovarian hyperstimulation. While some authors consider it necessary, others do not observe a significant difference in the chances of pregnancy for urinary tract infections with or without OS, but accuse this stimulation of leading to many multiple pregnancies, in particular in this indication. The male etiology in "moderate sperm insufficiency" is a fairly good prognosis, with pregnancy rates per cycle of around 15%.

The Cochrane database studies are again critical of the methodologies used but conclude that IUI is effective in this indication. In the event of unexplained infertility, three meta-analysis grouping together around 10,000 cycles concluded at the end of the 1990s to the superiority of IUI based on scheduled sexual intercourse. However, a prospective randomized study and a recent meta-analysis conclude that IUI is of no benefit compared with treatment abstinence in cases of unexplained infertility. It is therefore more than ever necessary in this indication to take into account all the prognostic criteria before referring couples to AMP (IUI or IVF).

Rank of the attempt and prognostic factors

Most authors agree that the best pregnancy rates are obtained in the first cycles of treatment.

It is usually recommended, as we have seen, to have three or four IUIs before switching to IVF.

Age and duration of infertility

The patient's age is an essential prognostic factor, as in all ART. While some authors have found a practically linear relationship that is inversely proportional to age, others have observed success rates that persist up to age 40, before dropping beyond. The prolongation of the duration of infertility is a factor of poor prognosis for the majority of authors; however for others, the success rates seem to be little influenced by the duration of infertility.

Characteristics of stimulation

A recent Cochrane database study did not demonstrate the superiority of an ovulation stimulation protocol. The number of follicles $\geq$16 mm is one of the essential prognostic factors; in fact, the chances of pregnancy per cycle of IUI increase in parallel with the number of mature follicles visible in ultrasound. On the other hand, the same authors observe an increase, also parallel to the number of follicles, of large multiples. Some authors attribute a prognostic value to the estradiol levels obtained at the end of stimulation, but this value is less reliable than the ultrasound appearance of the ovaries. While the occurrence of an LH surge at the end of stimulation has been considered by some as an unfavorable prognostic factor, most authors have not demonstrated any difference in the chances of success, whether the trigger is related to an LH surge or induced by an injection of HCG.

Number of motile sperm inseminated (NMSI)

Many authors consider NMSI to be one of the essential prognostic factors. If we take into account the association of a + b mobility, the NMSI threshold above which the results are optimized is, for most European authors, 5×106. Some authors, in particular North Americans, recommend a threshold of 10×106. In all cases, an NMSI less than one or equal to million should direct couples to IVF, or even ICSI. For intermediate values of NMSI between one and five million, the use of double sperm collection allows on average to double the value of the initial NMSI and to optimize pregnancy rates per cycle. The IUI technique with double collection is simple and well accepted: it consists of having a second collection carried out 1 hour after the first. The two collections are then "pooled" and the sperm preparation is carried out on the whole. It should be noted, however, that some authors did not reach statistical significance for the NMSI parameter.

Sperm morphology

Recent meta-analysis and reviews of the literature on this subject show that it is difficult to give universally applicable thresholds for sperm criteria, and this is because of a lack of standardization of semen analysis. However, these studies confirm that sperm morphology using strict criteria and the NMSI, after preparation, are the two parameters that most influence the results of IUI. Other studies, using multivariable logistic regression analysis or ROC sensitivity/specificity curves reach the same conclusions. Using the morphologic study of spermatozoa, according to Kruger's strict criteria, it appears that the threshold below which the results collapse is 4%. If we use the morphologic study according to the strict criteria of David and Jouannet, this threshold is around 20%. Some data were able to show that the quantitative increase in the NMSI could partly compensate for the qualitative alteration of the sperm.

Conclusion

IUI is a simple, cost-effective, noninvasive first-line therapy for cervical factor, anovulatory infertility, moderate male factor, unexplained infertility, and immunological infertility with clinical pregnancy rates ranging from 10% to 20%. Controlled ovarian hyperstimulation with close monitoring of folliculogenesis and ovulation to avoid adverse complications, such as ovarian hyperstimulation syndrome and multiple pregnancies, may be used to obtain the adequate number of follicles.

IUI is the preferred conception-enhancing technique for women <35 years, with functional tubes, short period of infertility, and moderate male infertility, particularly in technology-limited settings, and four to six IUI cycles may be performed before considering alternate therapy such as IVF. It is the method of choice versus timed intercourse or natural cycle IUI.

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CHAPTER

20

Ovarian stimulation protocols

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Introduction

More than 40 years have passed since the birth of Louise Brown on July 25, 1978, the first baby conceived after in vitro fertilization (IVF) in a natural cycle. Since then, remarkable evolutions in reproductive medicine, in clinical and laboratory areas, have occurred. Ovarian stimulation (OS) is essential for modern *in vitro* fertilization treatment of infertility, aiming to the production of an optimal number of oocytes to be fertilized, with more embryos available for selection and transfer, both in fresh and subsequent frozen cycles, thus maximizing the possibility of pregnancy. OS is a complex procedure, involving the administration of exogenous gonadotropins to stimulate multifollicular development, the cotreatment with gonadotropin-releasing hormone (GnRH) agonists or antagonists for pituitary suppression and prevention of premature luteinization and ultimately the triggering of final oocyte maturation and oocyte retrieval. However, besides the conventional protocols used currently, novel concepts in OS for IVF have introduced different protocols in daily practice.

Conventional protocols

An essential part of OS in IVF/intracytoplasmic sperm injection (ICSI) cycles involves comedication for prevention of a premature luteinizing hormone (LH) surge and premature luteinization, which would disrupt both normal follicle and oocyte development, and finally result in no retrieval of oocytes. Without comedication, about 20% of women stimulated with gonadotropins could not reach oocyte retrieval due to an unpredicted surge of LH [1]. Indeed, during OS with exogenous gonadotropins, the multiple follicular development results in high estradiol (E2) levels in blood that may activate the positive feedback mechanism and the occurrence of an LH surge at the time when the leading follicles have smaller than optimum (e.g., 16–18 mm) diameter. Classically, the two approaches for the prevention of premature LH surge are the pituitary desensitization with prolonged daily administration of a GnRH agonist or the instant and immediate blockade of the LH secretion with a GnRH antagonist [1a].

GnRH agonist protocols

The GnRH agonists (GnRH-a) were introduced into IVF protocols in the 1980s as an effective approach for pituitary desensitization and prevention of a premature increase in LH, thus reducing cycle cancellation rate and improving treatment outcome [2]. The mechanism of action involves the binding of GnRH-a to pituitary receptors, the release of large amounts of follicle-stimulating hormone (FSH) and LH (flare-up effect), and an increase in the number of GnRH receptors (upregulation). With the prolonged use of the GnRH-a, which requires at least 7 days, internalization of the GnRH-a receptor complex occurs, resulting in a decrease of the number of GnRH receptors (downregulation) and making the pituitary refractory to stimulation by GnRH. Therefore, the pituitary no longer secretes gonadotropins [3]. Until today, the GnRH-a protocols have gained popularity in clinical practice. These protocols, depending on the time the GnRH-a is administered, include the short and long protocols.

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In the short protocol, the GnRH-a is usually administered from day 1 (day 1 being the start of the menses) or day 2 of the cycle until the day of human chorionic gonadotrophin (hCG) administration for triggering final oocyte maturation. The gonadotropins for OS and multiple follicular development are given from day 1 or 2 of the cycle (ultrashort protocol) or 2–3 days after GnRH-a initiation (short protocol), until the day of hCG administration. In the short protocol, the GnRH-a exhibits the initial stimulatory effect on pituitary gonadotropins release (the flare-up effect) promoting follicular development. Following this effect, the pituitary is downregulated with subsequent inhibition of LH secretion.

In the long protocol, the GnRH-a is given at least 2 weeks before starting stimulation, to attain pituitary downregulation and suppression of endogenous gonadotropin secretion, and it is continued until the day of hCG administration. After the suppression of the pituitary-ovarian axis is confirmed with measurement of low LH and E2 serum levels, OS with exogenous gonadotropins starts and continues concomitantly with the GnRH-a until the day of hCG administration. The long protocol may start from either the second day of the menstrual cycle (long follicular protocol) or the midluteal phase (21st day) of the previous cycle (long luteal protocol). In the long luteal protocol, but not in the long follicular protocol, following pituitary downregulation, menses will occur. In clinical practice, the long protocol may improve the routine patient treatment schedule [4].

A Cochrane meta-analysis compared the effectiveness of long GnRH-a protocols and short GnRH-a protocols in women undergoing IVF treatment [5]. The authors did not find conclusive evidence of a difference in live birth and ongoing pregnancy rates, but there was moderate quality evidence of higher clinical pregnancy rates in the long protocol compared with the short protocol. This meta-analysis also compared other modifications of the GnRH-a protocol. There were no differences in efficacy in the following comparisons: long versus ultrashort GnRH-a protocol, short versus ultrashort GnRH-a protocol, long luteal versus long follicular GnRH-a protocol, in the long GnRH-a protocol the continuation versus the stopping of GnRH-a at start of stimulation, in the long agonist protocol the continuation of same dose versus the reduced dose of GnRH-a until trigger [5]. The European Society of Human Reproduction and Embryology (ESHRE) Guideline Group on OS [6] suggested that if GnRH-a is used, the long GnRH-a protocol is probably recommended over the short or ultrashort GnRH-a protocol.

However, the long GnRH-a protocol has been associated with some disadvantages, such as a long treatment period until the occurrence of desensitization, the increased risk of ovarian hyperstimulation syndrome (OHSS), cyst formation, and occurrence of side effects due to hypoestrogenemia [7]. Furthermore, there is a risk of about 4% of an unknown pregnancy to be exposed inadvertently to the GnRH-a that its administration commences in the luteal phase of the cycle [8].

GnRH antagonist protocols

The mechanism of action of GnRH antagonists (GnRH-ant) is different than agonists. GnRH-ant binds competitively to GnRH receptors, so endogenous GnRH is incapable of stimulating the gonadotrophs, and secretion of gonadotropins is decreased. The action of GnRH-ant is immediate, with cessation of gonadotropins secretion within hours after its administration, while there is no flare-up effect. The competitive blockade of the GnRH receptor by the antagonist is dose dependent, based on the balance between the quantities of endogenous GnRH and the antagonist. On the other hand, following the discontinuation of GnRH-ant administration, the recovery of pituitary is rapid with resumption of gonadotropins secretion [3]. Therefore, the introduction of GnRH-ant in assisted reproductive technology to prevent the premature LH surge resolved some major disadvantages of GnRH-a. Indeed, the IVF cycle become more "patient friendly," since the immediate and profound suppression of the pituitary by the GnRH-ant resulted in a shorter duration of injections compared with the GnRH-a long protocol and disappearance of the side effects related to hypoestrogenemia. Another advantage of the GnRH-ant mechanism of action, offering an alternative to the hCG triggering of final oocyte maturation, is that the pituitary remains responsive to a GnRH-a, provided that the GnRH-ant treatment utilized standard doses [9].

Depending on the dose of the GnRH-ant used, two different protocols have been developed: the multiple dose protocol, in which 0.25 mg of GnRH-ant is administered daily from day 6 of stimulation until the day of hCG triggering [10] and the single dose protocol, where a 3-mg dose of GnRH-ant is given on cycle day 7 during OS [11]. In cases when hCG administration was delayed, daily doses of 0.25 mg of the GnRH-ant could be added 4 days after the single 3-mg antagonist dose.

Depending on the time the GnRH-ant is administered, there are two protocols, the fixed and the flexible. In the fixed protocol, the antagonist administration starts always from stimulation day 6, whereas in the flexible

protocol the antagonist administration starts when a dominant follicle ≥14 mm is found. A meta-analysis showed a nonsignificant trend for lower pregnancy rate in the flexible compared to the fixed protocol [12].

The criterion for triggering final oocyte maturation, both in agonist and antagonist protocols, is usually the leading follicles size. The triggering is achieved traditionally by a single dose of hCG administered 36 h before oocyte retrieval, and in most studies, hCG was given when at least three follicles reached the diameter of 17 mm. However, it was found that triggering with hCG when the leading follicle was 18 or 22 mm had no effect on the live birth rate, although in the 22 mm group the ongoing pregnancy rate was higher and significantly more oocytes were retrieved [13]. The ESHRE Guideline Group on OS [6] suggested that the decision on timing of triggering in relation to follicle size is multifactorial, taking into account the size of the growing follicle cohort, the hormonal data on the day of pursued trigger, the duration of stimulation, the patient burden, the financial costs, the experience of previous cycles, and organizational factors for the center. Usually, the triggering is performed at sizes of several of the leading follicles between 16 and 22 mm. However, the ESHRE Guideline Group did not recommend triggering the final oocyte maturation based on E2 levels alone.

Comparison of GnRH-a and GnRH-ant protocols

In 2016, a meta-analysis evaluated the effectiveness and safety of the GnRH-ant protocol compared with the long GnRH-a protocol for OS [14]. There was moderate quality evidence showing no difference in live birth rate between GnRH-ant and long GnRH-a protocol (OR 1.02, 95% CI 0.85−1.23). However, the use of the GnRH-ant protocol was safer, since it was associated with lower incidence of any grade of OHSS than the long GnRH-a protocol (OR 0.61%, 95% C 0.51−0.72, moderate quality evidence). Furthermore, the cycle cancellation rate due to high risk of OHSS was lower with the GnRH-ant protocol (OR 0.47, 95% CI 0.32−0.69). Finally, there were no significant differences in miscarriage rate between the two protocols (OR 1.03, 95% CI 0.82−1.29, moderate quality evidence). Another meta-analysis [14a] found similar results to the previous meta-analysis. Based on these data, the ESHRE Guideline Group on OS [6] provided clinical recommendations for GnRH analogs protocols selection according to the patients' predicted response to stimulation. For PCOS women and non-PCOS high responders, the GnRH-ant protocol is recommended over the GnRH-a protocols with regard to improved safety (less OHSS rate) and equal efficacy (similar live birth rates). For normal responder patients, since live birth rates between the GnRH-ant and GnRH-a protocols were comparable and there was a significant decrease in the risk of OHSS with the GnRH-ant protocol, the GnRH-ant protocol is recommended. For predicted poor responders, there was no differences in safety and efficacy between the GnRH-a and GnRH-ant protocols, and both are equally recommended. However, the GnRH-ant protocol is associated with a shorter length of treatment compared with the long GnRH-a protocol.

Regarding the prevention of OHSS in predicted high responders (PCOS patients and women with high ovarian reserves as estimated by high anti-Müllerian hormone [AMH] and antral follicle count [AFC] values), the GnRH-ant protocol provides the opportunity for triggering with a GnRH-a instead of hCG, since the agonist displaces the antagonist from the receptor, resulting in a surge of both LH and FSH. Traditionally, the hCG is used as a surrogate for the midcycle LH surge since it binds to and activates the same receptor as LH (LH/hCG receptor). However, hCG is also the triggering factor of OHSS (mainly via secretion of the vascular endothelial growth factor [VEGF]), and its prolonged half-life results in stimulation of the corpora lutea for up to 1 week. On the other hand, the GnRH-a triggering induces a shorter LH surge. This GnRH-a-induced LH surge differs from the midcycle LH surge of the normal menstrual cycle since the LH surge of the natural cycle has three phases with a total duration of 48 h, while the LH surge after GnRH-a triggering has two phases with a duration of 24−36 h [15]. Therefore, the lower amount of LH in the luteal phase after GnRH-a triggering results in rapid luteolysis with decrease in estrogen and progesterone levels and deficient luteal phase. This luteolytic effect also decreases granulosa cell secretion of VEGF (the key factor for OHSS development) compared with hCG-triggered patients, providing the basic mechanism for the prevention of early OHSS. However, this rapid and early luteolysis significantly lowers the probability of pregnancy compared to hCG triggering [16] in patients undergoing OS for IVF with GnRH-ant. To overcome this problem and proceed with a fresh transfer after GnRH-a triggering, several ways for luteal phase support have been suggested, including the administration of hCG in various regimens or higher doses of exogenous E2 and progesterone. On the other hand, a safer practice to exclude the possibility of early and late OHSS after GnRH-a triggering is the cryopreservation of all embryos and their transfer in subsequent frozen-thawed cycles [17].

Gonadotropins

COS with exogenous gonadotropins is fundamental for IVF success since it enables multiple follicular development. Physiologically, FSH is the main regulator of antral follicle growth, but LH also participates in promoting steroidogenesis and in the development of the leading follicle. There have been major advances in technology to develop preparations that are safe and effective for clinical use [18]. The first generation of gonadotropins was human menopausal gonadotropin, produced from the urine of menopausal women (hMG, a combination of FSH and LH in a 1:1 ratio). The second generation of urinary gonadotropins was purified FSH (p-FSH), which contains less than 1 IU of LH per 75 IU of FSH. The third generation of urinary gonadotropins was highly purified FSH (hp-FSH) with less than 0.1 IU of LH per 75 IU of FSH. The fourth generation of gonadotropins was produced using recombinant DNA technology, i.e., recombinant FSH (rFSH), recombinant LH (rLH), and recombinant hCG (rhCG), and these products have high purity and high biological potency.

Considering the results in live birth rates of a Cochrane meta-analysis [19] and later published randomized controlled trials (RCTs), the ESHRE Guideline Group on OS [6] stated that the use of rFSH and hMG for OS is equally recommended. However, for GnRH-ant cycles, PCOS patients, and women of advanced age the evidence was less extensive, showing no significant differences in live birth rate between hMG and rFSH. The same group also concluded that the using rFSH versus purified FSH (p-FSH) and versus highly purified FSH (hp-FSH) for OS in GnRH-a protocols is equally recommended.

Although the addition of rLH to rFSH is mandatory for ovulation induction in hypogonadotropic hypogonadal women (WHO group I anovulation), it has been questioned whether this combination compared to rFSH alone may be beneficial in some patients undergoing OS for IVF. A Cochrane meta-analysis did not find a difference in live birth rate in patients treated with rFSH + rLH compared to those treated with rFSH only [20]. However, in patients treated with the GnRH-a protocol, although no difference was found in live birth rates, a higher ongoing pregnancy rate has been observed in the rFSH + rLH group compared to the rFSH only group. The meta-analysis did not find any difference in the OHSS rate between the two groups, but in patients treated with GnRH-a, a lower rate of OHSS has been observed with rLH addition [20]. Nevertheless, a more recent RCT in patients treated with the long GnRH-a protocol who had a 50% or greater reduction in LH levels 6 days after rFSH initiation, did not find differences in live birth and clinical pregnancy rates with rLH supplementation to rFSH [21]. It has been suggested that some groups of patients undergoing OS may benefit from the supplementation with rLH [22,22a]. The rLH supplementation may increase the number of oocytes retrieved and the implantation and clinical pregnancy rates in women with a hyporesponse to FSH monotherapy in a GnRH-a protocol [22a,23]. However, it has not been investigated whether the rLH supplementation may have any effect in hyporesponders undergoing OS in a GnRH-ant protocol.

Hyporesponse is the hyposensitivity to exogenous FSH, presented as an initial slow response or stagnation in follicular growth, resulting in the administration of higher FSH doses and/or the need to supplement with LH during OS. Hyporesponse is different from the poor response because the hyporesponders have adequate number of oocytes recruited, although the doses of gonadotropins are elevated, and the ovarian reserve tests (AMH and AFC) are normal. On the other hand, in poor responders, the number of oocytes retrieved is low, although the consumption of gonadotropins may be high, and their AMH and AFC values are low. Therefore, it has been suggested that in hyporesponders the rLH supplementation (75–150 IU) starting from day 7–10 of OS can compensate for the initial slow response more efficiently than increasing the dose of rFSH. Also, in cases when a hyporesponse was retrospectively identified, such as a history of excessive consumption of FSH, the rLH supplementation starting from day 7 or 8 of stimulation may improve the outcome [22a,23]. Another group of patients that may benefit from rLH supplementation are women of advanced age, 35–39 years old. Some, but not all studies, showed that the addition of rLH to rFSH may increase implantation and pregnancy rates in these women, treated with either long GnRH-a or GnRH-ant protocol [22,22a].

Regarding the source of LH bioactivity for OS, it is currently provided by HP-hMG and rLH. However, in HP-hMG, the LH molecules are lost during the purification process, and the LH bioactivity is provided by hCG. Most relative studies are not RCTs, and a small RCT [23a] in GnRH-a cycles showed that hMG and rhFSH + rLH appear to result in similar implantation and pregnancy rates, while data in antagonist cycles are missing [24].

Novel protocols

The design of the conventional protocols in IVF has been based on the traditional concept of folliculogenesis, that a single wave of antral follicles may be cyclically recruited, during the late luteal phase of the preceding menstrual cycle and the early follicular phase of the next cycle, under the intercycle rise of FSH levels (FSH

window). Usually, a single follicle is selected, while the others undergo atresia. In IVF cycles, the exogenous administration of FSH widens the FSH window, resulting in the recruitment and selection of multiple follicles [18]. Interestingly, it has also been shown that, even during the early stages of a viable intrauterine pregnancy, with OS, it is still possible to recruit follicles and retrieve mature oocytes that can be fertilized and cleave [25]; this finding indicates that pregnancy and the high progesterone levels do not render the ovaries refractory, and there are responsive follicles able to grow [25]. Furthermore, recent studies suggest that there are multiple waves of follicle recruitment within a single interovulatory period (two and even three waves), and some antral follicles in the late follicular or luteal phase may be in the early stages of follicular development [26]. This novel concept was the basis for the development of new OS protocols, in which ovarian stimulation starts not only at the early follicular phase but also during the middle, the late follicular, and in the luteal phase. Therefore, the random start and the double stimulation (dual stimulation or duostim or Shanghai protocol) protocols have been developed.

The random start protocol allows the initiation of OS at any time of the cycle, and its main indication is the fertility preservation with oocyte or embryo freezing for oncological patients. In these patients, the chemotherapy and/or radiotherapy are gonadotoxic and may result in infertility. However, the need for oncological treatment is urgent, and the "conventional" protocols are not suitable since they may be related to treatment delay. Indeed, the long GnRH-a protocol, requiring downregulation, may delay treatments up to 6 weeks. Also, with the "conventional" protocols that start on day 2 of the cycle, depending on the cycle day the patient is presented, the oocyte retrieval may take between 2 and 6 weeks. In the random start protocol, gonadotropin administration starts in any phase of the menstrual cycle, including the late follicular or luteal phase, and a GnRH-ant is given to prevent a premature LH surge, as used in the GnRH-ant protocols. In fertility preservation for oncological reasons, the GnRH-ant protocols also offer the possibility of final oocyte maturation with GnRH-a triggering instead of hCG, in cases of high ovarian response, reducing the risk of OHSS that, otherwise, would significantly delay oncological treatment. Many studies have shown that random start protocol has similar results regarding oocyte yield and maturity, allowing the patients to proceed with the cancer gonadotoxic treatment in 2–3 weeks after their presentation. Furthermore, in cases of estrogen-sensitive cancer, such as breast cancer, cotreatment with letrozole or tamoxifen simultaneously with OS is usually used to lower the E2 levels to physiological levels [6]. Since in the present book there is a chapter on fertility preservation, this issue will not be presented extensively here.

The double stimulation protocol involves two stimulations and two oocyte pick-ups within the same menstrual cycle. The first stimulation takes place in the follicular phase as usual, and after triggering (with hCG or GnRH-a), the first oocyte pick-up is performed, while the second stimulation occurs in the luteal phase of the same cycle, starting (immediately or 2–5 days) after the first oocyte pick-up, and after triggering (with hCG or GnRH-a), ultimately ending at the second oocyte pick-up [27]. Therefore, the first stimulation starts during the early follicular phase and the second begins the day after the first oocyte retrieval. This protocol has been suggested as a choice in poor responders or for emergency fertility preservation in oncological patients, aiming to maximize the number of oocytes retrieved in a single menstrual cycle. However, the freeze-all strategy is mandatory in this protocol. Also, in cases of urgent fertility preservation, the first stimulation may start randomly during the menstrual cycle (the so-called double random stimulation protocol) and the second stimulation can begin the day after the first oocyte pick-up [28].

So far, there are no randomized studies to compare the efficacy of the double stimulation protocol to two consecutive conventional protocols. Most studies are retrospective and observational, comparing the number of oocytes and embryological results of embryos produced in the follicular and luteal phase. Most of these studies showed that there were no significant differences in the number of metaphase II (MII) oocytes retrieved, fertilization, and euploid blastocyst rates between the follicular and the luteal phase stimulations. Therefore, the double stimulation protocol finally increases the number of euploids blastocyst that are available for transfer in only one menstrual cycle compared to the single follicular stimulation [29].

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CHAPTER

21

Oocyte retrieval

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Introduction

In the first decade after the pioneering attempts of in vitro fertilization (IVF) procedures in human reproduction, oocyte retrieval was one of the more complicated parts of IVF procedure. Women had to be under general anesthesia since the oocyte retrieval was done through laparoscopic procedure or even during laparotomy. After that, oocyte retrieval was done percutaneously and through the full urinary bladder using ultrasound guidance [1], which was also inconvenient, painful, and sometimes dangerous. After the invention of vaginal ultrasound probes, the procedure became simpler, and the transvaginal approach has been the gold standard for oocyte retrieval until now [2].

Transvaginal ultrasound-guided oocyte retrieval is a standard method for women undergoing IVF procedures. The method was described by Wikland et al. [3], and it is preferred to laparoscopic or transabdominal oocyte retrieval since it is less invasive. Nevertheless, cases of bleeding [4], infection [5], and injuries of the adjacent organs [6] have been described after this procedure. The advantages of the transvaginal approach are better visualization of the ovaries, shorter distance of ovary from the transducer, the use of local anesthesia for sedation instead of general anesthesia, decreased costs for patients, decreased risk of intestinal trauma, short learning curve, and quick postinterventional recovery. However, in some patients, transabdominal access is still preferred, especially when the ovaries were transposed or are enlarged above the pelvic brim. Transabdominal-guided oocyte retrieval continues to be used at some centers for rare patients who have ovaries inaccessible to transvaginal ultrasound-guided oocyte retrieval [7].

Setting and equipment

Oocyte retrieval is carried out in the operating theater or in semi-operating room with the equipment and drugs necessary for resuscitation and treatment of anaphylactic shock. The first necessary equipment is a gynecological operating table with adjustable leg holders. There should be an ultrasound machine with good resolution and utility for biopsy line, high-frequency vaginal ultrasound probe, and optionally, abdominal ultrasound probe. There should be a vacuum aspiration machine with the ability to adjust aspirating power between 50 and 200 mm Hg. It is useful to have an additional vacuum aspiration machine available nearby in case of malfunction of the original machine. Other necessary equipment include adjustable table on wheels for instruments, sterile instrument sets with sterile gauzes or tampons, disposable or reusable speculum, sponge holders, tenaculum forceps and needle guide to be attached to the vaginal probe, disposable sterile ultrasound probe covers, and sterile ultrasound gel. A test tube warmer for tubes with aspirated follicular fluid should be ready at 37°C. Translucent sterile test tubes are usually 15 mL volume. Single-lumen 17- or 18-gauge disposable needles are usually used for oocyte retrieval. At our center, we use 17-gauge (1.5 mm diameter) follicle aspiration needles of two different lengths: 320 and 240 mm. Double-lumen needles can also be used, allowing oocyte collection media to be infused into the follicle at the same time the follicular fluid is being aspirated. Optional equipment is a fully equipped anesthesia machine, when oocyte retrieval is done under sedation or general anesthesia. A vaginal surgery set with absorbable sutures should also be available nearby.

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Preparation of the patient for oocyte retrieval

Vaginal ultrasound should be done before inclusion of every patient to IVF procedure. The assessment of antral follicle count is important to decide the treatment protocol and gonadotrophin daily dose. Pelvic ultrasound examination is also important in cases of anatomical irregularities of certain patients. Congenital malformations, such as unicornuate or uterus didelphys can result in different position of the ovaries. Previous pelvic surgery, due to endometriosis, presence of uterine myomas, or other acquired uterine or ovarian pathology can cause adhesions and displacement of the ovaries. The accessibility of the ovaries and any potential complications or difficulties during previous oocyte retrieval should be clearly documented in the patient case notes, for the team to be prepared.

Screening for vaginal infection is done in some centers during the diagnostic evaluation before inclusion of the patient to IVF. Routine screening before every IVF procedure is not necessary; however, vaginal swab and causative treatment is necessary in symptomatic patients.

Taking full patient history is important to find out potential comorbidities and to take actions to prevent any possible associated complications. All patients should be asked about the use of medications, especially about the use of blood thinning agents (such as aspirin), relevant previous surgeries, and any relevant disease or deficit of coagulation factors. Aspirin should be discontinued at least 5–7 days prior to oocyte retrieval and low molecular weight heparins at least 12–36 h before oocyte retrieval. Verbal and written information should be provided to all patients, explaining the procedure, the risks and their incidence. Written informed consent for treatment should also be obtained from all patients.

Controlled ovarian stimulation is achieved by conventional protocols and followed by serial ultrasound examinations as it has been described in previous chapters. It is important, that the oocyte retrieval is precisely timed after the application of medication for triggering oocyte maturation. To ensure optimal yield of mature (MII) oocytes, oocyte retrieval should be carried out 36–38 h after triggering injection [8].

On the day of triggering injection, the couple receives the information about ejaculation abstinence prior to providing the semen sample for IVF/intracytoplasmic sperm injection (ICSI). Ideally, there should be 1 day of abstinence. All couples for IVF/ICSI cycles with fresh semen sample are provided with sterile cup for semen sample. At our center, the semen sample is usually collected at home, since it is more convenient for the couple and it does not affect the outcome of a fresh IVF/ICSI cycle [9].

On the day of oocyte retrieval

The team for oocyte retrieval should ideally consist of one operator performing the oocyte retrieval and two nurses or assistants. Although, the absolute minimum number of team members is two: one operator and one nurse. At least one member of the team should be trained in advanced life support. In cases when oocyte retrieval is performed under sedation or general anesthesia, the anesthesia specialist and anesthesia nurse should also be present.

Patients are asked to take proper care about intimate hygiene before the oocyte retrieval. They are also asked to remove jewelry and/or piercings. When they come to the center, they must all present valid personal ID with photograph. The male partner disposes the semen sample with personally signed document to the laboratory. The exact time of triggering injection should be checked again to ensure that the timing of oocyte retrieval is accurate (ideally 36–38 h after the application of triggering injection). At our center the female partner is given an oral tranquilizer (alprazolam or similar) and painkillers (naproxen, tramadol, paracetamol, or combination of them) approximately 60–30 min before the procedure. In the IVF operating room, she must again present valid personal ID with photograph. Personal identification is done by the nurse.

Technique

During the procedure, the patient is positioned in lithotomy position at the edge of the gynecologic table with legs adducted and supported. The operator can stand or sit on a chair between the patient's legs. The sterile gloves for the operator and nurses should be without talcum, as it can be toxic for gametes and embryos.

In cases of endometriosis with ovarian endometrioma, history of pelvic inflammatory disease, congenital or acquired immune deficiency, or other risk factors for infection, it is advisable, that broad spectrum antibiotic prophylaxis (e.g., 2 g of cefazolin i.v.) is used before or during the oocyte retrieval.

The vulva is washed with warmed normal saline or sterile water. Sterile cloths or compresses are put under the patient and on her legs to ensure a proper sterile surgical field. In some centers, the vagina is also washed with warmed saline to prevent possible infection spread from the vagina during oocyte retrieval [10]. At our center, the vagina is washed only in symptomatic patients, who are also prophylactically treated with antibiotics during oocyte retrieval. A gynecological speculum is inserted, the vagina and cervix are visualized for

anatomic irregularities, and the posterior vaginal fornix is infiltrated with 10 mL of 1% lidocaine (Xylocaine). Alternatively, 20 mL of 1% lidocaine (10 mL for the right vaginal fornix and 10 for the left) can also be used in cases where more than 15 follicles are expected to be aspirated. After few minutes, allowing the local anesthesia to work, follicular aspiration can begin. A vaginal probe, covered with a sterile cover, containing sterile ultrasound gel and with needle guide attached is then inserted into the vagina. Immediately before the insertion of the needle into the guide, needle patency and aspiration ability should be tested by aspiration of IVF media, warmed to 37°C, into the test tube. The proper attachment of the tubing system to the vacuum aspiration machine and to the aspiration needle should also always be checked before the oocyte retrieval.

For right-handed operators, the vaginal ultrasound probe is held with the left hand and the needle with the right hand. The vaginal ultrasound probe is gently introduced into the vagina and then held firmly to the vaginal wall, so the ovary is positioned next to the vaginal wall. The needle should be inserted to the guide, after the ovary has been positioned centrally above the vaginal ultrasound probe. Extreme caution should be used to avoid insertion of the needle through the bowel loop or through the urinary bladder. The patient should empty her bladder completely before the oocyte retrieval. If the bladder is still full, a single-use urinary catheter should be used to empty the bladder. The needle is carefully pushed into the follicle and the aspiration begins. The pedal for the vacuum aspiration machine can be controlled by the operator or by assistant nurse. We use a constant aspiration power of 180 mm Hg during oocyte retrieval at our center. Ideally, the aspiration of multiple follicles is done with one needle puncture. Avoid multiple penetrations of the ovarian cortex to reduce the chance for abdominal bleeding. The tip of the needle should be visualized throughout the procedure. Move the needle to the next follicle, when the follicular walls collapse, to ensure all follicular fluid is emptied into the test tube. Curetting of the follicle with clockwise and counterclockwise rotation of the needle is useful to ensure that the oocyte has been aspirated. When the needle path has to be adjusted, the needle is retracted from the ovary, to avoid laceration of the ovary and subsequent bleeding. Also, the needle has to be retracted when we move to the other ovary. It is advisable that the needle is flushed between the two ovaries to prevent blockage caused by potential blood clots. Transabdominal pressure on the side of the oocyte retrieval can be applied by the patient or assistant to stabilize the ovary during follicular aspiration. At the end of aspiration, the needle is flushed with IVF medium to ensure that no oocytes remain in the tubing system. The vagina is cleaned with a small tampon to remove any residual blood. If there is active bleeding from the vaginal wall, the speculum is inserted and the bleeding site should be visualized. Pressure with a big tampon for approximately 2 min usually stops the bleeding. Vaginal packing with gauze for 1 h is also another option. If there is still active bleeding after these interventions, a hemostatic suture should be placed at the bleeding site.

Anesthesia during oocyte retrieval

The transvaginal oocyte retrieval can be done under local anesthesia. The patient receives oral tranquilizer and painkillers before the procedure. Then local anesthetic is used to infiltrate the vaginal walls. We usually use 10 mL of 1% lidocaine. Local anesthetic is infiltrated on the vaginal side walls, most commonly at 4 and 8 o'clock, approximately 1 cm away from cervix. At our center, we perform approximately 1300 fresh cycles of oocyte retrieval yearly. In 2016, we prepared questionnaires regarding pain during oocyte retrieval for our patients. We included 166 consecutive patients. A total of 76.5% of patients, who had oocyte retrieval under local anesthesia, reported that the procedure was not painful, or that it was even less painful than they had expected. A total of 80.7% of patients would chose local anesthesia again, if another procedure would be needed. A total of 13.3% of patients would rather have chosen intravenous analgesia and sedation, and 6% of patients would like to be under general anesthesia.

We try to respect patients' preferences for pain management during oocyte retrieval, so the doctor at the ultrasound office counsels every patient individually, on the day when the time for triggering injection is being scheduled. If there are less than 10 follicles in both ovaries to retrieve, we offer them local anesthesia. If there are more than 20 follicles, we suggest the oocyte retrieval to be done under intravenous analgesia and sedation. Oocyte retrieval under intravenous analgesia and sedation should also be offered to the patients after operative treatment of severe, infiltrating endometriosis or similar bigger operative procedures. For some special patients, such as oncological patients or pediatric patients for fertility preservation, we suggest general anesthesia.

Verbal anesthesia is also very important, when oocyte retrieval is done under local anesthesia or under intravenous analgesia and sedation (conscious sedation). This means that verbal distraction is used to comfort patients, provide a friendly atmosphere, and therefore reduce pain, anxiety, and stress. It is very important that the procedure is explained preoperatively to the patient. Verbal anesthesia begins with calm conversation during the patients' invitation to the operating room. The operating room must be a calm environment, preferably with dimmed light, comfortable temperatures, cheerful images on the walls, and comforting music played in background.

When oocyte retrieval is done under intravenous conscious sedation or under general anesthesia, the anesthesia team, consisting of the anesthesia doctor and anesthesia nurse, must be present at the procedure. Pulse oximetry and blood pressure monitoring must be used when intravenous drugs are used. Conscious sedation is the preferred option for oocyte retrieval since the recovery time is shorter, the patient requires less medication, and the procedure is cheaper. Recommendations for personal safety and equipment necessary to optimize patient safety for the administration of intravenous sedation in IVF have been published recently [11]. All patients scheduled for oocyte retrieval under intravenous sedation or general anesthesia are asked to fast for at least 6 h from food and at least 2 h from fluid. An intravenous line should be inserted prior to the procedure. Systemic analgesic, sedation, and anesthetics therapy is decided by the analgesia team. At our center, we generally use local anesthesia along with intravenous sedation or general anesthesia since it has been shown that postprocedure pain is reduced in this way [12]. Speculum examination and infiltration of vaginal walls with local anesthetic is avoided in pediatric population and virgins.

Oocyte recovery

During oocyte aspiration, tubes are held in a test tube warmer or heat block, maintaining the temperature at body temperature, approximately 37°C. At the end of aspiration, the heat block with test tubes is transferred to the laboratory. If the aspirated fluid seems clear and yellowish, the test tubes should be transferred to the laboratory immediately. Laboratory staff should inform the operator if no granulosa cells or oocytes are present in the first examined test tubes. The correct application of triggering injection should be checked again if no cells are present in the aspirated fluid. In cases of hCG trigger, urine, serum, or follicular fluid pregnancy test should be performed. If pregnancy test is negative, the patient had not injected the trigger. In cases of gonadotrophin-releasing hormone agonist trigger, serum or urine luteinizing hormone (LH) peak should be checked. If serum LH is not elevated or urine LH test is negative, the patient has not had the trigger. The triggering injection should be applied on that day and the oocyte retrieval performed again after 36 h. If the triggering injection had been injected and the time interval was too short, the oocyte retrieval should be delayed. If premature ovulation is suspected due to abdominal fluid or corpora lutea seen on ultrasound, peritoneal fluid from the pouch of Douglas could be aspirated to recover oocytes.

After the oocyte retrieval

Patients should remain in bed resting and under supervision at the center for approximately 30–60 min if the retrieval has been done under local anesthesia. If intravenous drugs have been used, the bed rest must be prolonged to 2–3 h and they should be monitored (pulse oximetry and blood pressure). After sufficient bed rest, patients are asked to urinate and check the pad for bleeding. If urine is clear and no larger bleeding is seen on the pad, she can be discharged. Avoiding physical activity and sexual intercourse is advised for 2–5 days.

Complications

Centers performing IVF are obliged to report their results to the ESHRE IVF monitoring (EIM) registry. According to a recent analysis, complications during oocyte retrieval were reported in 0.17% of cycles [13]. The most common complications are bleeding (0.11% of cycles) and infection (0.013%); other complications are rare [13]. If the oocyte retrieval is done under intravenous sedation or general anesthesia, some complications can be related to medications used to achieve analgesia, sedation, and anesthesia.

The most common complication of oocyte retrieval, vaginal bleeding, can usually be stopped with compression or vaginal tamponade; rarely, hemostatic suture is needed. Intraabdominal bleeding is a rare, but more serious complication. The patient with intraabdominal bleeding complains about abdominal pain, sometimes with tachycardia and low blood pressure. In cases of severe bleeding with hypovolemia, laparoscopy with lavage and electrocoagulation of bleeding sites at the ovaries or suturing of the bleeding site in the pelvis is necessary to stop the bleeding. Infection or peritonitis is more common in patients with endometriosis, previous pelvic inflammatory disease, dermoid cyst of the ovary, or immune deficiency. Prophylactic antibiotic therapy is mostly a successful measure to avoid infection after oocyte retrieval. Caution should be applied at all times to avoid unintentional puncture of a bowel loop since this can result in serious peritonitis. In cases of infection after oocyte retrieval, antibiotic therapy is needed. In cases of infection, it is also advisable that the embryos are frozen and transferred after a few months.

Other complications are rare and most reports on serious complications after oocyte retrieval have been published in case reports. Reported complications include urinary tract injury, and a case report about ureteral injury following oocyte retrieval from our center has also been published [14].

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CHAPTER

22

Oocyte quality evaluation and cryopreservation

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Human oocyte quality evaluation

General aspects

The human oocyte has a diameter of approximately 150 μm and is surrounded by a membrane called the oolemma. It is surrounded by a glycoprotein envelope called the zona pellucida. The zona pellucida has a thickness of 15–20 um, and its main function is to protect the oocyte during transport, fertilization, and cellular development until the blastocyst stage. The area between the plasma membrane of the oocyte (oolemma) and the zona pellucida is called the perivitelline space. A "good quality" MII oocyte can be defined as the one with clear (or moderately granular) cytoplasm, small perivitelline space, with a clear and homogeneously layered zona pellucida.

When an oocyte is fully capable of fertilization, the secondary oocytes will temporarily pause their maturation stage at metaphase II of meiosis. Nuclear maturation accompanies full cytoplasmic development, characterized by an increase in the number of scattered organelles in the whole oocyte cytoplasm. At this stage, the visualization of the first polar body is generally accepted as the sign of nuclear maturation. In addition to the requirements for nuclear maturation, after the extrusion of the first polar body, it takes a short period for the oocytes to obtain total cytoplasmic capacity. Thus, both nuclear and cytoplasmic maturity is necessary for the oocyte to show its actual reproductive capacity. It is now well known that oocytes with asynchronous cytoplasmic maturation usually result in fertilization and early cleavage abnormalities later in the course of development.

During the in vitro fertilization process, oocytes are collected from the ovary and evaluated under the microscope. Evaluation of oocyte quality by morphologic assessment is one of the main issues in assisted reproductive technologies (ART) treatment cycles since multifolicular development induced by controlled ovarian stimulation regimens usually creates numerous oocytes with different characteristics. At the time of follicular aspiration of in vitro fertilization treatment, in picked-up oocytes, a cell line encircles the oocyte and is called the cumulus oophorus. Collected oocytes are surrounded by multiple layers of "cumulus oophorus" cells and are therefore termed "cumulus-oocyte complexes" (COCs). Cells located in the cumulus are functional throughout gap junctions, providing nourishment to the oocyte during development and probably transferring regulatory factors required to continue the meiosis [1]. Structural and morphologic visualization of COCs during oocyte collection are hence considered to be the early signs of the developmental competence of an oocyte. The corona or coronal layer is the innermost layer of the oocyte. This layer shows structural changes when oocytes mature either with exogenous hCG or a midcycle luteinizing hormone (LH) surge. The coronal layer unfolds and presents a radial shape. Close to the time of ovulation, as a result of the expansion of the coronal layer, cumulus cells get far from the zona pellucida, and probably cellular-oocyte communication diminishes. Oocytes with limited proliferative cellular change near ovulation showed decreased implantation potential, although fertilization and development were regular in vitro [2].

Studies have shown that a variety of cytoplasmic and extracytoplasmic abnormalities, called "oocyte dysmorphisms" exist in human oocytes. They were first described in 1992 by Van Blerkom and Henry [3]. Since then, many groups have investigated the origins, genetic mechanisms, and/or physiologic pathways that lead to the production of such dysmorphisms. It is now widely

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accepted that a high percentage of human oocytes in fact carry at least one form of dysmorphism and do not form implantation-competent embryos both in vivo and in vitro. Identification of certain oocyte characteristics to evaluate their "quality" or "developmental competence" that can be used to predict laboratory and clinical outcome is therefore, undoubtedly, very valuable. Historically, oocyte quality has been evaluated by observing its morphologic properties under different microscopic approaches. This approach is still the major approach in in vitro fertilization (IVF) clinics worldwide. On the other hand, numerous researchers have also investigated the presence of "molecular" markers that can be used in conjunction with oocyte morphology. Nowadays, as we experience digital transformation in many fields of modern medicine, a branch of reproductive research is now focusing on developing novel approaches that are based on "artificial intelligence" and machine learning algorithms to objectively evaluate and determine the oocyte quality.

Technologic advances and modern medicine are now reshaping fertility preservation approaches as well. Introduction of vitrification technique and increased public awareness have created considerable improvements in oocyte cryopreservation programs and have made this option widely offered and used worldwide. In this sense, oocyte quality evaluation has become extremely important and valuable to predict the feasibility as well as the possible outcome of oocyte cryopreservation for future fertility preservation. In this work, we aimed at summarizing the current state of oocyte quality evaluation and its impact on cryopreservation by current literature involving standard morphologic assessment under light microscopy, polarized microscopy, follicular fluid dynamics, studies utilizing molecular genetics approaches to find molecular markers, as well as artificial intelligence-based approaches.

Oocyte quality assessment by morphology under light microscopy

Evaluation of maturity

The reason for assessing the quality of an oocyte is that it is directly associated with fertilization capacity and fetal development. Therefore, several factors are considered in oocyte quality assessment, and in the following part, we will summarize these factors.

In the fetal life and prepubertal period, oocytes stop their development at the diplotene stage of the first meiotic prophase. This stage is also called the germinal vesicle (GV) stage. After LH surge, oocytes continue meiotic maturation and then have a second arrest at meiosis following out of the first polar body.

On the other hand, cytoplasmic maturity is evaluated with the expansion and radiance of the cumulus-corona complex [4,5]. The oocyte is categorized as mature when there is an expanded and luteinized cumulus matrix and a radiant or sunburst corona radiata. A less expanded cumulus-corona complex means an intermediate stage of maturity, and when there is no expanded cumulus, the oocyte is probably immature. The nuclear maturity of the oocyte and cellular maturation of the cumulus are different entities. As a result of this discrepancy, researchers developed a maturation score system. The size of the follicle, expansion of the cumulus mass, the radiance of the corona cells, size/cohesiveness of associated granulosa cells, and shape/color of the oocyte are considered. On the other hand, forthright perception of the oocyte and its GV or first polar body can be endeavored by fanning out the cumulus mass or eliminating it with the guide of compounds.

If apparent or stripped of cells, oocytes are categorized according to the status of first polar bodies and GVs:

metaphase II (MII): first polar body present, no GV
metaphase I (MI): no first polar body, no GV
prophase I (PI): GV present

Morphologic parameters of oocyte quality

Morphologic evaluation of oocyte quality is based on the situation of COCs, polar bodies (PBs), and spindles. However, several scoring systems have been developed; morphologic characteristics as predictors of oocyte quality are controversial, but most in vitro fertilization laboratories use these parameters to choose high-quality oocytes.

Cumulus-oocyte complex morphology

On the evaluation of COCs, ooplasm characteristics and cumulus compactness are evaluated. However, ideal quality criteria for ooplasm characteristics are not described because various species have various levels of cytoplasmic transparency [6].

Cumulus compactness is evaluated with the number of cumulus cell layers [3,7], but this is not easy because surrounding cumulus and corona cells harden accurate assessment of both the maturity and morphology. As a result, COCs morphology is not used alone for the evaluation of oocyte quality.

Cytoplasm and polar body morphology

Human oocyte cytoplasmic morphology is typically arranged by the presence or absence of granularity, coloration, inclusion, and regions of organelle clustering [8–10]. Still, the actual effect of these features on pregnancy rates is controversial.

First, PB morphology can show the postovulatory age of the oocyte [11]. Second, the shape, size, surface, and integrity of PBs are all evaluated in the prediction of oocyte quality [12–14]. Also, perivitelline space and the zona pellucida can be assessed, but the support of these structures on oocyte quality evaluation is unclear [15,16].

Meiotic spindle evaluation

The meiotic spindle is pivotal for exact chromosomal arrangement and segregation during meiosis. Several studies showed the effect of spindle characteristics on aneuploidy, balanced oocyte maturation, and quality of the preimplantation embryo [17–19]. After these studies, several attributes of the spindle are used to evaluate the oocyte's quality.

Between 60% and 70% of the oocytes obtained from the same cohort of oocyte retrieval can carry at least one morphologic variation [20]. Current literature indicates that such morphologic variations among human oocytes can be the outcome of certain intrinsic (e.g., age, metabolism) or extrinsic factors (e.g., stimulation protocols, culture conditions, diet). Early studies reported that there exists an association between certain oocyte morphologic features (such as COCs, the polar body, the zona pellucida, the perivitelline space, and ooplasm) and fertilization outcome, zygote formation, embryonic development, and implantation potential [21–25]. In one study, extracytoplasmic dysmorphisms were on the other hand accepted as phenotypic variations [26]. Although for many years there have been attempts to establish an oocyte grading system that can be used in an ordinary IVF laboratory setting, lack of a wider acceptance and yet subjective grading in different laboratory settings have so far resulted in conflicting outcomes for oocyte evaluation by morphology [27,28]. Results in oocytes with more than one dysmorphisms are also found to be contradictory. While Balaban and colleagues found no association between multiple dysmorphisms and embryo quality, others have reported that there exists a significant impairment on developmental potential of the resulting embryo [9,29,30].

Oocyte quality assessment by polarized light microscopy

Studies indicate that timely and optimal function of meiotic spindles are also vital for production of oocytes with high embryo development and implantation potential [31,32]. From this perspective, studies have recently documented possible association between the spindle characteristics (presence/absence, shape, size, and position) and ART outcomes by using polarized light microscopy [28,33]. Although several indicated that there exists a significant and positive correlation between spindle visualization, fertilization rates, and embryo quality [34–38], in others, no correlation in the implantation and pregnancy rates was observed [30,39]. From the published literature, one can conclude that analyzing spindle visualization in oocytes can in fact help to determine oocytes with high fertilization and implantation capacities. However, it should also be noted that visualization of spindles decreases with age, and the potential benefits of using polarized light microscopy on oocyte quality evaluation to improve laboratory and clinical outcome can be hindered due to varying levels of operator experience as well as variable technical instrumentations [40].

Oocyte quality assessment by follicular fluid dynamics

Follicular fluid (FF) carries important messages about oocytes inside it, and FF characteristics are evaluated by research groups to identify possible predictors of oocyte quality. The results of these studies are reviewed in the following part.

Follicular fluid hormones

Besides effects on follicular growth, gonadotropins also control the secretion of some substances by follicular cells, and these substances affect oocyte maturation and development. Oocytes with a high chance of fertilization had high FF levels of FSH, hCG, and LH [41–43].

Many studies evaluated estrogen, progesterone (P), and androgen levels in FF, but the results were conflicting. For example, in some studies, high FF levels were associated with a more advanced maturation stage and a higher chance of pregnancy [44–48]. Still, on the other hand, the same effect is not reproducible in other studies [49,50].

The effect of FF P levels on oocyte quality seems dose dependent [51], but the optimal threshold for follicular P level is not defined. Nearly similar controversies exist for the FF androgen levels. Some androgen is essential for oocyte competence, but the ideal amount is a question [52,53].

Growth factors of the transforming growth factor-beta (TGF-beta) superfamily in follicular fluid

Serum inhibin B and anti-Müllerian hormone (AMH) levels are used in ovarian reserve testing. Scientists also evaluated FF levels of inhibin B and AMH in the prediction of oocyte quality. Unfortunately, all the scientists did not find the same result. In some studies, inhibin B was a good marker for oocyte quality [54,55], but this result was not supported in other studies [56,57].

Conflicting results such as those for inhibin B exist in the reports about AMH values and oocyte quality [58,59].

Insulin-like growth factors in follicular fluid

Insulin-like growth factors I and II (IGF-I and −II) are influential in cell proliferation and differentiation. They show their effects through IGF-binding proteins (IGFBP-1 and-6). In several studies, IGF-I, IGF-II, IGFBP-1, IGFBP-3, and IGFBP-4 were all found positively correlated with oocyte quality [60−62]. However, Asimakopoulos et al. did not observe the exact correlation; further studies are needed to define the effect of IGFs and IGFBPS on oocyte quality [63].

Reactive oxygen species in follicular fluid

Oxidative stress can cause damage in the DNA of the oocyte, and after injury, apoptosis starts. Hypoxia damages both the oocyte and the embryo [64]. Reactive oxygen species (ROS) levels were higher in patients who became pregnant after IVF [65], but supraphysiologic levels cause defects in embryo development [66]. Therefore, researchers also evaluated antioxidant levels in the FF. Two endogenous antioxidant, superoxide dismutase (SOD) and selenium-dependent glutathione peroxidase (SeGPx), levels were studied. High FF SOD concentrations were associated with low fertilization rates [67], whereas high SeGPx levels were protective against fertilization failure [68].

Metabolomics of follicular fluid

In general, scientists study the effect of a limited number of proteins, hormones, or other substances on FF. The metabolomic analysis examines a detailed analysis of all the metabolites in the FF. As a result, metabolic research shows the actual functional status of the FF complex. Laboratories can use several methods in metabolomics analysis, but the preferred method is mass spectrometry techniques either alone or in combination with chromatography or electrophoresis. A group of scientists evaluated fatty acids, sugars, or amino acid levels in FF of animal models [69−71]. The antral follicle's metabolic profile was more stable than smaller follicles, reflecting the relationship between the biochemical status and oocyte maturity [72].

Perhaps soon metabolomic analysis will take the place of the conventional morphologic assessment, but nowadays, we need more studies to define precisely metabolomic quality predictors.

Oocyte quality assessment by molecular approaches

By employing either as a single selection tool or in combination with the data from oocyte morphologic evaluation under microscope, recent studies have also investigated several oocyte-related gene expressions, proteomic or metabolomic markers for their prospective potential in oocyte quality assessment, as recently reviewed by Fischer et al. [73]. Many of these studies could show that their markers of interest have the potential to predict laboratory performance; however only a few demonstrated the potential of their analyzed biomarkers for predicting live birth [74,75]. Finding and employing potential genomics- or proteomics-based markers in oocyte quality evaluation could be expected to minimize user subjectivity and help the scientists optimize the clinical outcomes in the near future.

Oocyte quality assessment by AI

Like other branches of modern medicine, the potential of artificial intelligence-based gamete and embryo selection algorithms has recently started to be investigated by numerous studies [76]. Such an approach would be expected to abolish the main criticism regarding operator-based subjectivity and provide improved validity of the oocyte selection process [77]. Preliminary results indicate that AI-based algorithms and machine learning approaches show considerable promise and fill the current gap created by subjective oocyte quality assessment methodologies, as well as contradictory results, and even perform superior to experienced embryologists [78,79]. On the other hand, the main challenge involving these novel approaches is the absolute need for an efficient digital transformation of the data to be investigated.

Metaphase II (MII) oocyte dysmorphisms

Definition of the high-quality metaphase II oocyte is described as sheer, slightly granular, homogenous, and translucent cytoplasm without inclusions, small perivitelline space (PVS), clear, colorless, and regular zona pellucida, perfect spherical shape, and an intact first polar body (PBI) [15,80−82]. It is not always possible to obtain an ideal oocyte at the time of oocyte pick-up. Oocytes generally show morphologic abnormalities. These abnormalities are classified into two categories: intracytoplasmic and extracytoplasmic abnormalities.

Cytoplasmic abnormalities

The first studies evaluating the effect of cytoplasmic abnormalities on the clinical outcome were made nearly 30 years ago. According to their results, oocytes with severe cytoplasmic abnormalities like dark cytoplasm, dark incorporations, spots, refractile bodies, single or

multiple vacuolization, and granulation in the cytoplasm affected fertilization and embryo quality [15,80,81].

Cytoplasmic maturity has a vital function in the fertilization process. Therefore, defects in this step negatively affect oocyte quality even in the presence of euploid genetic material [10,83]. In the literature, a significant number of cytoplasmic defect types were defined. However, some are the severe defects are certain types of fluid-filled vacuoles, organelle clustering or centrally located granulation, and the appearance of smooth endoplasmic reticulum clusters. Besides these abnormalities, differences from normal cytoplasmic appearance are accepted as normal oocytes with a phenotypically heterogeneous cytoplasm [12].

Extracytoplasmic abnormalities

Extracytoplasmic abnormalities can be observed on cumulus cells, zona pellucida, and perivitelline space. Several researchers evaluated the relationship between extracytoplasmic abnormalities and the clinical outcome of IVF treatment. According to their results, no significant association was observed between these parameters [84,85]. Instead, cytoplasmic abnormalities seem more effective on embryo development [15].

Factors affecting oocyte quality

Several factors can affect oocyte quality, as seen in Table 22.1. The most commonly encountered factors affecting oocyte quality negatively are endometriosis, aging, and polycystic ovary syndrome (PCOS).

Endometriosis

Endometriosis can cause infertility with several mechanisms, and one of the causes for infertility in endometriosis patients seems like poor oocyte quality.

TABLE 22.1 Factors affecting oocyte quality.

Endometriosis
Age
Polycystic ovary syndrome
Obesity
Follicular fluid environment
Reactive oxygen species (ROS)
Oocyte secreted factors
Ovarian stimulation factors

Therefore, researchers have evaluated the effect of endometriosis on IVF outcomes with oocyte donation cycles, and oocytes obtained from donors with endometriosis had lower pregnancy rates when compared with oocytes from donors without endometriosis [86,87].

Endometriosis causes damage to the ovaries with the release of inflammatory cytokines and increases in oxidative stress levels and ROS, and it causes vascular dysfunction and fibrosis in ovarian stroma. In addition, insufficient antioxidant capacity and chronic inflammatory state are responsible for DNA damage and chromosomal instability. Besides the effect on DNA, endometriosis affects all the oocyte components, either cytoplasmic or extracytoplasmic, negatively [88–91].

Age

Aging causes a decline in a woman's reproductive potential with two mechanisms: loss of ovarian follicles continuously and a decrease in oocyte quality [92]. Several mechanisms are proposed for the age-related decline in oocyte quality, but researches continue.

When we look deeper, the most known adverse effect of aging on oocyte quality is impairment in genetic stability [93]. Other less known but significant mechanisms for decreased oocyte quality in patients with advanced maternal age are mitochondrial dysfunction, shortening of the telomeres, cohesin dysfunctions, and spindle instability [94].

Polycystic ovary syndrome

PCOS is characterized by oligoanovulatory ovarian dysfunction, polycystic ovarian morphology, and/or biochemical or clinical hyperandrogenism. Two out of three features are required for PCOS diagnosis. Although, as a result of this definition, not all patients carry full disease features. Four different PCOS phenotypes are defined. In PCOS patients, need for assisted reproduction is higher than for healthy controls [95]. PCOS patients produce more oocytes after exogenous gonadotropin administration, but treatment outcomes are not superior or even worse [96]. One of the reasons for a worse outcome in PCOS is probably the effect of PCOS on oocyte quality. Meta-analyses were performed to search for the impact of PCOS on oocyte quality. In PCOS, the hormonal milieu is somewhat different, resulting in inappropriate development of dominant follicle and ovulation with an abnormal ovarian microenvironment. Another critical problem in PCOS patients is oxidative stress [97], a known disruptor for ovarian development. In addition to the mentioned factors, researchers identified other abnormalities in the extra- and/or intra-ovarian factors that may affect the

interaction between granulosa cells and oocyte, oocyte competence, and embryonic factors [98,99].

Oocyte cryopreservation

Cryopreservation is suggested to preserve the cells and tissues at subzero temperatures, stopping all biologic activity, to use in the future. Cryopreservation is one of the main milestones along the developmental pathway of IVF practice. The first human births from frozen sperm and frozen embryo were reported in 1953 and 1984, respectively [100–102]. Although sperm and embryo cryopreservation has been performed for a long time, oocyte cryopreservation has largely been highlighted in the past few years. Cryopreservation of sperm, embryo, and oocyte definitely makes fertility preservation a reality for women at high risk for infertility. Oocyte cryopreservation (OC) has recently become a clinically established technology for fertility preservation options to protect and preserve reproductive potential for women.

However, it is more difficult to cryopreserve oocytes because oocytes have more susceptibility to cryodamage because of their structural complexity when compared with sperm or embryo. Studies recently have paid attention to developing a reliable way with modification in cryopreservation protocols and evaluating success rate of human oocytes cryopreservation. While the use of frozen oocytes as an alternative method for infertility treatment was allowed by The Human Fertilization and Embryology Authority in the United Kingdom in 2000, the American Society for Reproductive Medicine suggested that IVF with vitrified/warmed oocytes could produce similar fertilization and pregnancy rates when compared to IVF with fresh oocytes after publishing the results of four randomized controlled trials in 2013 [103–107].

The current technologies for oocyte cryopreservation

There are still two basic protocols for human oocytes cryopreservation: slow-freezing and rapid-cooling vitrification. Several parameters including oocyte survival, fertilization rates, and pregnancy rates can predict the success rate of these procedures. The success rate of human oocytes cryopreservation is historically increased with revolutionizing protocols and technologic developments. The cryodamage of oocytes generally results from higher intracellular ice formation and/or uncontrolled dehydration during the freezing or thawing process [108,109]. These issues may also be dependent on aging oocytes, cryopreservation technique, and duration of storage. Several revolutions of cryopreservation protocols reduced cryodamage by prevention and/or minimizing of ice crystal formation in the past few years. Vitrification seems to be superior to slow-freezing in terms of reducing ice crystallization and to be noninferior to fresh oocytes in terms of good results [110]. Vitrification markedly contributes to improving the success rate of human oocytes cryopreservation.

It remains a challenge which technologies will be best to establish efficient, safe, and successful cryopreservation of human oocytes despite improved protocols, but vitrification is currently recommended as the best approach for human oocytes cryopreservation. In 2013, the National Institute for Health and Care Excellence updated guidelines stated, "In cryopreservation of oocytes and embryos, use vitrification instead of controlled-rate freezing if the necessary equipment and expertise is available" [111]. There are two basic methods of vitrification: open and closed vitrification. There is yet no consensus which vitrification protocol is optimal [112,113]. Potential infectious transmission in reproductive tissues may be considered a challenge for open vitrification protocol.

The recommendations for clinical applications of OC as fertility preservation

Who may be appropriate for OC is determined by fertility preservation counseling (Table 22.2) [114,115]:

- Elective cryopreservation for age-related fertility loss to defer childbearing with age-specific information and counseling: OC for age-related fertility loss especially contributes to protecting fertility against the natural biologic clock of women in current modern society. Appropriate counseling may raise the possibility of fertility preservation. However, the success rate to achieve a pregnancy after OC for age-related fertility loss definitely depends on the number

TABLE 22.2 The recommendations for clinical applications of oocyte cryopreservation.

Elective cryopreservation for age-related fertility loss
Patients with cancer who undergo gonadotoxic treatments
Patients with other medical diseases who undergo gonadotoxic treatments
Transgender men or lesbian women
Women who undergo IVF that are unable to cryopreserve embryos
Oocyte donation process
Patients who undergo oophorectomy because of benign or malign diseases
Women diagnosed with premature ovarian failure

of thawed MII oocytes and the woman's age when oocyte retrieval is performed. The cumulative live birth rate could be high when oocyte retrieval is performed before 35 years old and ≥20 thawed MII oocytes [116]. A retrospective observational multicenter study, including 1468 elective OC patients for nononcologic reasons, 137 of whom returned to use their vitrified oocytes, indicated that pregnancy rates were associated with age at oocyte retrieval time, and their suggestion for optimal number of stored MII oocytes for these cycles was at least 8–10 [117]. OC could ideally be done at a relatively early age (prior to the age of 35), but if it will be done at >38 years old, the increased risk of aneuploidy associated with advanced age should be counseled. The age limit at around 50 would seem to be reasonable for the stored reserve because of the risks of aneuploidies associated with aging oocytes and adverse perinatal outcomes related to advanced maternal age [118].

- Patients with cancer or other medical diseases undergo gonadotoxic treatments: The prevalence of cancer in reproductive-aged women recently increased, and the numbers of survivors also increases with improvement treatment protocols. OC allows them to preserve their reproductive potential previous to gonadotoxic treatments because gonadotoxicity as a late side effect of cancer treatment becomes definitely important. At the time of a cancer diagnosis before gonadotoxic treatments, patients should be informed of the negative impact of gonadotoxic treatments on fertility, fertility preservation, and their future fertility. Women with autoimmune disease and women diagnosed with premature ovarian failure are also candidates for OC.
- Patients undergoing oophorectomy because of benign or malign disease (such as women diagnosed with gynecologic malignancy, or women undergoing prophylactic salpingo-oophorectomy because of BRCA mutations)

The clinical outcomes of oocyte cryopreservation

There are many intrinsic and extrinsic determinates of OC outcomes. Intrinsic factors are associated with biologic and developmental features of the oocyte such as cumulus oophorus cell, oocyte size and stage, subcellular organelles, and zona pellucida, while extrinsic factors are related to the cryopreservation process. New technologies could protect sperm, embryo, and oocyte by minimizing cellular damage related to cryopreservation and thawing.

There are four randomized controlled trials that compare the outcomes of IVF cycles with cryopreserved and fresh oocytes in the literature. These studies suggest that the oocyte survival rate, fertilization rates, and implantation rates of IVF/ICSI with vitrified/warmed oocytes ranged between 90% and 97%, 71% and 79%, and 17% and 41%, respectively. However, according to their results, clinical pregnancy rates per embryo transfer presented between 36% and 61%. Their results also demonstrated that the outcomes of IVF/ICSI with vitrified/warmed oocytes in terms of fertilization and pregnancy rates are similar to those of IVF/ICSI with fresh oocytes [104–107]. Recent studies suggested that vitrification is superior to slow freeze protocol, and the use of vitrification makes the results including oocyte survival, fertilization, and pregnancy rates better. Therefore, there is a trend toward the use of vitrification. A recent meta-analysis of five studies from the United States evaluated the outcomes of IVF/ICSI with fresh, slow-freezing, and vitrified oocytes. It showed that vitrification is superior to slow-freezing in terms of oocyte survival rate, fertilization rate, top-quality embryo rate, and embryo cleavage rate, while there was no difference between vitrified and fresh oocyte for all parameters [119]. But it should be taken into consideration that the majority of studies included a highly selected population consisting of healthy and young (<30) oocyte donors with shorter vitrification duration that were performed in experienced centers for vitrified/warmed. Therefore, these results may not be generalized for other clinics with different populations, such as older women, by the use of different cryopreservation protocols. The success rates of IVF/ICSI with frozen oocytes should be considered clinic specific. Otherwise, the large multicenter observational studies coming from Europe concluded that IVF/ICSI with frozen oocytes may have lower implantation and pregnancy rates when compared with IVF/ICSI with fresh or frozen embryos [120].

There are limited data to evaluate the impact of duration of storage on the results of OC. A multicenter study that assessed these relationships concluded that human oocytes can be safely cryostored for several years [121].

The cryoinjuries of the oocyte during the freezing or thawing process may arise: premature zona pellucida hardening, damage to parthenogenesis, intracellular organelles, and the meiotic spindle apparatus, DNA fragmentation, and in vitro oocyte aging. Natural unique features of oocytes such as membrane permeability, oocyte size, the location of DNA material, and arrangement of meiotic spindle are different at different developmental stage (GV versus MII). Several characteristics such as the absence of the meiotic spindle, smaller size, and less developed zona may decrease

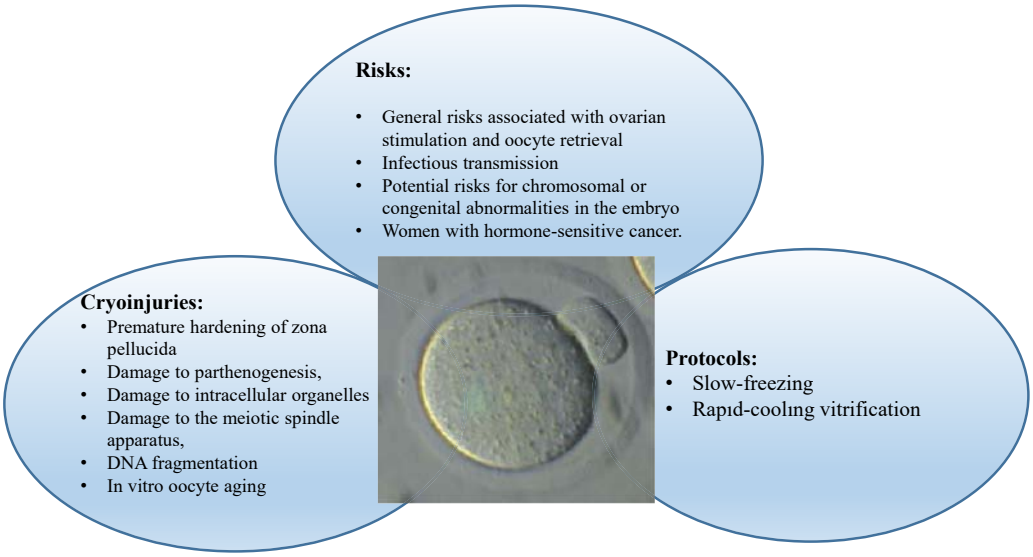
vulnerability of oocytes to cryoinjury and make the oocyte a better candidate for OC.

The risks of oocyte cryopreservation

Regarding potential risks for chromosomal or congenital abnormalities in the embryos obtained from OC, there are limited data related to definitive analysis of pregnancies and perinatal outcome derived from cryopreserved oocytes. According to the results of a review including 936 live births between 1986 and 2008 in the United States obtained from 58 cryopreservation studies, the incidence of major structural congenital anomalies was 1.3%, and there was no difference when compared to the results of naturally conceived infants in terms of congenital anomalies [122]. Moreover, a study that compared the results of 165 vitrified oocyte pregnancies (2.5%) to the results of fresh IVF pregnancies found that there was no difference in congenital anomalies between both groups [123]. There is also no definitive data to show the increased risk of embryonic aneuploidy obtained from OC. A retrospective cohort study including 33 patients who underwent OC and preimplantation genetic screening between 2011 and 2014 indicated that there is no difference in the number and percentage of euploid blastocysts [124]. There is no published data to show long-term follow-up of children from vitrified oocyte pregnancies.

Regarding infectious transmission with the use of open vitrification, there is no data on observing infectious transmission in reproductive tissues from this technique [125].

For general risks associated with ovarian stimulation and oocyte retrieval, thrombosis, hemorrhage, and infection associated with oocyte pick-up should be considered in women who undergo this procedure, and the risk of thrombosis may particularly be increased in special cases such as malignant conditions or autoimmune or rare diseases. However, some cases, such as with leukemia or lymphoma, could be at high risk of hemorrhage and/or infection. The risk of ovarian hyperstimulation syndrome is very low because of no embryo transfer, but it should also be considered in young or high responder women [126]. The potentially deleterious impact of ovarian stimulation due to supraphysiological estradiol levels should be kept in mind especially in women with hormone-sensitive cancer. The use of aromatase inhibitors for these special cases may minimize this risk.



Conclusion

According to the current data and evidence from the literature, it appears that laboratory and clinical outcome data on the possible influence of different oocyte morphologic abnormalities are still controversial, and no firm conclusion can be drawn regarding the relative impact of oocyte quality evaluation on laboratory and clinical outcomes. Novel and objective tools to evaluate oocyte quality and embryo developmental performance for both fresh as well as cryopreserved oocytes are needed. Recent studies involving molecular genetics

as well as artificial intelligence and machine learning approaches seem to be promising candidates to fulfill this promise.

To establish an objective oocyte grading system, there exist several challenges that have to be overcome by the clinics. One is the need of an establishment of a single oocyte-embryo tracking culture system, so the developmental and clinical performance of each oocyte with distinct morphologic or biomarker characteristics can be tracked. Most of the clinics nowadays prefer using group culture strategies, and changing their current system can increase the cost, require extra investment, and create a need for additional resources (time, personnel, and devices), therefore making it very difficult to implement such a tool in every clinic. Digital transformation is another challenge that many clinics will soon be facing. According to the current research trend as well as the potential of AI-based systems that are already implemented in certain areas of clinical services, most clinics will soon be transforming their paper-based, manually driven systems into digital data tracking and management forms. Until such challenges are resolved, oocyte morphology evaluation will be a subject of controversy.

The number of OC treatments is on the rise and will most likely be increasing in the near future due to expanding indications as well as increase of public access to such treatment options. Current data on the possible effect and impact of oocyte morphologic evaluation on cryopreservation outcome indicate that the outcome may not be associated with oocyte morphology. However the number of studies is still very limited, and there exists insufficient data on clinical outcome.

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CHAPTER

23

Ovarian hyperstimulation syndrome

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Introduction

The first cases of ovarian hyperstimulation syndrome (OHSS) and related deaths are repetaorted from 1960 when Lunenfeld in Europe first utilized urinary human menopausal gonadotropins for ovulation induction and, subsequently, when Van de Wiele and Turksoy reported their experience in the United States (US) [1]. In 1966, Melvin Taymor and Somers Sturgis formally described ovarian hyperstimulation during the 22nd Annual Meeting of the American Fertility Society in Chicago (IL) and published it in a peer-review journal for the first time [2]. They detailed ovarian hyperstimulation as a syndrome closely associated with high estrogen levels (assessed through cervical mucous arborization) and doses of gonadotropins over a prolonged period of time, more common in patients with polycystic ovaries, and they considered early induction/triggering of the ovulation as option to manage the syndrome [2].

After more than 50 years, albeit the pathophysiology is not still fully understood, many advances have been made [3]. This chapter will discuss the definition, the epidemiology, the pathophysiology, the risk factors, the clinical presentation and evaluation, and the prevention and treatment strategies for OHSS using, wherever possible, evidence-based data.

Definition

OHSS is generally considered a potentially life-threating iatrogenic complication of the early luteal phase and/or early pregnancy caused by an excessive response to ovarian stimulation [4]. It is characterized by cystic enlargement of the ovaries, abdominal distention and pain, and fluid shift from the intravascular space to the third space, which may eventually result in ascites, pericardial and pleural effusions, and in generalized edema (see below). OHSS may be asymptomatic, but in the severest cases, it may lead to hypovolemia, hemoconcentration, electrolyte imbalances, and coagulation disorders. Several life-threatening complications such as hemorrhage from the rupture of an ovarian cyst, adult respiratory distress syndrome, thromboembolism, and acute renal failure may be present [4,5]. However, at the moment, there is no consensus about its formal definition because it is a postovulatory syndrome due not only to drug-induced but also spontaneous triggering of multiple follicles [4]. In fact, in the literature are described spontaneous OHSS cases not associated with any ovarian stimulation [6]. Thus, it cannot be universally defined as an "iatrogenic complication."

Epidemiology

Beyond semantic concerns, the syndrome is generally due to ovarian stimulation with gonadotropins. Furthermore, over the years, the use of gonadotropins has been strongly reduced to induce ovulation in natural cycles with or without timed intercourse or in intrauterine insemination (IUI) cycles [1], changing the epidemiology of the syndrome. In fact, OHSS is extremely rare in infertile patients who receive oral ovulation inductors, and its risk is now thought to be low also in case of ovulation induction with gonadotropins for non-*in vitro* fertilization (IVF) cycles. At the moment, OHSS is mainly due to ovarian stimulation with gonadotropins for IVF cycles.

Based on these considerations, it is clear that the true incidence of OHSS is difficult to delineate because it is extremely changing and variable [7]. Available data are biased and confounded by many factors that include the population studies, the criteria adopted for the diagnosis, and so on. The incidence of mild OHSS is poorly reported, whereas data regarding essentially moderate and severe OHSS is mainly in hospitalized patients. Generally, the reported overall incidence of the

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syndrome ranges from 0.5% to 33%, respectively [8,9]. According to American Society for Reproductive Medicine classification (see below) [7], moderate to severe OHSS occurs in approximately 1%–5% of IVF cycles with an incidence of up to 20% in high-risk women [8,9]. It is interesting that many OHSS patients seek initial care in the emergency departments. From 2002 to 2011 in the US, there were 11,562 hospitalizations for OHSS, and about 4.4% of these cases experienced life-threatening events [5]. A mortality rate of 3/100,000 after IVF cycles has been estimated in Europe [10].

However, recent and complete data on the incidence of OHSS are not available, and probably, the real incidence of that reported is lower in consideration of the large use of mild stimulation, single embryo transfer, and new protocols for triggering ovulation followed by embryo cryopreservation.

Risk factors

Several risk factors have been identified that, alone or in concert, can increase the overall risk for OHSS [7–9]. In Table 23.1 the main risk factors potentially related to OHSS development are detailed.

Demographic characteristics, such as younger age, anovulation, black race (particularly African-American women), tubal factor, and unexplained infertility were all associated with an increased risk of OHSS in IVF population [7–9]. The main risk factor is younger age; in fact, more than 60% of women who develop OHSS are less than 35 years old, and this is probably due to the high number of gonadotropin receptors available in a younger ovary, making them more susceptible to stimulation [7–9]. Finally, a low BMI may also be related to increased risk to develop OHSS [7–9].

Several markers for ovarian reserve, mainly the serum anti-Müllerian hormone (AMH) levels and the antral follicle count (AFC), have been also used to assess risk of OHSS, but clear-cut points have not been validated in the literature. In the IVF population, serum AMH concentrations higher than 3.36 ng/mL can be effective for the prediction of OHSS (significantly better than age and BMI) and directly related with the risk to develop OHSS [7–9]. AFC is also predictive of OHSS before gonadotropin stimulation for IVF/intracytoplasmic sperm injection (ICSI). In particular, the risk of OHSS increases about fourfold in patients with more than 24 AFC in comparison with those with less than 24 AFC [7]. Interesting, the diagnosis of polycystic ovarian morphology (PCOM) is performed also with a number of antral follicles of at least 12 for ovary [11].

The main ovarian responses to stimulation, such as follicular development, serum estradiol levels, and oocytes retrieved, should be taken into account to predict the risk for OHSS during ovarian stimulation [7–9]. Moreover, as for the ovarian reserve markers, well-established and generally accepted, clear cutoffs are not available in the literature. The number of growing follicles is directly and independently related to OHSS development, and with a clinically significant risk in presence of 20 or more follicles during ovarian stimulation for IVF/ICSI cycles [4]. On the other hand, the risk-benefit ratio seems to be unbalanced for more than 15 follicles [7]. The number of oocytes retrieved, as well as the high and rapidly increasing estradiol concentrations, is also a predictor of OHSS [4]. A number of more than 24 oocytes or estradiol levels higher than 3500 pg/mL are strong markers for OHSS development [7].

Another crucial factor is a diagnosis of ovulation disorder or polycystic ovary syndrome (PCOS). The hyperresponse to ovarian stimulation in patients with PCOS may be due the presence of too many antral follicles at the beginning of the stimulation cycle but also to abnormal sensitivity to gonadotropins. In PCOS, the antral follicles are closely synchronized and respond to stimulation in concert with limited intraovarian self-inhibition [12]. Hyperinsulinemia and hyperandrogenism, two other features of PCOS, promote, alone and in concert, early folliculogenesis (and PCOM) and frequently a multifollicular response following the ovulation induction increasing the sensitivity to follicle stimulating hormone (FSH) [12]. Insulin-like growth factor 1 (IGF-1) and insulin, frequently altered in women with PCOS, may stimulate vascular endothelial growth factors (VEGF) production, and an increased expression of VEGF within the thecal stroma of women with PCOS may be responsible for their higher risk of OHSS [12]. However, several features commonly present in PCOS patients, such as insulin resistance, hyperandrogenism, PCOM, and/or high antral follicular count, may be considered risk factors also in non-PCOS patients [13].

TABLE 23.1 Main risk factors for OHSS.

Ovarian stimulation with gonadotropins (including hCG for triggering ovulation)
Young age
Black race
Lean
PCOS/PCOM
Hyperinsulinemia
Hyperandrogenism
Elevated AMH values[a]
High AFC[b]
High peak of estradiol[c]
Multifollicular development[d]
High number of oocytes retrieved[e]

[a] *AMH values > 3.4 ng/mL.*
[b] *AFC > 24.*
[c] *Estradiol values > 3.500 pg/mL.*
[d] *Development of >18–20 follicles.*
[e] *> 24 oocytes retrieved.*

Finally, a genetic predisposition can also be a crucial factor increasing the risk for the syndrome. The presence of single nucleotide polymorphisms in the FSH receptor (FSHR) gene and/or FSH β subunit-encoding gene (FSHB) seems to significantly influence the ovarian response in predicted normal responders treated with recombinant FSH [14]. Further data about the role of genetics in OHSS risk have been detailed above.

Classifications

Numerous attempts have been made to categorize and classify OHSS [4]. Two modalities of classification have been described. The first is based on the timing of presentation, while the second on the severity of presentation.

Timing of presentation

According to the timing of presentation, it is possible to distinguish an early and a late OHSS form [15]. Early OHSS form typically occurs 3–7 days after ovulation triggering by human chorionic gonadotropin (hCG) and is caused by an excessive ovarian response to exogenous hCG [15]. Late OHSS form typically occurs 12–17 days after hCG administration and is due to excessive response to endogenous hCG from trophoblast during early pregnancy [15]. The early OHSS form is considered less clinically relevant when compared with the late OHSS form because is closely related to the hCG administration and half-life. On the other hand, the endogenous hCG production due to pregnancy is incremental and may achieve high serum concentrations, especially in case of multiple pregnancy [16].

Severity of presentation

Many classifications based on severity presentation have been proposed in the literature [4]. The most used classify OHSS into four stages based on clinical and laboratory features. In Table 23.2 is shown the classification of OHSS proposed by the Royal College of Obstetricians and Gyneacologists [9]. In particular, OHSS may be categorized into four classes, including mild, moderate, severe, and critical forms of the syndrome, on the basis of the severity of symptoms, signs, and laboratory parameters [9]. However, these grades are not strictly separated and can quickly transition.

Even if the bilateral enlargement of ovaries has been used in clinical classification, clinical evidences underline that their dimension is not related to OHSS severity [9]. The mild form is characterized by abdominal bloating and mild abdominal pain. The moderate form of OHSS is described by moderate abdominal pain, nausea and/or vomiting, and ultrasound finding of ascites. The severe form of OHSS is defined by clinical manifestation of ascites with or without hydrothorax, with abnormality findings like sodium, potassium, and osmolarity serum leading to decreased urine output and hypovolemic shock. Critical OHSS is characterized when there is tense ascites or hydrothorax, hematocrit of over 55%, white cell count over 25,000/mL, anuria, thromboembolism, or acute respiratory distress syndrome (ARDS) (Table 23.2).

TABLE 23.2 Classification of OHSS. OHSS may be categorized into four classes, including mild, moderate, severe, and critical forms of the syndrome on the basis of the severity of symptoms, signs, and laboratory parameters (Topo 2016).

OHSS stage	Features
Mild	Abdominal bloating
	Mild abdominal pain
Moderate	Moderate abdominal pain
	Nausea and/or vomiting
	Ultrasound evidence of ascites
Severe	Clinical ascites (with or without hydrothorax)
	Oliguria[a]
	Hematocrit >45%
	Hyponatriemia[b]
	Hypoosmolarity[c]
	Hyperkaliemia[d]
	Hypoproteinemia[e]
Critical	Tense ascites and/or large hydrothorax
	Hematocrit >55%
	White cell count >25.000/mL
	Anuria
	Thromboembolism
	ARDS[f]

[a] *<300 mL/day or <30 mL/h.*
[b] *Sodium <135 mmol/L.*
[c] *<282 mOsm/kg.*
[d] *Potassium >5 mmol/L.*
[e] *Albumin <35 gr/dL.*
[f] *Acute respiratory distress syndrome.*
The presence of all features are needed for mild and moderate stages, whereas for severe and critical stage, at least one feature is necessary.

Pathophysiology

Hypersensitivity to ovarian stimulation with exogenous gonadotropins is the most common cause of OHSS

[16–18]. Commonly, in case of OHSS, ovarian stimulation induces growth of a large number of follicles, and the administration of hCG to complete oocyte maturation triggers the syndrome. As hCG has a longer half-life than the endogenous luteinizing hormone (LH), sustained luteotropic activity will induce arteriolar vasodilation and increased capillary permeability that results in fluid shifting from intravascular to extravascular spaces (third space), and a state of hypovolemic hyponatremia [16–18].

The key molecules responsible for the high vascular permeability are VEGFs, mainly involved in the ovarian renin-angiotensin system [16–19]. VEGF is produced by the granulosa cells after stimulation with gonadotropins, and its production increases substantially after the administration of hCG [16–19]. VEGF appears involved in follicular and corpus luteum growth and function, angiogenesis, and vascular endothelial stimulation [16–19]. Even if VEGF is considered the main systemic mediator of hCG responsible for the increased vascular permeability of OHSS, other systemic and local vasoactive substances, including interleukin (IL)-2, IL-6, IL-8, IL-10, IL-18, angiotensin II, histamine, prolactin, prostaglandins, IGF-1, and transforming growth factor β, are also directly and indirectly involved in the pathogenesis of OHSS symptoms [16–19]. Recent data seem to demonstrate a crucial role of the receptors for the VEGFs for explaining the different risk for OHSS especially in cases of patients with predicted low risk. As already stated in the introduction, the OHSS may be not related to hCG administration, for example in gonadotrophin-releasing hormone (GnRH) antagonist cycles in which LH surge is induced by GnRH agonist, or to ovarian stimulation, as observed in familiar spontaneous OHSS cases [6]. In these cases, a genetic predisposition, regarding genetic variants of the genes for the receptors of the VEGFs, has been considered the pivotal cause of the syndrome [20].

The formation of the third space leads to depletion of the intravascular volume resulting in hypotension [16–18]. The large fluid shift can cause tension ascites that can be transmitted into the thoracic cavity leading to pleural effusions, other pulmonary manifestations, or pulmonary edema. Hypotension leads to decreased venous pressure and reduced venous return, and a potential decreased cardiac output that also affects organ function such as the kidney (decreased glomerular filtration rate) and for the liver (altered synthesis of proteins including anticlotting factors) because of the decreased perfusion [16–18]. These hemodynamic changes associated with OHSS are the same of the “abdominal compartmet syndrome”.

Clinical presentation

The signs and symptoms of OHSS are a result of ovarian enlargement and increased vascular permeability. Initial symptoms develop gradually with abdominal distention and mild abdominal discomfort due to the enlargement of ovarian cysts up to 25 cm. Increased capillary permeability leads to third spacing and subsequent intravascular volume depletion. As already underlined, the clinical features and severity are correlated with increasing organ system involvement [16–19].

The first clinical sign of OHSS is typically the development of ascites. Accumulation of ascetic fluid leads to intraabdominal hypertension (>12 mmHg), associated with abdominal distention and pain, up to abdominal compartment syndrome (>20 mmHg), associated with organ dysfunction/failure (affecting the renal, respiratory, gastrointestinal, cardiovascular, and hepatic systems). The increased intraabdominal pressure initially reduces the venous drainage, inducing edema and, subsequently, perfusion reduction and tissue hypoxia [4,5,16–18].

One of the initial signs of organ failure is oliguria, but the hepatic and intestinal injury can result in severe paralytic ileus, emesis, and diarrhea [5]. Elevated levels of aspartate aminotransferase and alanine aminotransferase are frequently observed (about one-third of cases) in patients with severe OHSS, although abnormal γ-glutamine transpeptidase and/or alkaline phosphatase levels may also be detected [5]. Hyponatremia (due to a low serum osmolality) and other metabolic abnormalities, including hyperkalemia and metabolic acidosis are frequent in severe cases and suggest an acute renal failure. Hyponatremia may lead to cerebral edema, altered mental status, and neurologic complications, whereas hyperkalemia may induce alterations of the cardiac conduction [5].

Leukocytosis, increased hematocrit, and thrombocytosis are signs of hemoconcentration and systemic inflammation [4,5,16–18]. The hypercoagulability due to hemoconcentration, pregnancy and/or high estrogen levels and/or genetic thrombophilia, frequently related to infertile patients, together with the pressure from enlarged ovaries and/or ascites on pelvic vessels, predisposes to thrombotic events, complicating up to 10% of severe OHSS cases [5]. The venous system (about 80% of cases) is commonly involved and regards, in order of decreasing frequency, the jugular, subclavian, lower extremity, upper extremity, cerebral, renal, and retinal veins. Arterial embolism is possible, but it is a rarer event occurring primarily in the pulmonary, cerebral, central retinal, coronary, upper extremity, and lower extremity arteries [5].

Patients with OHSS are also at a high risk for infection and about 80% of hospitalized patients report fever. In two-thirds of cases a pathogen is identified and the fever may be due to the increased endogenous production of proinflammatory cytokines. However, severe OHSS

TABLE 23.3 Main sites of infection identified in OHSS patients and microorganisms involved.

Sites of infections	Incidence (%)
Kidney/bladder	~20
Lung/low respiratory tract	~4
Upper respiratory tract	~3
Intravenous line	~2
Abdominal and gluteal puncture sites	~1.5
Postoperative wounds	~1.0
Microorganisms involved	**Incidence (%)**
Klebsiella pneumoniae	~25
Pseudomonas aeruginosa	~20
Proteus mirabilis	~18
Escherichia coli	~15
Proteus vulgaris	~10
Morganella morganii	~9

TABLE 23.4 Main questions to ask to patients for a correct diagnosis.

Have you had babies?
Are you infertile?
Have you received an infertility treatment?
Have you received an ovarian stimulation?
Can you provide me your ovarian stimulation plan?
Have you had PCOS?
When did IVF cycles start?
What drugs have you received?
How many follicles have been counted at ultrasound?
How many eggs have been retrieved at pick-up?
What are you taking?
Have you received an embryo transfer?
How many embryos have been transferred?
Have you performed a pregnancy test?

should be considered a relatively immunodeficient state with decreased levels of immunoglobulin A (IgA) and immunoglobulin G (IgG). Table 23.3 details the main sites of infection identified in OHSS patients and the microorganisms involved [5].

Critical patients generally present with combination of many signs and symptoms. Generally, hypovolemic shock is associated to shock due to infection, distributive shock for severe inflammatory state, and/or obstructive shock due to pericardial effusion with cardiac tamponade or massive pulmonary embolism [5].

Clinical assessment

History

The assessment of the risk factors (Table 23.1) and of the history is crucial for the diagnosis and to define the risk to develop a severe symptomatology (Table 23.4).

A history of infertility and previous/current ovarian stimulation is certainly of great help for the clinician. A diagnosis of PCOS-PCOM, the date of initial IVF cycle, the drugs and the doses used for ovarian stimulation or triggering (hCG or GnRH agonist), the number of follicles present at ultrasound before triggering, the number of eggs retrieved at pick-up, the previous and current therapies taken, the embryos transferred or not, and if transferred, when and how many embryos are all important information for a correct diagnosis. Information about other complications including presence of an associated infection, thrombosis, hemorrhage, ectopic, or heterotopic pregnancy should be also taken (see before) [7,9,18,21].

Physical examination

Body weight and abdominal girth should be taken daily and compared to previous measures. Vital signs (including heartbeat, systemic/diastolic arterial pression, respiratory frequency, and temperature) are crucial for the initial evaluation and further follow-up of OHSS patients. These are frequently normal in patients with mild and moderate OHSS syndrome, even if their normality cannot rule out a potential OHSS. On the other hand, hypotension and tachycardia are frequent in case of severe OHSS. In these patients, fever, tachypnea/dyspnea, or signs of hypoxia need complete cardiopulmonary evaluation since severe infection, pulmonary embolism, acute pulmonary edema, or pleural effusion may be present [5,18,21].

Evaluation of the abdomen should exclude or confirm the presence of peritoneal irritation/infection, masses, and ascites. General examination should search for hematomas or abscesses. On the other hand, pelvic examination should always be deferred in patients with moderate or severe OHSS because of the iatrogenic risk of ovarian cyst rupture with intraabdominal hemorrhage [5,18,21].

Laboratory and imaging tests

Laboratory and imaging studies are crucial to confirm the diagnosis, to evaluate/study the organ (dys)function, and to define an accurate prognosis. In Table 23.5 are listed the main laboratory and imaging tests to require in OHSS patients.

Laboratory tests include complete blood count and basic metabolic panel, venous blood gas, serum

TABLE 23.5 Main laboratory and imaging tests needed in OHSS patients.

Complete blood count
Basic metabolic panel (including electrolyte)
Venous blood gas analysis (including serum osmolality and lactate level)
CRP[a]
Procalcitonin[a]
Analysis and culture of urine, sputum, abscess, and peritoneal fluid[b]
Liver enzymes
Direct/indirect bilirubin
Albumin
Complete coagulation studies (including fibrinogen and antithrombin III)
Pregnancy test (serum β-hCG levels)
Blood type and screen
Electrocardiogram
Transvaginal and abdominal ultrasound[c]
Chest X-ray

[a]*In case of leukocytosis and thrombocytosis.*
[b]*If high CRP and procalcitonin levels.*
[c]*Consider doppler velocimetry of the ovarian pedicle if a torsion is suspected.*
Other specific imaging and laboratory tests should be required according to patient's clinical presentation, history, and underlying comorbidities.

osmolality, and lactate level. These are useful to evaluate for electrolyte and metabolic abnormalities. As detailed before, leukocytosis and thrombocytosis are frequently due to hemoconcentration, even if they may indicate an underlying infection. In these cases, the evaluation of serum C-reactive protein (CRP) and procalcitonin levels is useful for definitive diagnosis. Even if high CRP levels may be per se associated with the syndrome, it is suggested to require blood, urine, sputum, abscess, and peritoneal fluid cultures to exclude infection [5,18,21].

Other laboratory tests should also include liver enzymes, direct/indirect bilirubin, alkaline phosphatase, and albumin to investigate the liver function, and complete coagulation studies, including fibrinogen and antithrombin III. A pregnancy test and/or serum β-hCG should be obtained to clarify the outcome of the treatment cycle and to predict the prognosis. A blood type and screen should always be required for the risk intra-abdominal bleeding from hemorrhagic cyst rupture. An electrocardiogram should be performed in all patients with moderate or severe OHSS at hospital admission to have an initial cardiologic evaluation for the further management and for the risk of surgical intervention [5,18,21].

Transvaginal and abdominal ultrasound are useful to evaluate ovarian size, presence of ascites, or other associated conditions such as ovarian torsion, ectopic/heterotopic pregnancy, intraabdominal hemorrhage, or pelvic abscess. In this regard, the incidence of adnexal torsion and ectopic pregnancy is particularly high in patients with OHSS, whereas pelvic infections or abscess are rare events [5,18,21].

Other specific imaging and laboratory tests should be required according to the patient's clinical presentation, history, and underlying comorbidities.

Prevention

Prevention is the main strategy for reducing the global incidence of moderate to severe OHSS [7,9].

Identification of risk factors

OHSS may theoretically occur in any woman undergoing ovarian stimulation, especially after gonadotropin treatment. However, evidence indicates that there are some patients who are at a much higher risk. Identifying these women is essential to lowering, and potentially eliminating, the incidence of OHSS (see above) [7,9]. Thus, it is crucial to know the numerous risk factors contributing to the development of OHSS (Tables 23.1 and 23.4).

Unfortunately, at the moment, there is no specific algorithm designed to identify in the clinical practice potential high-responder patients, and data demonstrating the universal clinical effectiveness of the risk factors identifications for the reduction of the OHSS risk and severity are not formally available. However, common sense drives the need to identify risk factors for OHSS and use the best evidence-based strategies for minimize the risk.

Potential strategies or intervention before ovarian stimulation

Avoid gonadotropin for ovulation induction in non-IVF cycles

Several oral drugs are used to induce ovulation for patients with infertility anovulation with high efficacy and safety, and gonadotropins should be used only as second-line treatment both in patients with anovulatory infertility [22,23] and in patients with unexplained infertility [24,25].

The main oral drugs used for treating anovulation are clomiphene citrate (CC), letrozole, and metformin. Among these drugs, the risk for OHSS is probably higher in patients who receive CC. In fact, the risk of OHSS in metformin-treated patients is anecdotical and, probably, not different from normo-ovulatory women [26], whereas the risk in letrozole-treated is very low [27] as demonstrated by large clinical trials [28–30]. Letrozole is, at the moment, the first-line drug for inducing ovulation in PCOS patients, especially in young patients with PCOM [23,31].

In patients with unexplained infertility, if gonadotropins are used in low doses or with strict cancellation policies, there is no increase in live birth over oral agents [24,25]. These data suggest that gonadotropin (including hCG triggering) administration may be avoided in all IUI cycles including not only patients with unexplained infertility but also patients affected by HIV, anatomical problems, psychological disorders, or for semen donation.

Oral contraceptive (OC), progestogen or estrogen

Pretreatment with OC has been assessed in women with PCOS before IVF to improve the efficacy and safety of the treatments, reducing the local and systemic androgen levels, and to synchronize multiple cycles. Moreover, available data suggest that OC pretreatment did not reduce the risk of OHSS but increase the miscarriage rate and lowered the cumulative live birth, especially in GnRH antagonist cycles [32]. Thus, at the moment, OC administration before starting ovarian stimulation in patients at high risk for hyperresponse and OHSS is not suggested [33]. Similarly, it is not suggested to use any progestogen or estrogen pretreatment for ovarian stimulation IVF protocols for lack of data on OHSS risk [34].

Natural IVF cycles

The use of natural IVF cycles avoids the risk of OHSS because it does not involve gonadotropin administration for ovarian stimulation and oocytes triggering. Initially, a natural IVF cycle was utilized for reducing the overall costs when IVF efficacy was low and embryos cryopreservation not possible or effective. Unfortunately, meta-analytic data have showed an ongoing pregnancy rate ranging from 0% to 7% per cycle with a cycle cancellation rate higher than 50% [1,3]. Thus, there is no evidence to justify the use of natural cycle (or modified natural cycle) for ovarian stimulation in predicted high responders [33].

Gonadotropin starting dose for IVF cycles

Personalization

An optimal response to gonadotropin is generally considered a retrieval of 6–15 oocytes per stimulation cycle because live birth rate per fresh started cycle increases linearly [35]. On the other hand, considering the cumulative live birth rate, 12–18 oocytes are suggested as an optimal number of oocytes associated with maximal fresh live birth rate, whereas cumulative live birth rate continues to increase with the number of oocytes retrieved [35]. A high ovarian response is, however, associated with increased risk of OHSS. Thus, some authors think it is crucial to personalize the gonadotropin starting dose using patient characteristics (including age, BMI, and ovarian reserve tests, including FSH, AMH, and AFC) to minimize the risks, whereas others prefer to use the "segmentation" strategy (see below) to maximize the efficacy.

To mitigate the risk of OHSS in fresh IVF cycles, a starting dose of less than 150 IU of FSH in patients with potential or expected ovarian hyperresponse is always recommended. A large systematic review with meta-analysis [36] on 20 trials that evaluated the efficacy and safety of individualized gonadotropin dose using markers of ovarian reserve in women undergoing IVF demonstrated that a personalized treatment is effective and safe in predicted high responders because a dosage of gonadotropin lower than 150 UI daily reduces the likelihood of moderate or severe OHSS in high-risk patients [36]. However, the evidence was scarce for quality and number of studies. To this regard, the main evidence comes from the OPTIMIST trial in which the use of 100 UI daily as starting dose in the predicted hyperresponders (AFC >15) reduced the risk of mild and moderate OHSS in comparison with a standard dose of 150 UI/day, even if a reduced odds of live birth in young women may be observed [37,38].

In conclusion, a gonadotropin dose lower than 150 UI daily is suggested in predicted hyperresponders to reduce the risk of OHSS. The specific personalization of the starting dose for high-risk patients needs to be confirmed in the future.

Mild ovarian stimulation

Even if the concept of "mild stimulation," defined as the use of a starting dose ≤150 IU daily of gonadotropin in IVF cycles, has been developed in different contexts to demonstrate a best risk-benefit profile in predicted normo-responders, recent data [39] have confirmed a lower risk of OHSS with mild stimulation than with conventional stimulation in normal and hyperresponders, and live birth rates not different among normal, poor, and hyperresponders.

Choice of the gonadotropin for high-risk patients

No difference between gonadotropins for ovarian stimulation for IVF cycles has been demonstrated [35]. Thus, it is not possible to choose a specific gonadotropin with the aim to modify the risk of hyperstimulation or OHSS [33]. However, long-acting gonadotropin is associated with an overall risk for OHSS at least 30% higher in comparison with daily recombinant FSH [40]. Thus, the use of long-acting gonadotropin should be absolutely avoided in potential high-risk patients because it is associated with very high risk of OHSS when used in GnRH antagonist cycles [33,40]. Of interest, a recent

analysis [41] of global safety data from Merck KGaA (Darmstadt, Germany) reveals a very low incidence of thromboembolic events with the use of recombinant alpha FSH.

Regimens of ovarian stimulation

Several different regimens of ovarian stimulation have been tested for reducing the OHSS risk in predicted high responders.

Drug co-administrations

CC or letrozole

The addition of CC or letrozole to gonadotropins has been suggested to minimize the risk of OHSS. The mechanism of action is not totally known. Potentially, CC suppresses several little antral follicles, avoiding their growth, whereas letrozole could act by lowering the systemic estradiol levels [27].

Even if CC seems to reduce significantly the OHSS risk in comparison with non-CC protocols both in GnRH agonist [42] and antagonist cycles [42,43] in normal and poor responders, the effect of CC is confounded by different stimulation protocols with particular regard for mild or minimal stimulation protocols [33]. In addition, the higher incidence of cycle cancellations, as well as of reduction in the number of oocytes retrieved, in both the general IVF population and the poor responders underline the potential risks of worst reproductive performances in infertile patients erroneously considered at high risk for hyperresponse and OHSS [42]. Recently, a systematic review with meta-analysis [44] concluded that letrozole has no efficacy in reducing the risk of early OHSS.

Thus, at the moment, these schemas are not recommended in the clinical practice for reducing the risk of OHSS in predicted hyperresponders [7,33].

Metformin

Metformin is an insulin-sensitizing drug commonly used for treating type 2 diabetes and has been widely studied in patients with PCOS [26]. Several mechanisms have been suggested for its use in the prevention of OHSS, including reduction of intraovarian androgen level, normalization of FSH sensitivity on granulosa cells, and so on [26]. Evidence-based data [45,46] have demonstrated that metformin administration, especially also given pretreatment, at doses extremely variable (from 500 to 2000 mg), can reduce the risk of OHSS in high-risk PCOS patients by about 60%–80%. Moreover, even if there is good evidence that metformin decreases the risk of OHSS risk in PCOS patients [7], at the moment its use is limited because available data about its efficacy are limited to GnRH agonist cycles [33]. In fact, metformin administration has any effect in reducing the OHSS in GnRH antagonist cycles [47], so it may be suggested only in patients at high-risk scheduled for GnRH agonist cycles [33].

FSH dose decrease

Decreasing the FSH dose in mid-follicular phase during treatment may reduce the occurrence of OHSS in comparison with stable dosage [48]. However, most trials evaluating the dose adjustment in predicted hyperresponders are designed to assess individualization of the starting dose, confounding and making inconsistent the available findings [33]. In addition, the reduction of the gonadotropin dose can be not only ineffective but can be deleterious in patients with PCOM/PCOS. In fact, the arbitrary reduction of gonadotropin dosage below specific threshold values may arrest the follicular growth [12].

Coasting

Coasting is a strategy used to decrease OHSS risk by withholding gonadotropins during ovarian stimulation [49]. Commonly, it is performed for a variable number of days up to the significant reduction of serum estradiol levels; specifically, about 4 days are necessary for a clinically significant drop in serum estradiol levels. Moreover, evidence-based data and guidelines do not suggest its use in the clinical practice because it reduces the efficiency of the IVF cycles with a safety not different from other strategies, such as GnRH agonist oocyte trigger with or without a freeze-all strategy [7,33,49].

Strategies for controlling LH surge

The inhibition of the LH surge is one of the main steps for optimizing the safety and efficacy in IVF cycles. At the moment, GnRH agonists, GnRH antagonists, or progestogens are used for that aim.

GnRH analogs

In infertile women unselected for OHSS risk, long-acting GnRH agonist follicular protocol, when compared to GnRH antagonist protocol, is associated with a risk of OHSS higher than 60%, even if the live birth and the clinical pregnancy rates result improved 60% and 40%, respectively [50].

The use of GnRH antagonist protocol does not reduce only the risk of OHSS but also the severity of the syndrome [51]. Both fixed (on day 5 of stimulation) and flexible (mean follicle size of 12 mm) GnRH antagonist administration appear to achieve comparable results [52]. In particular, the incidence of severe OHSS in high-risk women who did not receive any form of luteal phase support is zero, whereas in patients who receive

hCG in addition to standard luteal phase support or to GnRH agonist for triggering ovulation, it is about 1% [51,52]. Unfortunately, in quantitative data synthesis are frequently included other complementary strategies, such as the "freeze-all strategy" (see below). A well-done phase IV, dual-center, open-label, RCT including 1050 patients demonstrated similar reproductive outcomes but a lower incidence of severe (5.1% versus 8.9%) and moderate OHSS (10.2% versus 15.6%) in the GnRH antagonist group compared with the agonist group [53]. In addition, fewer patients were admitted to the hospital due to OHSS (1.7% versus 3.6%) [53]. Of note, the population was composed of women less than 40 years of age nonselected for ovarian reserve and/or PCOS. This study is very interesting just for its clinical limitations due to use of freeze-all strategy and GnRH agonist triggering only in a small proportion of patients, making the results essentially secondary to GnRH antagonist effectiveness.

In conclusion, the use of GnRH antagonist suppression is strongly recommended for predicted hyperresponder patients at high risk of OHSS [7,33].

Use of progestin-primed ovarian stimulation

The use of progestin-primed ovarian stimulation is a new ovarian stimulation protocol to avoid the LH surge. In fact, oral administration of exogenous progestogen, such as medroxyprogesterone acetate (10 mg daily) and dydrogesterone (20 mg daily), from the early follicular phase can be used in combination with gonadotropins to prevent the activation and transmission phases of estradiol-induced LH surges [54]. In comparison with conventional GnRH analog downregulated cycles, progestin-primed ovarian stimulation protocols are not different in terms of efficacy but associated with about a 50% lower risk for OHSS [54], suggesting its potential effectiveness in the clinical practice when the cryopreservation of all embryos is scheduled, such as for fertility preservation, oocyte donation, preimplantation genetic testing, and oocyte donors [55].

Ovulation triggering strategies for high-risk patients

Since OHSS is generally a postovulatory syndrome due to spontaneous or iatrogenic ovulation triggering, specific strategies to trigger ovulation in assisted reproductive technology (ART) cycles are of crucial interest for the prevention of OHSS.

hCG

hCG administration is an excellent strategy for triggering oocyte maturation before oocyte retrieval in ART cycles and represents the golden standard in poor and normal-responder patients for autologous fresh cycles [35]. Moreover, its safety is not as good as the efficacy, especially in hyperresponder patients. In fact, hCG induces a sustained stimulation of LH receptors on the multiple postretrieval corpora lutea due to its long half-life. This prolonged stimulation may be effective in terms of luteal phase and endometrial competence but may result also in the development of OHSS [56].

It has been postulated that the use of recombinant, instead of urinary, hCG may reduce the risk of OHSS. However, meta-analytic data [56] have showed no significant effect on the use of recombinant hCG versus urinary hCG on OHSS risk. Another strategy used is to lower the dose of urinary hCG. In fact, a half (5000 UI) dose seems effective in terms of oocyte maturation, but data about its safety in terms of the reduction of OHSS risk are conflicting [7,33]. Moreover, this approach may be an option in GnRH agonist cycles in case of high risk for OHSS when the "freeze-all embryo" approach cannot be carried out [33].

LH

LH is the physiologic trigger for ovulation. Its injection has been experimented to trigger ovulation also in IVF patients, and its use has been suggested to reduce the OHSS risk thanks to its short half-life. However, scientific evidences demonstrated no benefit in terms of OHSS reduction of its administration when compared to hCG (urinary or recombinant) [56].

GnRH agonist

GnRH agonist administration permits to trigger final oocyte maturation in IVF cycles avoiding the use of hCG. This strategy can be used only when ovarian stimulation is performed in the context of cycles downregulated with GnRH antagonists because they inhibit daily and directly the pituitary function, permitting trigger of endogenous LH surge (about 34–36 h after its administration) thanks to the temporary displacement of the GnRH antagonists on their specific receptors [57].

Overall, clinical data have confirmed the efficacy of GnRH agonist trigger compared with hCG trigger for final oocyte maturation in lowering the risk of OHSS by at least 60% [51,58]. Furthermore, a lower live birth rate of 30%–70% has been observed in fresh autologous cycles probably for rapid and dramatic postluteal drop in hormonal LH support inducing an acute luteal phase insufficiency defect (contrarily to hCG) [58,59].

Many strategies have been used to minimize the lower pregnancy rates observed with GnRH antagonist cycles when GnRH agonists have been used as trigger for oocyte

maturation. Initially different regimen for "intensive" support for avoiding luteal phase insufficiency defect have been experimented. These include the administration of high doses of estradiol and progesterone with or without hCG during the luteal phase or of a co-trigger with low-dose hCG in multiple doses (1000, 500, or 250 IU every third day after retrieval, or 100 IU/daily) or in single doses (1500 UI) [60]. Even if the hCG use is associated with the best efficacy, directly related to the dose administrated in all therapeutic schemas, the risk of OHSS seems to be present in high-risk patients [51]. A strategy to cryopreserve all embryos and to transfer in a subsequent frozen embryo transfer cycle rather than performing a fresh embryo transfer ("freeze-all strategy") is the procedure recommended in these cases [33].

In conclusion, the use of a GnRH agonist to trigger oocyte maturation prior to oocyte retrieval reduces the risk of OHSS but also the live birth rates in fresh autologous cycles. Thus, it is recommended to freeze all embryos and transfer in subsequent cycles because data on the co-administration of low dose of hCG at the time of GnRH agonist trigger or on the "intensive" hormonal schemas for luteal support are not totally convincing in terms of efficacy and safety, and not supported by strong clinical evidence. However, the efficacy of the GnRH agonist trigger is the same in donor-recipient cycles so always recommended. Thus, GnRH agonist trigger is recommended for final oocyte maturation in women at risk of OHSS [33] and, probably, recommended in all GnRH antagonist protocols where no fresh transfer is scheduled or performed irrespective from the risk of OHSS for minimizing the residual risk of OHSS [33].

Kisspeptin

Kisspeptin acts by stimulating hypothalamic GnRH secretion from the hypothalamus and induces gonadotropin secretion [61]. In IVF cycles, a bolus of kisspeptin-54 induces an LH surge of 12–14 h of duration and is effective to induce oocyte triggering. Initial data seem to suggest that its administration may reduce significantly the OHSS rates [62]. At the moment, only a few clinical trials have been published, limiting its use in the clinical practice.

Elective cryopreservation

Elective cryopreservation (also called "freeze-all strategy") is the cryopreservation of all embryos with their transfer in subsequent nonstimulated cycles (also known as "cycle segmentation"). That strategy should be used only in hyperresponder patients and not as a strategy to improve reproductive outcomes [63,64]. In fact, it prevents late-onset OHSS symptoms and duration in patients at high risk for OHSS due to the endogenous hCG rise in pregnant patients who underwent IVF/ICSI cycles. Even if that strategy can virtually avoid the risk of late OHSS, evidence-based data demonstrate a reduction of about 70% in the risk for OHSS [64]. At the moment, that strategy is recommended as a method to reduce OHSS risk only in GnRH antagonist cycles where the triggering has been performed with GnRH agonist [7,33].

Cancellation cycle

In selected cases at high risk for OHSS the cancellation of the cycles remains an option [7,33]. In particular, a cycle may be cancelled in GnRH agonist cycles before ovulation triggering (withholding hCG) or in GnRH antagonist cycles when the elective cryopreservation is not possible or the risk is still very high with also scheduling a GnRH analog triggering.

Elective single embryo transfer (eSET)

Patients with hyperresponse are considered patients with good prognosis. In these patients, an eSET policy followed by a further transfer of SET in fresh or frozen cycle is effective such as double embryo transfer reducing multiple pregnancies [65]. As detailed before, however, the risk and severity of OHSS is closely related to hCG levels that are significantly higher in multiple pregnancy. Based on these considerations, the risk of early OHSS is presumable lower in case of single pregnancy in homologous fresh cycles [66]. Thus, an eSET is strongly recommended for predicted hyperresponders that are considered patients with good prognosis [67].

In vitro maturation (IVM) of oocytes

Even if the term IVM is controversial, it generally refers to the maturation of the retrieved immature oocytes in a special culture environment generally in untreated patients [68,69]. Exogenous gonadotropin stimulation, FSH and/or hCG, for short courses seems to improve the ultrastructure of the oocytes expected to mature in *in vitro* conditions [68,69].

In some subgroups of women at high risk of ovarian stimulation, such as those with PCOS-PCOM, IVM of oocytes has been considered alternative to classical IVF for these women because the risk of OHSS is virtually zero [68,69]. Furthermore, recent data [70] demonstrated that IVM is significantly less effective that IVF in terms of live birth per transfer (−8%) and of cumulative ongoing pregnancy rates (−18.7%). Based on these considerations, IVM should be considered still an

experimental procedure that has little clinical role in the antagonist era [71].

Other treatments or procedures

Intensification of monitoring and surveillance

The intensification of monitoring and surveillance with a more frequent use of ultrasound examinations and/or serum estradiol assays has been considered in high-risk patients for OHSS. However, direct data seem to exclude advantages or benefits of a more aggressive monitoring and surveillance probably for the lack of efficacy of strategies to take during ovarian stimulation, such as gonadotropin dose reduction or coasting [72]. Thus, no recommendation can be given in regard and the timing should be defined case by case [33]. Ultrasound monitoring is always suggested up to a follicular size ranging from 16 to 22 mm, whereas the estradiol assays did not improve the safety of the surveillance [7,9,33].

Dopaminergic agonists (cabergoline, quinagolide)

Because the pathophysiology of ovarian OHSS is mainly related to an increased vascular permeability of the ovarian and peritoneal capillaries caused by ovarian hypersecretion of VEGF, dopaminergic agonists, including cabergoline, have been suggested as effective therapies for the prevention and treatment of OHSS via blockage of VEGF expression. Cabergoline has been administrated at dosages of 0.5 mg daily starting at the time of hCG trigger resulting in being effective for the prevention of moderate to severe OHSS in comparison with no treatment or placebo, whereas less data is available about its effects on reproductive outcomes [73]. No efficacy in reducing OHSS in comparison with other preventive intervention has been proven [73]. At the moment, cabergoline as an additional preventive measure for OHSS is suggested only in GnRH agonist cycles, whereas it is not recommended when a GnRH agonist is used for triggering final oocyte maturation in GnRH antagonist cycles [33].

Few data regarding quinagolide, another non-ergot-derived dopamine agonist, an RCT [74] demonstrated that quinagolide (at dosages of 50, 100, 200 μg/day) is effective in reducing the risk of moderate or severe early OHSS in a dose-dependent manner.

Aspirin

Aspirin inhibits cyclooxygenase-1 (COX-1) in the platelet and results in an antiplatelet effect. Its administration may alter the pathological cascade secondary to VEGF and be used as a preventive measure for reducing platelet activation due to VEGF levels, and thus the release of substances such as histamine, serotonin, platelet-derived growth factor, or lysophosphatidic acid that can further potentiate the severity of OHSS. Notwithstanding available data showing a reduction in OHSS incidence using 100 mg aspirin (with or without corticosteroids) [75], the available evidences are not of good quality to suggest the routine use of aspirin in the clinical practice [33], even if in high-risk patients it can be suggested also for reducing the risk of thromboembolic events [7].

Corticosteroids (methylprednisolone)

Corticosteroids, and in particular methylprednisolone, have been tested as prophylactic agents for OHSS development in consideration of their potent antiinflammatory action. They have been administrated alone or in combination with other interventions, such as glucocorticoids or intravenous albumin infusion, with significant benefit in terms of risk reduction [75]. In consideration of the clinically small reduction of OHSS risk (about 20%–30%) and of the new available strategies, corticosteroids are not recommended as a preventive measure for high-risk patients [7].

Calcium infusion

The increase in serum calcium level may inhibit cAMP-stimulated renin secretion and decrease angiotensin II synthesis and VEGF production. Based on this rationale, the intravenous administration of calcium, given as 10 mL of 10% calcium gluconate in 200 mL normal saline, on the day of oocyte retrieval and days 1, 2, and 3 after oocyte retrieval has been studied to decrease the risk of OHSS. Furthermore, the results obtained are mixed, and the evidence is fair to suggest calcium infusion as a preventive measure for OHSS [7]. The infusion of calcium is not better than cabergoline in terms of reduction of OHSS [73].

Ketoconazole

Ketoconazole is an inhibitor of steroidogenic P450 enzymes in the adrenal cortex and the gonads. Sparse data have examined the potential clinical use of ketoconazole for attenuation of ovarian response to gonadotropin treatments. A double-blind placebo-controlled RCT demonstrated that ketoconazole (50 mg every 48 h) starting on the first day of gonadotropin administration does not prevent OHSS in patients with PCOS [76].

Diosmin

Diosmin is a natural flavonoid commonly used for treating chronic venous diseases. Recent data has indicated that diosmin possesses several pharmacological activities, including antiinflammation and antioxidation activities. An RCT has showed no difference in OHSS risk between diosmin and cabergoline [73]. Diosmin is reported as also effective to reduce the severity of the syndrome [77].

Luteal GnRH antagonist administration

The administration of GnRH antagonist during the luteal phase has been experimented as an intervention to prevent early OHSS and to reduce the severity of the syndrome [78]. GnRH antagonist, administrated daily using subcutaneous injections of 0.25 mg, suppresses LH release and induces a significant decline of VEGF [79]. The efficacy of luteal GnRH antagonist administration is an effective intervention as well as the volume expansion therapy [80] and has been studied in multiple-interventions strategies (including addition of cabergoline to GnRH agonist triggering with subsequent addition of GnRH antagonist for 5 days in the luteal phase) [81]. More recently, GnRH antagonist administration was effective to prevent moderate and severe OHSS, and to induce a faster regression of OHSS symptoms [82]. At the moment, those data need to be confirmed in large, well-powered RCTs.

Volume expanders

A number of clinical studies with conflicting results have reported on the use of plasma expanders such as albumin, hydroxyethyl starch (HES), mannitol, polygeline, and dextran as a possible intervention for the prevention of OHSS [56,72].

Since albumin increases plasma oncotic pressure and binds to vasoactive substances, a potential role of its administration has been suggested to counteract the permeability related to angiotensin II and to block factors related to the renin-angiotensin system and VEGF. Even if initial studied have showed that the intravenous administration of 20% human albumin around the time of oocyte retrieval decreased the incidence of moderate to severe OHSS compared with no treatment or placebo, more recent data have demonstrated that albumin does not prevent OHSS and may reduce the pregnancy rate [56,72]. The lack of efficacy of albumin is due to insufficient oncotic pressure generated to prevent OHSS because the albumin itself leaks into the extravascular space, whereas the compromised pregnancy rate after albumin administration is probably due to binding to other molecules involved in implantation. In consideration of these inconclusive data and that albumin is expensive and a blood-derived product, and can lead to allergic/anaphylactic reactions, and the transmission of viral or unidentified diseases, the use of albumin cannot be recommended or suggested to reduce the risk of OHSS [7,33].

Other volume expanders seem to have an influence on pregnancy rates but safety data are sparse and inconclusive [56,72]. HES is much cheaper and is a nonbiologically derived colloid fluid and is free from the risks detailed above for albumin. HES is effective with a risk 80% lower than placebo [56,72]. Unfortunately, some studies report an increased risk of mortality in patients with sepsis and an increased risk of kidney injury requiring dialysis in critically ill patients treated with HES [4].

Luteal phase support

Progesterone and synthetic progestogens represent the gold standard treatments for luteal phase support after IVF [35]. In particular, new evidences demonstrate the best efficacy of oral dydrogesterone in IVF cycles [83]. This treatment is strongly recommended for hyperresponder patients. On the other hand, hCG administration for luteal phase support after classic hCG ovulation triggering should be avoided for the high risk of OHSS [84]. hCG in addition to standard progesterone luteal phase support or after GnRH agonist triggering increases the overall risk of OHSS closely related to doses and times of administration [51].

Treatment

The main principle for a correct management of OHSS is to individualize the treatment, avoiding the standardization also in patients with the same severity (OHSS stage) [7,9,85].

OHSS is a self-limiting condition in patients who do not conceive, and it typically resolves at the time of the next menstrual period. On the contrary, in patients who do become pregnant, rising hCG levels continue to stimulate the ovaries and symptoms may extend through the end of the first trimester. Generally, the management of OHSS is dependent on severity and presence of comorbid conditions [85].

Patients with mild to moderate OHSS may be managed on an outpatient basis, while severe OHSS always requires hospitalization. In particular, patients presenting with severe abdominal pain or distention, intractable emesis, hemoconcentration (hematocrit >45%), abnormal liver function studies, intraabdominal hypertension, oliguria or anuria, hypotension, tachypnea, dyspnea, syncope, and/or electrolyte disturbances should be always strictly monitored in a specialized setting [7,9]. The same patients with a symptomatic moderate syndrome that are not able to do the self-monitoring should be also managed as inpatients and hospitalized. The treatment of OHSS is primarily supportive, and in most cases, OHSS follows a self-limited course that parallels the decline in serum β-hCG [7,9,85].

Several treatments, such as GnRH antagonist administration (250 mcg daily) [82], cabergoline (0.5 mg daily) [73] or their combination [86], or diasmin [77] may be useful to induce a faster regression of OHSS symptoms. However, it is not possible to recommend any of these for the lack of adequate well-done clinical data.

Outpatients

Mild and moderate OHSS may be treated on an outpatient basis with symptomatic relief, monitoring, and close follow-up in 2–3 days. These patients should be counseled about the need to monitor fluid intake and output, body weight, abdominal girth, and the necessity of avoiding nephrotoxic medications, including nonsteroidal antiinflammatories [7,9]. Analgesic and antiemetics may be used in women with OHSS [9]. A fluid intake of approximately 2 L of water daily is commonly suggested [5,18]. To avoid injury to the enlarged ovaries, patients are advised to avoid strenuous physical activity and coitus. On other hand, they should be instructed to mobilize and avoid strict bed rest [18].

Daily communication with the woman is recommended. Specifically, the woman is advised to contact her health provider with any of the following: an increase in weight of 1 kilogram or in abdominal girth of 2 cm, increasing pain, increasing abdominal distension, subjective oliguria, symptoms suggestive of thrombosis, or reduced mobility [7,9,18].

Thromboprophylaxis with low-molecular weight heparin doses is suggested especially in case of pregnancy at standard doses (4000 UI/day) [7,9,18]. Also antiembolism stockings may be suggested [9]. The patients must self-assess the symptoms, the body weight, and the urine output. In case of symptom worsening, weight gain of 1 kg/day or more, and urine output less than 20–30 mL/h, hemoconcentration is very probable. There is not strong evidence for suggesting or contraindicating paracentesis of ascitic fluid on an outpatient basis [9]. All laboratory tests should be repeated according to symptomatology assessed every 2–3 days [9].

Even if some authors have suggested to perform paracenteses for the management of OHSS in an outpatient setting, the evidence for its efficacy and safety is fair at the moment [7,9,18].

Inpatients

In case of severe OHSS the main clinal alteration is the hypovolemic hyponatremic state that is usually managed with fluid replacement to maintain intravascular perfusion and supportive care [5]. Hospitalized patients should have daily abdominal palpation, abdominal girth measurements at the level of the umbilicus, daily weight recorded, chest auscultation, and peripheral oxygen saturation levels checked every 2–8 h. All relevant clinical data (including history and clinical assessment), as well as the imaging and laboratory tests required, should be carefully reported on the clinical chart in longitudinal fashion to compare new data with previous to detect a potential improving or worsening of the syndrome [18].

A strict fluid balance is recommended [18]. Urine output should be always obtained via a urinary catheter, whereas only in selected cases a naso- or orogastric tube placement may be useful to improve the abdominal pression. In many cases, the addition of vasopressor therapy may be needed to maintain adequate perfusion. To this regard, norepinephrine and dopamine are two potential options, and for the reasons detailed before, dopaminergic agonists are preferred [5].

Correction of severe electrolyte abnormalities plays an important role in OHSS management. Hyperkalemia in these patients should be managed in the usual fashion [5]. Salt or water restriction are not recommended because they do not improve the patient's weight, peripheral edema, intravascular volume status, nor abdominal circumference [85]. On the contrary, hypertonic saline solutions (administrated at doses of 100–150 mL over 5–10 min), alone or in combination with colloid solutions, result in reduction in intraabdominal pressure, expansion of intravascular volume, and correction of hyponatremia [85].

Pulmonary support may involve thoracentesis, oxygen supplementation, and noninvasive ventilation, whereas if ARDS develops, mechanical ventilation is needed [5]. The presence of ARDS makes the fluid management more and more complicated and personalized to maintain systemic perfusion and adequate renal perfusion [5].

Diuretics should be used only in specific cases (pulmonal edema, oligo-anuria, etc.) [5,7,9,18]. In general, they should be avoided because they may worsen hemoconcentration and hypovolemia, increasing the risk of venous thromboembolism, and should be administrated only in combination with colloid solutions (including human albumin). Similarly, the use of volume expanders alone for the treatment of OHSS is not supported by clinical evidence [7,85]. On the other hand, in oliguric patients, an aggressive regimen including volume expanders (25% albumin 250 mL) with diuretic (furosemide 20 mg or bumetanide 1 mg) and dopamine IV (2–3 mg/kg/min) every 8 h may be suggested [5,7] after failure of fluid infusion and paracentesis [9].

Glucocorticoids (methylprednisolone 30 mg/kg) may provide some benefit in the treatment of ARDS in the setting of OHSS [5]. Suggestions about albumin infusion are variable. In fact, albumin has been recommended when the serum albumin level is < 20 g/dL, or <30 g/dL, when the hematocrit is $>45\%$ or when severe ascites is present (see below) [18]. At the moment, there is no role for angiotensin converting enzyme inhibitors (also for their teratogenicity) nor for antihistamines [18].

Paracentesis may be performed transvaginally (culdocentesis) or transabdominally under ultrasound control under either local anesthesia or light sedation. Paracentesis may be suggested only in case of severe symptomatology due to ascites (such as dyspnea, abdominal pain for distention, and oliguria), for evaluating spontaneous bacterial peritonitis, or in presence of intraabdominal pressure higher than 20 mm Hg [5,7,9,18]. The transabdominal approach is preferred because it is considered safer in terms of infections and complications. It is not possible to define the optimal volume of peritoneal fluid to be removed, even if about 1000 mL may generally be an appropriate initial amount to remove [5]. The procedure should always be performed under ultrasound guidance and slowly for avoiding any vascular injury or puncture of the small bowel and/or large ovarian cysts, and the rapid reaccumulation of ascites with lost proteins in the intravascular compartment [5]. During the procedure, human albumin (20%) should be always infused to maintain intravascular volume [5,9,18].

Surgical management is indicated in the presence of ovarian torsion, pregnancy termination, intraabdominal hemorrhage, ectopic/heterotopic pregnancy, or ruptured cysts. All abdominal procedures may be performed laparoscopically following the usual surgical steps, even if an expert surgeon is always needed for the high risk of complications [9,18]. In critical cases, pregnancy termination may be suggested to reduce the risks due to high and prolonged levels of β-hCG. A full written consent form should be prepared case by case. Generally, these cases regard multiple pregnancies and can be treated with medical therapy (mifepristone and mifeprostole) or uterine suction under local or general anesthesia.

If infection is suspected, waiting for the results of the cultures, an empiric antibiotic therapy that has broad coverage against the most common bacteria should be initiated. An antibiotic regimen including a third or fourth generation cephalosporin in combination with metronidazole may be suggested [5]. After 48 h, alternative agents including imipenem-cilastatin, meropenem, doripenem, and piperacillin tazobactam may be started in case of worsening of the clinical conditions and suspected resistance [5].

Conclusions

At the moment, OHSS, specially in its moderate to severe forms, is a rare condition, especially if evidence-based data are carefully followed. However, all clinicians should know the syndrome and remain alert about the possibility of OHSS in all women undergoing fertility treatment [9]. In fact, before starting a fertility treatment including gonadotropins, each clinician should provide verbal and written information concerning OHSS to all women undergoing fertility treatment and ensure close liaison and coordination with referral units where their patients may be managed [9]. In fact, OHSS is also a largely unpredictable condition because genetic predisposition play a crucial role.

Available data suggest that all efforts should be made to reduce the use of gonadotropins for ovarian stimulation in anovulatory patients and for IUI cycles. Great attention should be given to the presence of risk factors for hyperresponse and high risk for OHSS. Initially (before start of stimulation), the ovarian response may be predicted according to all the patient's characteristics since AFC and AMH determinations alone cannot be totally useful. In potential high responders, the LH surge suppression should be managed with a GnRH antagonist. and the gonadotropin starting dose should be lower than 150 IU daily, irrespective from the kind of gonadotropin. Only as a second choice is it suggested for LH suppression with GnRH agonist, but in these cases the gonadotropin starting dose should be still lower (for example, 125 IU daily).

Even if no evidence-based data are available about the monitoring and the potential interventions during ovarian stimulation, a new reassessment of the risks should be made at term of ovarian stimulation. If the OHSS risk is low to moderate, oocyte triggering can be done with hCG at full or half doses. In these cases, cabergoline treatment may be useful to reduce further the risk. If the OHSS risk is high (for example, in presence of more than 18 follicles) a new counseling with the couple is needed. In case of patients who received GnRH antagonist protocol, LH surge should be triggered with GnRH agonist (triptorelin 0.1−0.4 mg) and all oocytes/embryos frozen. In case of patients who received GnRH agonist protocol, the use of a half dose of hCG plus cabergoline is recommended [33] followed by embryo freezing or embryo transfer according to clinical choice. Progesterone and synthetic progestogens represent the gold standard treatments for luteal phase support after IVF, and hCG should be not used because it increases enormously the risk of OHSS. In all patients at high risk for severe OHSS, the cancellation of the cycle is always a potential option.

The crucial concern is that the risk profile of the patient does not predict always the OHSS risk, and preventive measure are not systematically adopted, and the syndrome can develop. All available reviews, guidelines, and recommendations suggest managing patients with OHSS case by case since the procedures and interventions are largely based on expert opinion rather than strong evidence. Outpatient management is appropriate for women with mild or moderate OHSS. However, in severe to critical cases hospitalization is needed. Clearly,

in these cases the management is multidisciplinary and severe to critical patients with OHSS should be admitted in specific centers with knowledge in the pathophysiology and treatment of the syndrome. Thus, the development of diagnosis and treatment with local protocols within each referral hospital is a priority for all clinicians.

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CHAPTER

24

Sperm quality evaluation and cryopreservation

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Sperm quality evaluation

Semen quality evaluation forms an integral part of assessment of the infertile couple. The semen sample must be assessed using standardized and reproducible techniques and ranges to ensure integrity of both clinical care and research. It is important that the patient is provided with clear, written information regarding the process of sample collection, handling, and storage [1].

Sample collection

The World Health Organization (WHO) *Laboratory Manual for the Examination and Processing of Human Semen* (2021) provides clear guidance on the collection of a semen sample [1]. Typically a sample is collected via masturbation into a clean container in a private room in close proximity to the lab. If collection via masturbation is not possible, alternative options include vibratory therapy or the wearing of a specially designed condom during sexual intercourse. If the sample is collected in a location away from the lab, during transport the temperature must remain between 20 and 37°C. The ejaculate needs to be completely collected and any loss of a portion of the sample must be reported by the patient. The sample should be provided after a minimum of 2 days and a maximum of 7 days of ejaculatory abstinence. Ejaculates may contain dangerous infectious agents and should therefore be handled as a biohazard. Some assessments of semen quality, e.g., macroscopic appearance, assessment of liquefaction, and assessment of vitality, must be undertaken within 30–60 minutes of collection. Other tests, e.g., assessment of concentration, must be performed within 3 hours. Assessment of sperm morphology is not under such strict time constraints and can be established within a few days. This allows the laboratory to organize an efficient workflow without jeopardizing examination quality [1].

Reference ranges

The WHO reference limits (Table 24.1) were produced from studying the semen parameters of 1953 men across 3 continents [1]. Only men who had a recently proven record of fertility (their partner had conceived within the last 12 months) were included. The fifth centile was calculated based on the parameters of these men and used as the lower reference limit [2]. This remains controversial as the reference ranges were based on fertile men so cannot necessarily predict infertility [3]. Increasingly the total motile sperm count grading (*sample volume × density × percent of A and B motility spermatozoa*) is gaining favor as a superior way to classify semen compared with the WHO classification [4].

TABLE 24.1 WHO reference ranges [1].

Parameter	Lower reference limit
Semen volume	1.5 mL
Total sperm number	39 million
Semen concentration	15 million/mL
Total motility	45%
Progressive motility	32%
Vitality (live spermatozoa)	58%
Morphology (normal forms)	4%

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Semen analysis

Basic assessments

Basic assessments of sperm quality would be expected to be carried out by all labs that investigate human ejaculate (Box 24.1).

Ejaculate volume

Ejaculate volume is calculated by weight in a preweighed container using an established value for semen density (1 g/mL) [5]. As sperm, produced in the testes, travels through the reproductive tract, fluid is added from the accessory glands of the seminal vesicles, prostate, epididymis, and periurethral glands [6]. An adequate volume of ejaculate is required to transport and nourish the sperm through the female reproductive tract [7]. Low semen volume can be artifactual, psychogenic, pathological, or idiopathic [7]. Artifactual causes include incomplete collection or a short abstinence period. Psychogenic causes include anorgasmia. Anorgasmia can be identified via careful history to avoid unnecessary and burdensome investigations for patients who might instead require psychosexual counseling. True pathological causes of low semen volume include retrograde ejaculation, ejaculatory duct obstruction, congenital absence of the vas deferens, and hypogonadism [7]. Congenital absence of the vas deferens can be associated with cystic fibrosis and may be associated with other genitorurinary abnormalities [7]. If retrograde ejaculation (semen passing into the bladder at ejaculation) is suspected, a sample of postorgasmic urine should be assessed for the presence of spermatozoa [8].

BOX 24.1

Basic sperm quality assessments

1. Assessment of ejaculate volume
2. Macroscopic assessment
   - **(a)** Macroscopic appearance
   - **(b)** Liquefaction, becoming thinner
   - **(c)** Ejaculate viscosity
   - **(d)** Ejaculate odor
   - **(e)** Ejaculate pH
3. Microscopic assessment
   - **(a)** Assessment of sperm clumping
   - **(b)** Cellular elements other than spermatozoa
   - **(c)** Sperm motility
   - **(d)** Sperm vitality
   - **(e)** Counting spermatozoa and other cells
   - **(f)** Sperm morphology

Macroscopic assessment

A normal liquefied ejaculate is creamy-grey in color, becoming more yellow with abstinence due to carotene pigment [1,9]. Discolouration can suggest pathology such as hemospermia, drugs, jaundice, or contamination with urine (bladder neck dysfunction) [1,9]. If the ejaculate appears viscous, totally clear, and colorless, then it may be preejaculate from only the Cowper's glands in a patient who did not orgasm [1]. There is interobserver variation in who can or cannot smell semen, but a strong odor of urine or infection may be of clinical significance [10]. Odor is generally produced due to sperm oxidation [9]. Normal seminal pH is within the range of 7.2−8.2 and is a balance between the alkaline contribution of the seminal vesicles and the acidic contribution of the prostate [9]. As such, changes in pH usually reflect inflammation in the accessory glands [9]. Ejaculate viscosity is measured after liquefaction by allowing the semen to drop with gravity from a pipette [1]. Viscosity represents resistance to flow that can affect sperm motility, antibody coating of spermatozoa, and concentration [9]. The clinical significance of semen that fails to liquefy is unclear [11].

Microscopic assessment

Pregnancy rates via both natural conception and intrauterine insemination decline in the presence of low sperm concentration [12,13]. Azoospermia describes the absence of sperm in the seminal plasma, while oligozoospermia refers to a concentration of <20 million/mL [1]. Other descriptors of semen quality assessment can be found in Table 24.2 [14]. The number of spermatozoa in the ejaculate is calculated from the concentration of spermatozoa and the ejaculate volume, and it is a functional measure of the testes [1,15]. In comparison the concentration of sperm is not a measure of testicular function as it depends upon the amount of fluid added by the accessory glands [16].

There are several types of sperm motility, but it is rapidly progressive motility that propels sperm through the cervical mucus and thus is related to pregnancy rates (Table 24.3) [1,17,18]. Abnormalities in sperm motility may reflect abnormalities in the accessory glands [9]. Abnormalities in sperm motility are a predictor of fertilization success and thus an important variable in decisions regarding mode of fertility treatment [18]. The total number of progressively motile spermatozoa is calculated by *the total number of spermatozoa in the ejaculate x the percentage of progressively motile cells* [1]. If <40% of the spermatozoa are progressively motile,

TABLE 24.2 Terminology [14].

Aspermia	No semen or ejaculate produced
Normospermia	Normal sperm count, motility and morphology
Oligozoospermia	Decreased total number of sperm
Asthenozoospermia	Decreased percentage motility
Teratozoospermia	Decreased percentage normal forms
Oligoasthenozoospermia	Decreased percentage of motility and normal forms
Oligoasthenteratozoospermia	Decreased number of sperm and decreased percentage of motility and normal forms
Azoospermia	No sperm in the ejaculate
Cryptozoospermia	No sperm seen in the ejaculate, but sperm found in the centrifuged pellet
Hematospermia	Presence of red blood cells in the semen
Leukocytospermia	Presence of white blood cells in the semen
Necrozoospermia	Decreased percentage liver sperm and increased percentage immotile sperm

TABLE 24.3 Categories of sperm movement [1].

Rapidly progressive (25 μm/s)	Spermatozoa moving actively, either linearly or in a large circle, covering a distance, from the starting point to the end point, of at least 25 μm (or ½ tail length) in 1 second
Slowly progressive (5 to < 25 μm/s)	Spermatozoa moving actively, either linearly or in a large circle, covering a distance, from the starting point to the end point, of 5 to < 25 μm (or at least one head length to less than ½ tail length) in 1 second
Nonprogressive (<5 μm/s)	All other patterns of active tail movements with an absence of progression—i.e., swimming in small circles, the flagellar force displacing the head less than 5 μm (one head length), from the starting point to the end point
Immotile	No active tail movements

then an assessment of sperm vitality should be undertaken to discriminate between immotile dead sperm and immotile live sperm [1].

Sperm morphology can be assessed via the WHO classification or Kruger's strict criteria. WHO classifies sperm based on abnormalities in the head, tail, and mid-section [1]. In Kruger's strict classification, all borderline forms are considered abnormal [19]. The clinical implications of poor sperm morphology remain controversial and should not be used as an isolated parameter; indeed pregnancy is thought to be possible with low morphology scores [20].

Extended assessments

There are a variety of extended assessments of semen quality offered only by specific labs (Box 24.2). However the clinical value of some of these tests is yet to be fully elucidated [21,22,23].

Sperm DNA fragmentation

Sperm DNA fragmentation (SDF) is an area that has garnered great interest in recent years. SDF is thought to be increased by infection, hormonal disruptors, and lifestyle factors such as smoking [23]. Evidence suggests that the spermatozoa of subfertile men have greater DNA damage than their fertile counterparts [24]. Sperm DNA damage is associated with increased rates of miscarriage, recurrent miscarriage, birth defects, and poorer assisted reproductive techniques (ART) outcomes [25,26]. There are various ways to quantify sperm damage; the most common methods are abnormal sperm chromatin packaging assessment and sperm nuclear DNA integrity assessment (Table 24.4) [24,27,28]. DNA integrity can be assessed either directly using reagents that attach directly to damaged areas and are viewed under fluorescence or light microscopy, or indirectly using protein denaturation in an acidic solution [27,29]. The most commonly known direct measure is the TUNEL (terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling)

BOX 24.2

Extended assessments [1]

1. Indices of multiple sperm defects
2. Sperm DNA fragmentation
3. Genetic and genomic tests
4. Immunology tests
5. Assessment of interleukins (marker of male genital tract inflammation)
6. Assessment of immature germ cells in ejaculate
7. Testing for antibody coating of spermatozoa
8. Assessment of accessory gland function (biochemical assays)
9. Assessment of sequence of ejaculation

TABLE 24.4 Tests for sperm DNA fragmentation [24,27,28].

Sperm nuclear DNA integrity assessments	Direct	TUNEL assay (terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling): Attachment of deoxyuridine triphosphate (dUTP) to single- and double-strand DNA breaks
		In situ nick translation (ISNT): Attachment of biotinylated dUTP to single-strand DNA breaks
	Indirect	Sperm chromatin structure assay (SCSA)
		Sperm chromatin dispersion (SCD) assay
		Comet assay
Sperm chromatin packaging assessment	Staining using methyl green, aniline blue, toluidine blue, and chromomycin A3	

assay; pregnancy is considered unlikely if SDF is >12% [30]. Indirect measures include sperm chromatin structure assay (SCSA), the Comet assay, and the sperm chromatin dispersion assay (SCD). Definitive references ranges remain elusive for some of these tests, and cost limits their widespread use [24].

Genetic and genomic tests

It is becoming increasingly clear that genetic or genomic abnormalities underpin the pathophysiology of a significant proportion of male infertility. Sperm aneuploidy, the presence of more or less than the usual haploid chromosome number, can be tested for using fluorescent-in-situ-hybridization. There is an association between sperm aneuploidy, especially sex chromosome aneuploidy, and impaired spermatogenesis, increased levels of DNA fragmentation, recurrent implantation failure, and recurrent pregnancy loss [31,32,33]. The clinical rationale for testing for sperm aneuploidy is that these men can be offered preimplantation genetic diagnosis and IVF/intracytoplasmic sperm injection (ICSI) to enhance live birth rates [34].

Testing for antibody coating of spermatozoa

Sperm agglutination, evaluated in the basic semen analysis, can represent infection, antisperm antibodies, or ascorbic acid deficiency [35]. Furthermore, patients with sperm agglutination often report prior scrotal surgery or trauma [35]. Sertoli cells form a blood-testis barrier that offers immunological protection from sperm antigens [9]. In the event of disruption of this barrier, antisperm antibodies can develop and can be cytotoxic to spermatozoa, causing immobilization, agglutination, and cell death [10]. These can be tested for in specialist units.

Assessment of accessory gland function (biochemical assays)

Semen quality can be impaired by malfunction of the accessory glands [1]. Spectrophotometric assays allow measurement of the secretory function of the accessory glands. For example the amount of fructose reflects the secretory function of the seminal vesicles, and as such, low fructose suggests ejaculatory duct obstruction, retrograde ejaculation, or bilateral congenital absence of the vas deferens [36].

Advanced assessments

There is a selection of advanced assessments carried out only in specialized centers (Box 24.3).

BOX 24.3

Advanced assessments [1]

1. Quantification of reactive oxygen species (ROS)
2. Assessment of acrosome reaction
3. Assessment of sperm chromatin
4. Transmembrane ion flux and transport in sperm
5. Computer-aided sperm analysis (CASA)

Quantification of reactive oxygen species

Reactive oxygen species (ROS) have been suggested to impair sperm parameters and increase SDF, thus its association with miscarriage and infertility [37]. Sperm ROS can be measured by incubating semen with luminol and measuring the light emitted using a luminometer [38]. This offers a promising advance; however currently standardized reference ranges are not established [39]. Currently the European Association of Urology does not endorse routine measurement of ROS in the investigation of the infertile male [3].

Computer-aided sperm analysis

Computer-aided sperm analysis (CASA) involves a computer system with a high-resolution camera

attached to a microscope. It carries the advantage of automatically capturing the data for subsequent reassessment and is thus more reproducible and time efficient than manual semen analysis [40]. CASA has a precision of at least 97% in assessing sperm morphology, and evidence suggests computer-aided analysis of sperm morphology can predict fertilization and pregnancy likelihood [41]. Furthermore, CASA allows assessment of sperm motion that cannot be assessed by standard, manual semen analysis [42]. These kinematic parameters include linearity, amplitude of lateral head displacement and assessment of velocity curvilinear velocity, average path velocity, straight-line velocity and linearity [9]. However, CASA is far less accurate than manual semen at measuring sperm concentrations and number of immotile sperm as it has limited ability to differentiate between debris and immotile sperm [43].

Sperm preparation and selection

Spermatozoa need to be separated from seminal plasma for use in ART. Preparation for sperm used in ART aims to ensure good quality sperm are used [1]. It has been suggested that one cause for the relatively low success rate of ART is the lack of an optimum sperm selection process [44]. This is in contrast to the physiological selection of superior sperm during natural conception. Furthermore, the use of defective spermatozoa may lead to long-term health implications on the offspring [45]. There are a variety of techniques available to prepare and select sperm for use, some more experimental than others (Table 24.5).

Routine techniques

1. Simple washing:
   Simple sperm washing involves the use of a media supplemented with human serum albumin (HSA) and centrifuged to remove seminal plasma. It does not remove debris and leukocytes [1]. If the semen sample is of high quality, it provides a high yield of spermatozoa [1].
2. Direct swim-up:
   To separate motile from nonmotile spermatozoa a "swim-up" technique can be used. This involves placing a layer of culture medium over the semen and motile spermatozoa will swim into the culture medium supplemented with HSA, leaving the nonmotile spermatozoa behind. Centrifugation should not be used prior to swim-up as this may induce peroxidative damage of the cell membrane [46]. This process provides a lower yield of spermatozoa but is valuable in a sample with a significant proportion of immotile sperm [1].
3. Discontinuous density gradients:
   This method describes centrifugation of semen over a density gradient to provide a small sample of highly motile sperm, separated from leukocytes, debris, and nonvital germ cells [1].

TABLE 24.5 Sperm preparation and selection techniques [46,47].

Routine techniques	• Simple washing • Direct swim-up • Discontinuous density gradients
Advanced techniques	• Physiological intracytoplasmic sperm injection (PICSI) • Magnetic activation cell sorting (MACS) • Intracytoplasmic morphologically selected injection (IMSI) • Microfluidic sperm sorting (MFSS)

Advanced techniques

1. Physiological intracytoplasmic sperm injection (PICSI):
   This technique uses hyaluronic acid to select quality sperm for use in ICSI. Hyaluronic acid is a major component of the oocyte extracellular matrix, and it has been suggested that sperm able to bind to hyaluronic acid are adequately developed and mature [48]. PICSI identifies sperm capable of binding to hyaluronic acid-coated dishes. However a 2019 Cochrane review did not find an association between PICSI use and increased live birth rates [49].
2. Magnetic activation cell sorting (MACS):
   Sperm with DNA damage may undergo apoptosis, which manifests in the early stages as externalization of phosphatidylserine [50]. Coating magnetic nanoparticles with molecules that have a high affinity for phosphatidylserine, e.g., annexin V, leads to binding of apoptotic spermatozoa. With the use of a strong magnetic field, this can allow separation of sperm to provide a subpopulation of spermatozoa without evidence of apoptosis [51]. However a 2019 Cochrane review did not find an association between MACS use and increased live birth rates [49].
3. Intracytoplasmic morphologically selected injection (IMSI):
   During microscopy at standard optical resolution and magnification (x200−x400), sperm morphologic defects may not be identified. IMSI uses greater optical magnification (x600−x6600) to allow the embryologist to assess sperm morphology in greater detail to select the optimum sperm for use in ICSI.

However a 2020 Cochrane review was unable to ascertain if IMSI offers statistically significant benefits over standard ICSI [52].

4. Microfluidic sperm sorting (MFSS), e.g., Zymot:
   Zymot, also called a chip, is a membrane filter designed to mimic aspects of natural conception. The sperm are required to actively swim through the filter. By selecting sperm based on motility, it is thought to select for sperm with less DNA damage [47].

Cryopreservation of spermatozoa

Sperm cryopreservation ("sperm freezing") describes a process to preserve male gametes. The process involves the collection of a semen sample followed by cooling with an agent such as nitrogen vapor and storing for future use [53]. Sperm cryopreservation is most commonly used for fertility preservation in those whom future fertility might be compromised, e.g., prior to cancer treatment or gender reassignment, or in those with existing fertility concerns during ART. Other indications include altruistic donation of gametes for heterologous use and to reduce the transmission of blood-borne disease.

Sample collection

Samples are produced by masturbation as standard. In some cases surgical sperm retrieval may be needed, e.g., obstructive and nonobstructive azoospermia. Retrieval can be via percutaneous epididymal sperm aspiration or testicular sperm extraction depending upon the underlying cause [54].

Cryopreservation techniques

The biochemical processes that lead to cell death are stopped at −196°C, the boiling point of liquid nitrogen [3]. There are various protocols for cryopreservation, but none have been perfected as freezing and thawing sperm still risks cell damage [3]. Damage is often caused by ice crystal formation and dehydration. Cryoprotectants, most commonly glycerol mixed with egg yolk, are used to reduce sperm damage [55]. Prior to freezing a cryoprotectant solution is add to the semen sample. This mixture is then aspirated into straws, heat-sealed, and then frozen with liquid nitrogen. The sample is stored in the straw, and when required for use thawed at 37°C [1].

Indications

Fertility preservation

Cancer treatment

The gonadotoxic action of systemic cancer therapy makes pretreatment fertility preservation important for all oncology patients but none more so than children and adolescents. The prognosis of most childhood cancers is now good, and long-term survivors often report concern regarding their fertility as adults [56,57]. Indeed, when compared with their siblings, cancer survivors are approximately half as likely to biologically father a child [58]. The degree of risk of infertility depends upon cancer type, with testicular and hematological malignancies carrying an especially high risk [59]. The American Society of Clinical Oncology advises clinicians should offer sperm cryopreservation to all postpubertal males of reproductive age receiving cancer treatment [60]. However a UK survey revealed only 38% of patients receive this [61]. It is vital that samples are produced prior to the onset of treatment as sperm is at risk of genetic damage following cancer treatment [62]. There is little evidence to support downregulation of testicular function by gonadotrophin-releasing hormone antagonists in a bid to reduce cytotoxic damage [63]. For men who do not regain testicular function the cryopreserved semen can be used, up to 40 years after its collection, with the aid of ART to conceive [64]. Even in the setting of testicular cancer, 50% of patients will have recovered spermatogenesis at 2 years [65]. If spermatogenesis has recovered prior to the wish to conceive the patient will require extensive counseling regarding their options; posttreatment sperm may be inferior to the pretreatment sample; however the use of the cryopreserved sample requires invasive and expensive ART [66].

Gender reassignment

Male-to-female gender reassignment treatment can render an individual reversibly or irreversibly infertile by virtue of estrogen therapy or bilateral orchidectomy [66]. Increasingly individuals are undergoing such procedures at a life stage where starting a family is not a priority, but this might otherwise become an unmet need in later life [67]. As such the American Society of Reproductive Medicine recommends offering gamete cryopreservation to all patients undergoing gender reassignment [68].

With assisted reproductive techniques

Autologous use

Surplus sperm retrieved for IVF/ICSI may be banked as a "back-up" in patients for whom its future use is

anticipated such as those with severe oligozoospermia or intermittent presence of motile spermatozoa [66]. Sperm can also be banked in those for whom a future need for ART is assumed, for example in patients with Klinefelter syndrome for whom a semen sample can be collected at puberty [69]. Some men will be unable to provide a fresh sample to coincide with his partner's egg collection so will use a previously cryopreserved sample. This includes posthumous use of semen, in men for whom the production of a sample is psychologically challenging, or men who require elective surgical sperm extraction such as those with nonobstructive azoospermia or those rendered anejaculatory secondary to a spinal cord lesion [70]. Of importance there is considered to be no difference in success rates when using cryopreserved or fresh sperm during ART, but there is evidence of increased sperm DNA fragmentation in the cryopreserved group [71,72,73]. Furthermore, in severe male factor, ART with the partner's sperm can be much less successful than ART with donor sperm [74].

Heterologous use

Donor sperm can be stored in sperm banks for use during ART. Indications for the use of donor sperm include prevention of transmission of infectious or heritable disease, severe male factor infertility, and fertility treatment for single women or women in same-sex relationships [55]. Rigorous testing to exclude infectious or genetic disease is a requirement prior to sperm donation, and the process is bound by strict legal guidance [75].

Risks

Evidence suggests that cryopreservation impairs semen quality. DNA sperm damage and the proportion of abnormal sperm motility and morphology parameters are increased following thawing of a cryopreserved sample [76]. However, by virtue of many of the indications for sperm cryopreservation, studies often include patients with preexisting abnormal sperm parameters prior to freezing. A 2021 retrospect cohort study of >6000 men with normal sperm parameters undergoing ICSI found that in normozoospermic men, cryopreservation had no deleterious effect on pregnancy or live birth rates [77].

Challenges

The legal and ethical aspects of sperm cryopreservation are manifold. The Human Fertilisation and Embryology Authority (HFEA) allows freezing for up to 55 years for those with irreversible infertility, though NHS funding may be limited to 5–10 years depending upon geographical location [66]. To reduce inadvertent consanguinity the HFEA recommends one sperm donor only contributes to a maximum of 10 families [78]. Following a 2015 policy change, sperm donors are no longer granted full anonymity; at the age of 18 the child is able to access certain identifying features of their genetic father [79]. This has led to as of yet unfounded fears that the donor pool may diminish [79]. Posthumous use of sperm is a highly contentious issue and is prohibited in the United Kingdom without prior written consent [80].

Future

Future horizons include the development of advanced freezing and cryoprotectant strategies. Another area of ongoing research is cryopreservation of stem cells and testicular tissue samples [81].

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CHAPTER

25

In vitro fertilization and embryo culture in time-lapse imaging

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Introducing time-lapse devices

The last decade has seen increasing interest and implementation of time-lapse imaging (TLI) systems within fertility clinics offering more continuous monitoring and incubation, compared with conventional practices. Most time-lapse devices combine imaging and incubation hardware with specialized software, providing multiple focal plane images of the developing embryos and the facility to automatically or manually record and time stamp developmental events.

When implementing time lapse within an *in vitro* fertilization (IVF) laboratory, there are many factors for consideration. These include reliability of the supplier and device footprint, ease of use, scalability, capacity, alarm system compatibility, training and support provision, and technical specification (e.g., number of focal planes, facility to humidify, whether it requires ready-mixed gas or performs gas mixing).

Once installed, as for all critical equipment, robust training of staff is vital to ensure safe and effective operation. Training is commonly provided by the device manufacturer, but it is also recommended to undertake training with expert users whose tips and tricks can be invaluable.

Standard operating procedures are required to incorporate equipment setup, maintenance, and troubleshooting, culture dish or slide preparation, recording of developmental events by automatic or manual annotation, and fertilization and embryo assessment and selection, which can differ from conventional practices due to the increased quantity of information generated [1].

New users may be daunted by the apparent relative complexity of the time-lapse device compared with standard incubation and static microscopy, but they may wish to consider the device as the incorporation into a single unit of an incubator, workstation, microscope, and data capture system. Time-lapse users often report benefits in workflow, and the vast number of time-lapse-related publications in medically assisted reproduction demonstrates the improvement in our knowledge and understanding of the preimplantation human embryo.

Embryo selection using time lapse

Depending on the device, setup, and laboratory protocol, the output from the time-lapse device can range from a simple series of timings at which the embryo reaches developmental milestones (morphokinetics) to detailed image and morphokinetic and metadata. Morphokinetic information is generated either automatically by the device using computer vision, or image recognition software, or by manual assessment and recording of these events by the embryologist, or annotation.

The use of embryo annotation for embryo selection can take time to prepare and implement. In the first instance, TLI can be used for simple deselection of embryos. There are well-documented abnormal division events that have been observed using TLI and linked to an embryo's capacity to implant. Implementation of deselection criteria can be utilized almost immediately following installation of TLI and does not require sophisticated annotation practices.

The observation of an abnormal division event can be indicative of reduced implantation potential, in particular multichotomous mitosis whereby a single cell in the developing embryo divides into three daughter cells instead of the expected two. A number of investigations have linked this form of abnormal division to

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implantation potential [2–6]. From these analyses the implantation potential of embryos exhibiting multichotomous mitosis was reported to be as low as 1.2%. Embryos that exhibit reverse cleavage, chaotic cleavage, or absent cleavage may also have a reduced chance of implantation [2,4,7] In addition to these "macro" division events, certain embryological features could indicate reduced developmental potential, such as the presence of vacuoles [8] or the completeness of the compaction of the morula [9,10]. Though these latter observations could be seen in standard incubation, the use of TLI allows detailed monitoring of the progression of vacuoles and the continued inclusion of cells in the resulting morula, all while the embryos remain undisturbed. Utilizing these deselection criteria can provide an instant benefit to the laboratory when implementing TLI.

When the laboratory is ready to introduce more detailed annotation practices, at which point these annotations take place should be considered. This is largely service dependent and driven by the goals of the laboratory utilizing the technology. One option, and one which many laboratories adopt at an early stage, is the exhaustive annotation of all embryos. This allows the collation of useful data that can be used to develop in-house annotation policies and embryo selection models, discussed later. However, this is often impractical for larger laboratories or those that adopt TLI as the standard method of incubation, meaning all embryos created are cultured in TLI. Perhaps the more pragmatic approach would be to annotate a selection of parameters shown to influence an embryo's implantation potential and only annotate these on the embryos that are being considered for utilization. Next, the laboratory must consider when in an embryo's development annotation should take place. Some opt to annotate a number of parameters on each day of an embryo's development. For example, from pronuclei appearance to time to four-cell on day 2 of development, all divisions up to the eight-cell stage on day 3 followed by the post-compaction parameters, such as blastulation, on day 5 of development, while others choose to perform all annotations on the day of utilization. The former of these practices can be time consuming and is better adopted in conjunction with an exhaustive annotation program where the laboratory has chosen to annotate all parameters in the interest of data collation. Annotation on the day of utilization is better adopted where the annotations will be used in real time to make decisions regarding the fate of the embryo and where only those that are in consideration for utilization are to be annotated.

Once annotation policies have been implemented, it is important to monitor the quality of the annotations being performed by the laboratory team. This is particularly important if the annotation data is to be used to develop in-house embryo selection models or if a "plug and play" embryo selection model is being used that relies on annotations to aid in decisions regarding an embryo's fate. To do this, a robust annotation quality assurance scheme should be developed. This should involve the annotation of the same embryo(s) by all operators at specified time intervals. The number of embryos to annotate and how often the annotations should be evaluated can be determined based on previous performance. However a regular evaluation is recommended in the early stages of implementation of annotation. It is recommended that at least three embryos be annotated by all operators each month. The selection of the embryos to be used for this exercise will depend on the development of the annotation program in the laboratory. For example, if the use of annotation is novel in the laboratory, then the embryos used for evaluation should be those that follow the expected timeline and do not exhibit any abnormal division events. Conversely, if the laboratory is demonstrably practiced at annotating straightforward embryos or is introducing a new annotation parameter into practice, the selection of embryos for evaluation with more discordant developmental patterns may be recommended. For data analysis, interoperator agreement should be assessed using a two-way, mixed intraclass correlation coefficient (ICC) for consistency. From this analysis, five categories of agreement can be designated based on the ICC score; very weak (0–0.2), weak (0.21–0.4), moderate (0.41–0.6), strong (0.61–0.8) and very strong (0.81–1.0). The result is that very weak or weak agreement between operators can be easily identified and retraining provided where necessary. Consideration should also be given to evaluating intraoperator agreement. This can be achieved by selecting embryos for evaluation that have been used previously. Individual operators can then be evaluated by comparing annotations on the same embryos but on separate occasions. This will identify if the operator annotations have drifted or if they have changed practice. As the evidence of strong interoperator agreement increases, the interval between evaluations can increase from every month to every other month. However, it is recommended that a quality assurance scheme for annotation be performed at least every three months on at least three embryos.

Once the quality of the annotations has been assured, the creation and implementation of a device-integrated or a published, algorithm application or development of an in-house embryo selection model can be considered.

Commercial algorithms are largely derived from diverse patient populations across multiple clinics with varying clinical protocols [11] (Vitrolife known

implantation data [KID] score day 5), and they can be easily adopted and hold the promise of instantaneous ranking scores to aid embryo selection. Replication of published in-house derived models [12–15] can provide an alternative starting point when embarking on designing a selection algorithm. Although there is increasing evidence that the predictive value of these models is not reproducible, with issues of transference due to the use of specific clinical and laboratory practices and the resultant effect on morphokinetic variables [2,16]. To assure the selection model will rank embryos effectively, validation of the model performance against known outcome data is advised before adopting any selection model in clinical practice.

Developing an in-house derived algorithm maximizes the predictive value of your model as it is built on your own data derived from embryos cultured using known clinical and laboratory practices following a strict quality assured annotation scheme. Before embarking on this exercise, determine what the algorithm is to achieve. Is it to predict whether an embryo will blastulate, distinguish between embryos' chromosomal competence, or be able to predict implantation or a live birth? This decision will direct fertility professionals toward the outcome data required to generate a robust algorithm.

KID is commonly used for model development, with known live birth (LB) outcome considered the gold standard for prediction. It is important to remember that utilizing data derived from implantation outcome is reduced when transferring more than one embryo, as the fetal heart or LB outcomes cannot be traced back to a specific embryo. Using LB as the end point, though, is the gold standard for prediction, and it requires time to develop a sizable data set. Establishing an algorithm to predict LB will be less predictive than an algorithm designed to determine the capacity of an embryo to cavitate, as apart from embryo quality, the determination of a LB is dependent on the transfer procedure, endometrial factors, and other variables during the establishment of the pregnancy. The diversity of embryo quality is often limited as all included embryos have already been determined suitable for transfer by the embryologist utilizing morphologic grade [17] and avoiding known erroneous events linked to poor outcome. These effects can only be overcome by maximizing the size of data to ensure the model's success. When designing a model to ensure robust prediction, the consideration of the size of the data included in this process is imperative, with smaller samples sizes of 100–200 embryos potentially affecting the performance of your final model [18].

The variables to include should be assessed on their correlation to the outcome measure and on the consistency and accuracy of in-house annotation (if utilized) by examining the ICC from a quality assurance program. Some variables may be less reliably annotated and may affect the success of the model. To date there are few algorithms that incorporate morphologic biomarkers alongside known kinetic markers despite the correlation of key morphologic features to implantation potential [16]. Inclusion of morphologic markers alongside defined kinetic indicators has been associated with improved outcomes including pronuclear morphology [19] and the morula (blast six abstract) or trophectoderm grade [16]. The use of morphologic markers should be balanced against the knowledge that they are less consistently annotated than kinetic events and subject to greater degrees of inter- and intrapractitioner variation [20]. The need for a thorough understanding of time-lapse data cannot be understated; complete, consistently annotated data linked to a known outcome is imperative for the successful generation of a robust in-house derived model, and this is only possible by employing continuous quality assurance to verify data quality.

Once there is a quality assured data set with a known outcome measure for prediction, the data set is split to allow part to be used to train the model and part that is independent of this process, held back for testing and validating the model performance. Varying methods of analysis can be adopted to investigate the data, from simple correlations of a single variable to the outcome measure, to hierarchic models where a number of variables are included with an expected value or range, weighted for importance by their position in the hierarchy to create a decision tree. Logistic regression analysis may be a preferred method as mathematically transformed morphokinetic parameters are determined from the data using an exploratory approach. Expertise from a data analysist may allow you to determine "the best fit" and highlight the limitations and benefits of different statistical approaches.

The success of a model is generally determined by plotting the sensitivity against specificity generating an area under the curve (AUC) from the receiver operating characteristics (ROCs) curve [21]; a model with no predictive value will have an AUC of 0.5 on average, and an algorithm with perfect prediction will have a value of 1. A model performing well for AUC on the training and validation set should be assessed further for consistent prediction against novel data sets. Cross-validation can test the model's ability to predict against new random data and can help to reduce the chance of overfitting or selection bias. Partitioning the data, using

either exhaustive methods of leave one out or nonexhaustive methods where the data is divided equally (k-fold cross-validation), provides a measure of fitness of the model by determining the average performance from each partition. Validating the model against a variety of laboratories, patient types, demographics, and treatment types can further assess the robust nature of the predictive value prior to its prospective clinical use.

The finalized model should have excellent prediction but, at least when starting out, also be simple to understand to ensure ease of use and continued accuracy and interpretation. A score is normally utilized to determine a ranking of the embryos. The number of integers is defined by the decision tree or increments of probability linked to the outcome measure. If based on KID, this relates to the propensity of the embryo to implant, and guidance should be provided to clinical staff and patients on the interpretation of the score and how to select embryos when the score contradicts the morphology assessment.

Patient perspectives and feedback

The offering of TLI by fertility clinics can have several benefits for patients. As well as the increasingly reported improved embryo selection and associated reduced time to successful outcome, TLI provides fertility professionals and patients the facility to observe short video footage of embryo development to scrutinize and compare anomalous and regular embryo development. These videos can be used as a consultation tool to help patients understand how their embryos develop and may provide some insights into the selection made by the embryologist and the outcome of the treatment. Patients have described how time lapse has aided their understanding of what takes place in the embryology lab and have reported positively about the facility to download the videos of their own embryos [1].

In addition, it seems that patients like that their embryos remain undisturbed within the time-lapse incubation environment and are not removed for static assessments associated with standard incubation and practice.

Information provision is a vital part of fertility treatment. An unpublished questionnaire, undertaken at CARE Fertility, where patients are provided with verbal, written, and time-lapse video information, asked 393 patients several questions relating to their experience using TLI during their treatment. A large majority (82%) confirmed that TLI had improved their understanding of what happens in the IVF laboratory. Some of the comments are shown in Fig. 25.1.

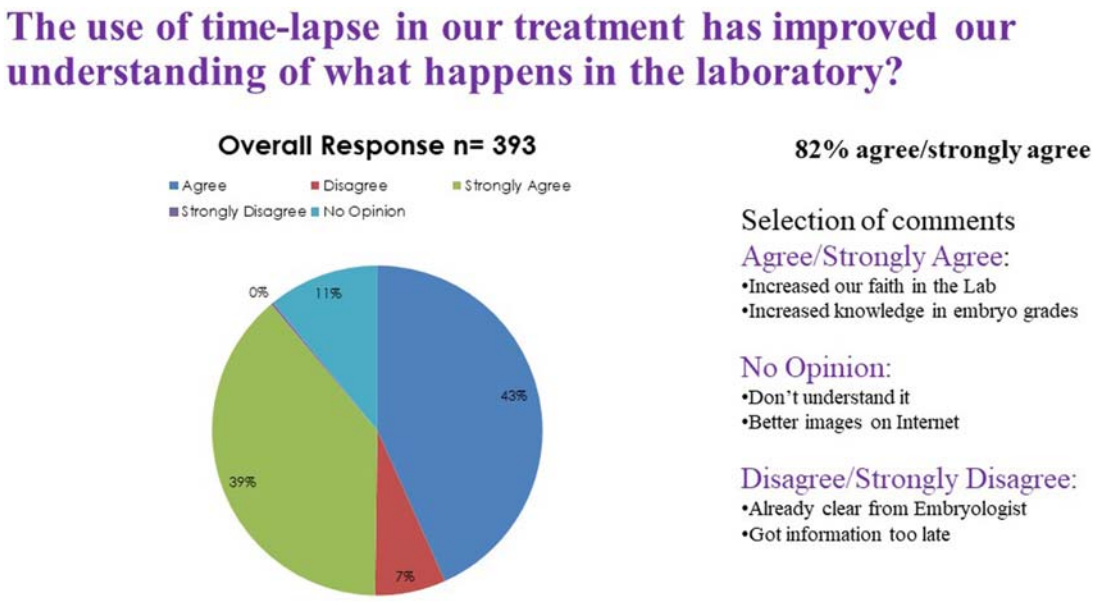
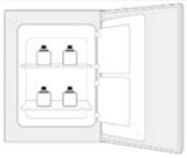


FIGURE 25.1 Patient feedback related to time-lapse imaging used within their treatment.

TABLE 25.1 Some examples of time-lapse devices, the dish or slide used, and their capacity.

Time-lapse device		
	EmbryoScope[a] (vitrolife): • Integrated time-lapse imaging • Six patient capacity • Uses a specific dish: embryoslide 	Patient culture • Six patient dishes with up to 12 embryos each • Four flushing rinsing wells • Sliding rack with small chamber capacity
	EmbryoScope + (vitrolife): • Integrated time-lapse imaging • Capacity for 15 patients • Uses a specific dish: EmbryoSlide+ and iC8[b] • Isolated handling port  	Patient culture • 15 patient dishes with up to 16 embryos each • Two distinct loading areas in the dish • Four flushing rinsing wells • Special barcode recognizes the patient details
 	Primovision[a] (vitrolife): • Equipment sits in a standard box incubator • One Primovision camera per patient • Uses a specific dish 	Patient culture • Culture in nine-well or 16-well dishes in group culture conditions • No flushing rinsing wells
	Geri (Merck): • Integrated time-lapse imaging • One patient per compartment • Humidified chamber • Uses a specific dish 	Patient culture • Six patients with up to 16 embryos • Three flushing rinsing wells • Group culture conditions • Individual CO_2 sensors per chamber
	EmbryoScope 8 (vitrolife): • Integrated time-lapse imaging • Capacity for eight patients • Uses the EmbryoSlide+ and iC8[b] (see EmbryoScope+) • Isolated handling port	Patient culture • Eight patient dishes with up to 16 embryos each • Two distinct loading areas in the dish • Four flushing rinsing wells • Special barcode recognizes the patient details

Continued

TABLE 25.1 Some examples of time-lapse devices, the dish or slide used, and their capacity.—cont'd

Time-lapse device		
	EmbryoScope Flex (vitrolife): • Integrated time-lapse imaging • Capacity for 24 patients • Uses a specific dish- EmbryoSlide Flex • Isolated handling port 	Patient culture • 24 patient dishes with up to six embryos each • Two distinct loading areas in the dish • Two flushing rinsing wells • Special barcode recognizes the patient details
	Miri TL (ESCO): • Integrated time-lapse imaging • Independent chambers • TL6, capacity 6 patients • TL12, capacity 12 patients • Uses Culturecoin dish 	• Six to 12 patients with up to 14 embryos • Optional continuous pH using SAFE Sens • Tri mixed gas

[a]*No longer produced. EmbryoScope+, EmbryoScope 8, and EmbroScope Flex are the latest or current time lapse systems from Vitrolife.*
[b]*iC8 EmbryoSlide dishes are designed for noninvasive testing and PGT-A.*

Anticipating the future

There is no doubt that TL has enlightened our understanding of embryo development by highlighting discrete morphologic and morphokinetic events otherwise unseen when embryos are cultured in a standard incubator. But as the number of such markers linked to embryo viability increases, will human interpretation continue to be effective? Artificial intelligence (AI), computer vision, and machine learning are already promising to reveal unseen markers of development by harnessing elusive information from the time-lapse embryo images. But can these technologies improve the value, viability prediction, and clinical outcomes associated with TLI as we know it?

TLI of preimplantation embryos using morphokinetic algorithms, as a potential advancement in embryo selection, remains hotly debated even after almost a decade of clinical implementation. AI has the potential to increase the indicators for classification by using image segmentation, deep neural networks, and convolutional neural networks to analyze images. AI is defined by a set of rules so has the capacity to improve reproducibility and accuracy and save time while reducing human bias.

Several published AI solutions focused on predicting morphologic grade to improve embryo selection have been reported to have high prediction accuracy compared with experienced embryologists. This approach may be limited, however, as the AI application is trained and built on subjective embryologist-defined data, and it mimics existing classification systems that are not strongly correlated to outcome [22,23].

A number of recently published AI models were trained on images from transferred embryos with a known clinical pregnancy (positive or negative) or on embryo ploidy status.

Using such defined outcomes, instead of subjective embryo grading, improves clinical applicability of an AI model. These studies reported that AI outperformed classification and prediction by embryologists and highlighted the ability for AI to select embryos according to their potential for implantation [24] or ploidy [25].

Automated annotation has the potential to save time and remove bias, and iDAScore (Vitrolife, Sweden) [26] successfully combines this new technology trained by deep learning, creating a reproducible output that can replace manual annotation in the AI algorithm. Using both static and dynamic markers of development, the model, without the need for manual annotation, performed as well as KID score day 5 (AUC 0.67 versus 0.66).

AI for embryo selection is an exciting prospect that should provide reproducibility, reduce bias, and possibly reduce cost and time, but with the caveat that the rationale for the embryo selection is unknown because it uses "black box" technology. The prediction

potential should be considered alongside the size, diversity, and quality of the training data, with inclusion of compiled data from multiple clinics with varying practices and patient cohorts to ensure transferability. Commercial AI models for embryo selection are emerging, designed for use with blastocyst culture and combined with metadata. Such AI models may enhance outcomes for IVF patients but require stringent evaluation prior to widespread adoption in IVF clinics.

Summary

TLI systems are becoming more common in IVF laboratories and, over the last decade, have resulted in an increased understanding of the developmental timings and patterns of the preimplantation human embryo. This, in turn, has led to more objective embryo selection associated with either the deselection of embryos, exhibiting anomalous cell divisions, or the active selection of embryos with preferable morphokinetics. The stable culture environment, relative to box or flatbed incubation with its interrupted (up to daily) microscopical assessment, may account for some of the improved results reported, but with time stamping of developmental milestones being reached, providing more data and information, algorithms have been developed to rank embryos within a cohort and to indicate their likelihood of blastulation, implantation, LB, and euploidy. The future is likely to see time lapse transition to a more automated offering utilizing AI, saving time and bringing further levels of objectivity and reproducibility to embryo selection (Table 25.1).

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CHAPTER

26

Intracytoplasmic sperm injection

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Background

In vitro fertilization (IVF) was first conceptualized in humans in 1934, when Gregory Pinchus and E.V. Enzmann discovered how to correctly culture and develop mammalian ova in vitro [1]. This led the way to Dr. John Rock and embryologist Miriam Menkin laying the groundwork for human IVF [2], a visualization that came to fruition with the birth of Louise Brown to a woman with bilateral tubal occlusion in 1978, through the incredible collaborative work of Bob Edwards and Patrick Steptoe [3,4].

This technique, although successful at first, soon appeared unable to grant fertilization in all couples, particularly those plagued by male factor infertility [5]. Despite the development of microdroplet insemination, in which oocytes were exposed to a high concentration of spermatozoa in minute amount of medium under oil, dysfunctional sperm cells still failed to achieve fertilization [6].

To treat these couples, embryologists began to manipulate the oocyte, starting at the zona pellucida (ZP). The first attempts involved the use of pronase or trypsin to soften the ZP, which resulted in consistent fertilization and poor embryo development [7]. Another oocyte manipulation technique that arose was *zona* drilling, exposing the oocyte to an acidic Tyrode's medium to create a hole in the ZP and allowing spermatozoa to enter the perivitelline space (PVS) [8]. This technique, while yielding a fertilization rate of 32%, was not ideal due to the damage to the oocytes created by exposure to the low pH. Partial zonal dissection was the evolution of this technique, in which a mechanical slit was created in the ZP of an oocyte with a microneedle under micromanipulation control. The fertilization of this technique was preferable to *zona* drilling, reaching 45%. However, abnormal fertilization was a major drawback, occurring at a more frequent rate (48%) than normal fertilization [9]. To reduce rates of polyspermy, as well as find a solution for couples experiencing asthenozoospermia or extreme teratozoospermia, still often resulting in fertilization failure, a technique dubbed subzonal injection (SUZI) was introduced. Through the use of an injection pipette, spermatozoa were placed directly into the PVS of an oocyte [10]. One study showed that this technique had increased fertilization rates to 30.9% in 43 couples who had previously had complete fertilization failure with standard in vitro insemination. Furthermore, the embryo cleavage rate was evidenced to be 80%. However, clinical pregnancy rates were between 2.9% and 16.3% [11,12].

The first case of intracytoplasmic sperm injection (ICSI) occurred in 1992: while the operator was performing SUZI, the oolemma of an oocyte was unintentionally pierced, allowing a spermatozoon to enter the cytoplasm. This oocyte survived and was successfully fertilized, though all the other SUZI-inseminated oocytes did not [13]. This embryo was eventually transferred on day 2 post-fertilization and a healthy child was born. The ICSI technique would later become routinely used in assisted reproductive technology (ART) laboratories across the world [11,12].

ICSI is the evolution of all prior micromanipulation methods and techniques. Its consistent fertilization, combined with the ability to use only one spermatozoon per oocyte, makes it the perfect technique to treat couples struggling to conceive due to suboptimal or extremely scarce male gametes [14–18]. Over the last 29 years following its inception, ICSI indications have broadened due to its versatility and consistency,

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granting a chance to conceive to infertile couples. Though initially designed to treat male factor infertility, including cryptozoospermic and even azoospermic men [16,17], ICSI has since become the most utilized ART treatment worldwide—building upon its repertoire of male factor indications based on overcoming male gamete defects [18].

Indications

Male factor

ICSI represents the ultimate option to conceive for men with suboptimal semen parameters. It is now accepted that men with compromised motility, morphology, and even in cases with extremely few sperm cells can be successfully treated with ICSI [15].

Ejaculated spermatozoa can be used even from men with normal spermatogenesis but concurring structural abnormalities of their gametes, such as globozoospermia, characterized by round-headed spermatozoa that lack an acrosomal cap and have an uncompacted chromatin. These cases require either identification of the few normal gametes, if present, or assisted gamete treatment (AGT) in cases with complete forms to successfully fertilize an oocyte [19]. Other structural abnormalities such as primary ciliary dyskinesia, such as Kartagener's syndrome, where genetic mutations hinder cilia and flagellar motion leading to immotile, albeit still viable, spermatozoa, are benefited by a direct injection into the oocyte [18].

Likewise, 5%−15% of infertile men present with positive antisperm antibodies (ASA) in the seminal fluid [20]. These antibodies are most frequently localized on the head of the spermatozoon and block its ability to properly enter and thus fertilize an oocyte. Men with positive ASA are also prone to abnormal semen parameters [21]. To overcome both the hindered semen parameters and reduced oocyte-penetrating ability of the spermatozoon due to the presence of ASA, ICSI is preferable. Furthermore, couples who are HIV or hepatitis-C discordant may conceive via ICSI, as proper sperm processing can shed the virus from the sperm cells. Highly active retroviral therapy also has been shown to negatively impact semen parameters, which would be obviated by ICSI [18,22].

ICSI has allowed even azoospermic men to conceive with the utilization of gametes retrieved directly from the genital tract through surgery. Azoospermia is observed in 10%−15% of men undergoing fertility treatments [23]. Obstructive azoospermia (OA) is caused by a blockage of the male genital tract at multiple levels. This includes structural abberations of the genital tract such as ejaculatory duct obstruction and unilateral or bilateral congenital absence of the vas deferens. Furthermore, the blockage can be acquired by vasectomy or unsuccessful vasoepididymostomy or vasovasotomy, or simple trauma to the genital tract. While some cases of obstruction can be repaired surgically and allow spermatozoa to return to the ejaculate, reconstruction does not always succeed. In these men with retained spermatogenesis, epididymal sperm aspiration, either microsurgical or percutaneous, can be successfully used in combination with ICSI. Nonetheless, testicular sperm extraction can be utilized in these patients, if the epididymal approach is not feasible [18].

Nonobstructive azoospermia (NOA), comprising hypospermatogenesis, maturational arrest, or germ cell aplasia, can only be remedied by the extraction of gametes directly from the seminiferous tubule. Microsurgical testicular sperm extraction (microTESE) seeks to target the most dilated seminiferous tubules within the testis to obtain these precious male gametes. Spermatozoa retrieved directly from the germinal epithelium often display poor motility and peculiar morphological characteristics, and thus benefit from ICSI [18].

Non-male factor

ICSI is increasingly being applied to couples that struggle to conceive even if not clearly affected by male infertility. These include cases of oocyte dysmorphism where ICSI has been shown to overcome morphological deficiencies of the female gamete to generate consistent fertilization and embryo cleavage, especially in couples where conventional insemination has failed [24,25]. Furthermore, cases with low oocyte yield or poor oocyte maturity are also frequently allocated to ICSI insemination, entailing cumulus removal and allowing the assessment of the first polar body extrusion [18,26].

Thawed oocytes are also frequently allocated to ICSI insemination. The cryopreservation and subsequent thawing processes hardens the ZP of the oocyte, and early studies showed an advantage with ICSI to obtain consistent fertilization with these cases [27]. In recent years, there has been a palpable increase for fertility preservation for social purposes, raising the utilization of ICSI to inseminate thawed oocytes [18].

Another technique aimed at treating women with polycystic ovarian syndrome is in vitro maturation, where the oocytes are retrieved from small follicles and matured in vitro by the exposure to maturation medium until the oocytes reach the metaphase-II (MII) stage. Studies on this technique have indicated ICSI to be the treatment method that grants consistent fertilization in these cycles [18,28].

Popularity

The versatility of ICSI has led to the technique being the most prevalent ART treatment worldwide. The global report from the International Committee for Monitoring Assisted Reproductive Technologies (ICMART) detailed 1,149,817 ART treatment cycles from over 2600 ART centers in 69 countries in 2012. This was an 18.6% increase from the number of ART treatments reported for 2011. ICSI was utilized for 68.9% of non-donor ART treatments for fresh aspiration cycles worldwide, a small increase from the 66.5% ICSI utilization from the previous year. Specifically, ICSI accounted for 88.4% of aspiration cycles in Africa, 56.6% in Asia, 69.4% in Europe, 85.2% in Latin America, 99.9% in the Middle East (excluding Israel, who did not report on ART technique used), and 73.5% in North America. The ICSI data gathered by the ICMART showed a pregnancy rate of 24.8% with a delivery rate of 18.0% per oocyte retrieval [29].

Likewise, the European IVF Monitoring Consortium (EIM) documented 563,224 ART treatments from 1347 IVF clinics within 40 European countries in 2016, of which 407,222 were ICSI (73.2%), which the authors noted was an increase of 1.2% from the previous year. These cycles resulted in a 25.0% clinical pregnancy rate and an 18.5% delivery rate per aspiration [30].

In the United States as a whole, a study was performed to analyze the increasing trend in ICSI utilization from 2000 to 2014 and divided the country into six distinct regions using data from the Department of Health and Human Services. The authors used this data to determine that ICSI allocation countrywide was rising in all regions, with an average increase of $23.7 \pm 6.7\%$ in ICSI utilization within these regions, a concurrent rise in clinical pregnancy rate of $3.3 \pm 2.9\%$ and live birth rates by $2.6 \pm 2.8\%$. They suggested that ICSI may be overused, especially over the latter 7 years of the study, as the rise in ICSI utilization over conventional insemination did not seem to correlate to an increase in typical ICSI indications; however, they did notice an increase in couples diagnosed with male factor infertility by $22.7 \pm 8.4\%$, supposedly attributed to the adoption of the Kruger strict morphological criteria [31]. The authors also argued that overutilization of ICSI did not correspond to an increase in pregnancy and live birth rate [31].

It is just fair to mention that at our center, ICSI is by far the most prevalent ART technique used. When the technique was first introduced in 1993, it was used for about 32.2% of all ART treatment performed. Just 2 years later, the utilization of the two techniques leveled, with ICSI reaching 48.8%. From that point, ICSI has been the main insemination method from 2012 onward, reaching 9:1 over standard in vitro insemination, resulting in a yearly utilization of over 95% [18].

Results

Ejaculated spermatozoa

During the last 27 years, we have utilized ejaculated spermatozoa in 39,215 ICSI cycles. Of these, only 6368 (16.2%) were cycles in which the semen parameters were within the normal threshold [31a]. A total of 340,392 oocytes have been injected with ejaculated spermatozoa, with 2.9% damage rate following injection. Of the 330,897 oocytes that survived injection, we have achieved a normal 2-pronuclei (2PN) fertilization of 78.3%, with 3.6% 3-pronuclei (3PN), 2.5% with 1-pronucleus (1PN), and the remaining 15.8% failing to fertilize.

To analyze the data, we allocated ejaculated spermatozoa according to the sperm source: ejaculate, retrograde ejaculation, or electroejaculate (EEJ). We reviewed the fertilization and clinical pregnancy rate (CPR), defined as the presence of at least one fetal heartbeat detected by transvaginal ultrasound (Fig. 26.1). The fewest number of cases were carried out with retrograde ejaculate specimens ($n = 64$). There was a slightly larger number of ICSI cycles with fresh EEJ ($n = 62$), and frozen EEJ were utilized in 26 cycles. This left over 37,000 cycles with normally ejaculated specimens.

We have performed ICSI in 2377 cycles with severe oligozoospermia, or an initial sperm concentration of $\leq 1 \times 10^6$/mL. These cycles were characterized by a mean concentration of $0.9 \pm 0.3 \times 10^6$/mL, a mean motility of $19.7 \pm 23\%$, and a $1.5 \pm 2\%$ normal morphology. In these cycles, we have been able to achieve a fertilization rate of 62.1%, as well as a 45.9% CPR.

In even more extreme cases where no spermatozoon was identified in an initial Makler® chamber, specimens were centrifuged at 3000 *g* in an attempt to pellet spermatozoa. In 371 ICSI cycles, we have identified spermatozoa to inject following by this high-speed centrifugation. In these cases, the final mean concentration was an evidenced 0.34×10^6/mL with $32.6 \pm 36\%$ motility. Injection of these precious spermatozoa have yielded a fertilization rate of 54.2%, resulting in 420 conceptuses replaced, yielding a 44.4% CPR.

Surgically retrieved spermatozoa

During the same time period (1993–2020), at Weill Cornell, we injected spermatozoa retrieved directly from the epididymis or through microdissection of the seminiferous tubule in 3170 cycles.

For men with OA caused by bilateral absence of the vas deferens, microsurgical epididymal sperm aspiration (MESA) was performed in 606 cases. For these

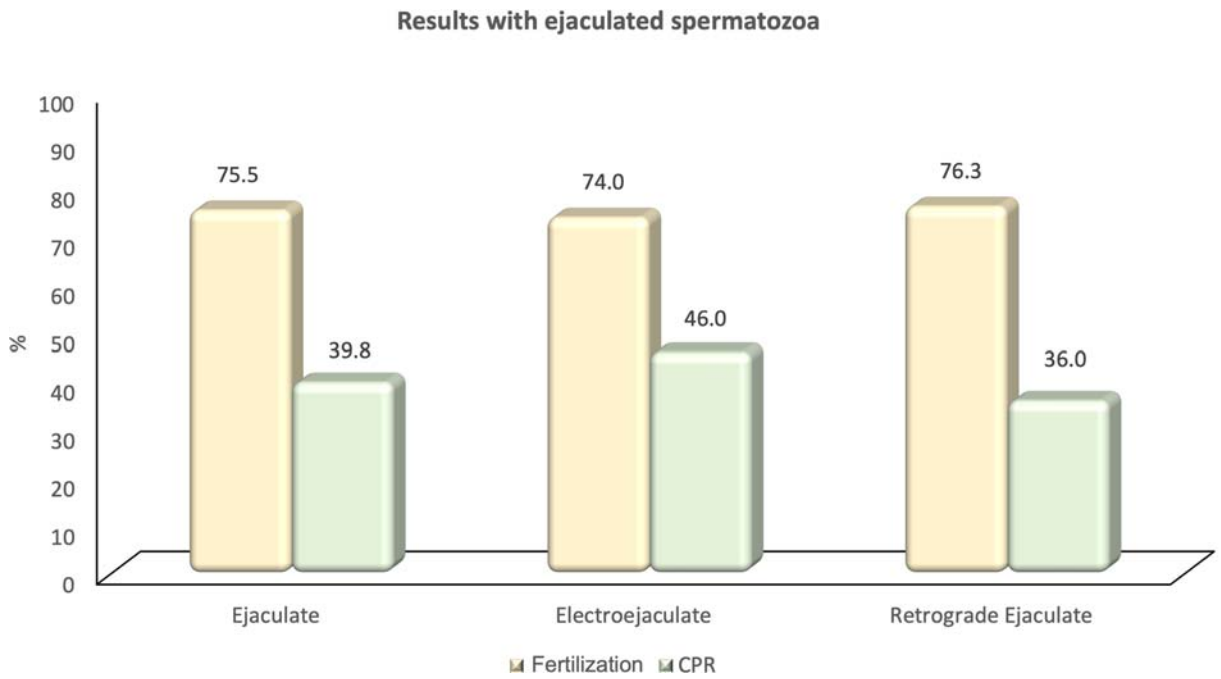

FIGURE 26.1 Fertilization and CPR in ICSI cycles that utilized ejaculate, electroejaculate, or retrograde ejaculate spermatozoa. There is no difference in either fertilization or CPR among these sperm sources.

cases, a fertilization rate of 72.2% was achieved as well as 319 clinical pregnancies (52.6%). We have similarly used fresh MESA-retrieved gametes in men with acquired etiologies for their obstruction in 620 ICSI cycles. These cycles have yielded slightly lower fertilization rates than congenitally obstructed patients, at 69.9% ($P < 0.01$) and a CPR of 42.7% ($P = 0.0005$). Furthermore, epididymal spermatozoa provide adequate fertilization and CPRs despite whether fresh or frozen gametes are used for insemination (Fig. 26.2).

The most challenging cases with surgically retrieved spermatozoa arise from couples who require microTESE. While the majority of these cases are performed in the event of NOA, we also infrequently retrieve testicular spermatozoa from men with OA if the epididymal approach is not available. At our center, microTESE has been successful in yielding spermatozoa in 61.6% of attempts. We have performed 302 ICSI cycles with fresh testicular spermatozoa in couples with OA, in comparison to 1646 ICSI cycles in couples affected by NOA. A comparison of fertilization rate and CPR is visible in Fig. 26.3. In summary, the fertilization rates ($P < 0.00001$) and CPR ($P < 0.05$) are higher when testicular spermatozoa are retrieved from OA men rather than NOA men. Testicular spermatozoa maintain similar fertilization profiles whether utilized fresh or frozen; however, the former generated higher clinical pregnancies than the frozen ($P = 0.05$; Fig. 26.4).

ICSI for difficult cases

Extreme male factor

A skilled ICSI operator is able to identify progressively motile and well-shaped spermatozoa for injection with ease. However, in some extreme cases, spermatozoa are incredibly rare and require dedicated searching by multiple embryologists to identify a number of gametes adequate for injection. This extended sperm search often requires sacrificing morphological selection and even the presence of motility of the spermatozoon, as the effort shifts exclusively to the identification of a spermatozoon within the sample.

We have performed a retrospective study on cases of extreme ICSI, whether with ejaculated or testicular spermatozoa. We considered control ideal cases with a search time of up to 29 minutes, and compared them to cases in which a search time required 30−60, 61−20, 121−180, and ≥181 minutes.

There were 2121 cases in the control group and 76 within the extended search group for cycles that utilized ejaculated spermatozoa. The required search time for ejaculated experimental cases ranged from 30 to 225 minutes, or 3.75 hours. The fertilization in the control group was reported as 75.6%, which was significantly higher than the fertilization in each of the groups requiring extended search ($P = 0.0001$). The delivery rate within the control group was 31.5%, which became

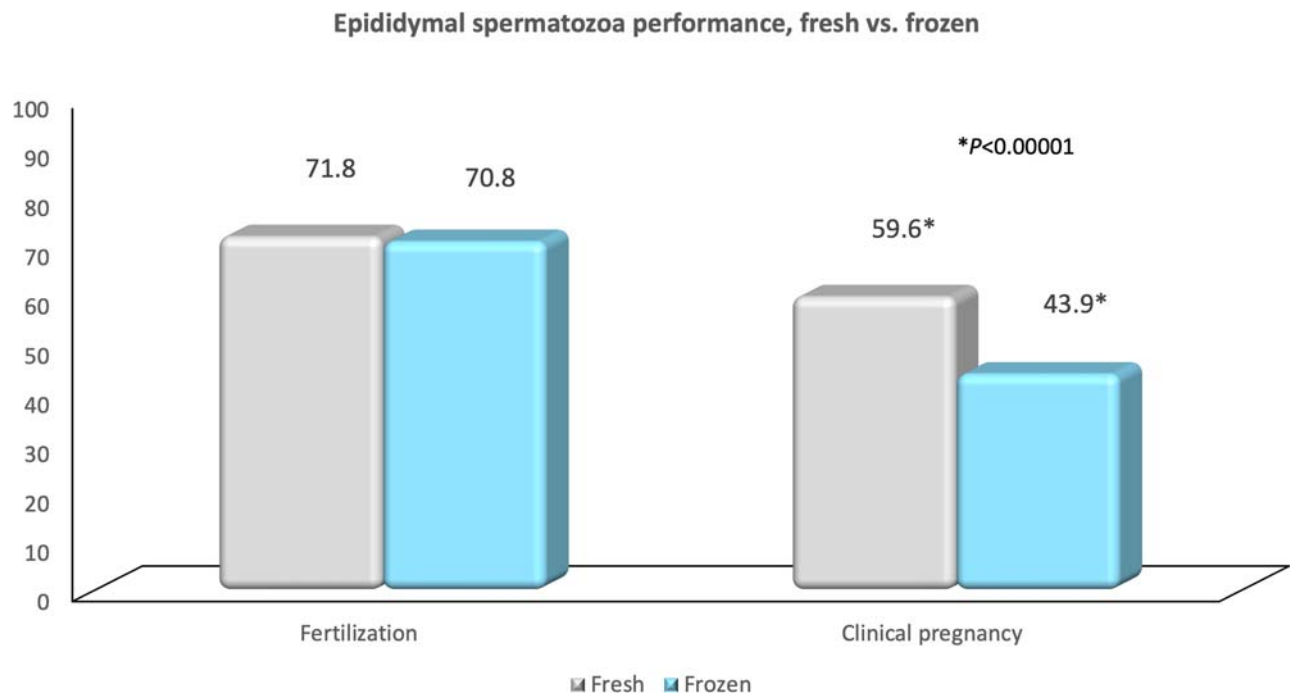

FIGURE 26.2 Fertilization and CPR from ICSI cycles that utilized fresh or frozen epididymal spermatozoa. Fertilization is consistent in these two groups. However, fresh epididymal spermatozoa performs much better in terms of generating a clinical pregnancy ($P < 0.00001$).

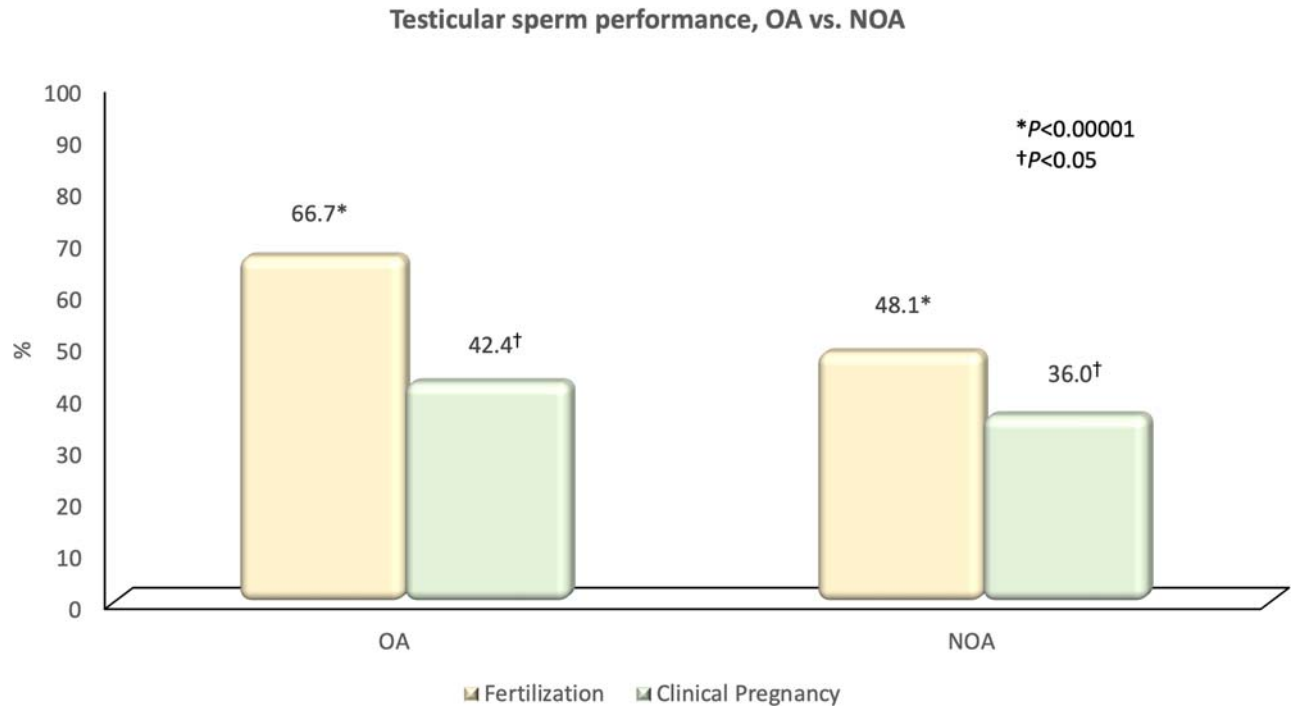

FIGURE 26.3 Fertilization and CPR from ICSI cycles that utilized fresh testicular spermatozoa based on the etiology of their azoospermia, OA, or NOA. The testicular spermatozoa of OA patients perform better than those from NOA patients, both in terms of fertilization and CPR.

27.1% following a search interval of 30–60 minutes and 42.9% in the 61–120 minute interval, though there were no deliveries in these cycles for cases requiring a search time of longer than 2 hours despite two clinical pregnancies in cycles that required searching for over 181 minutes.

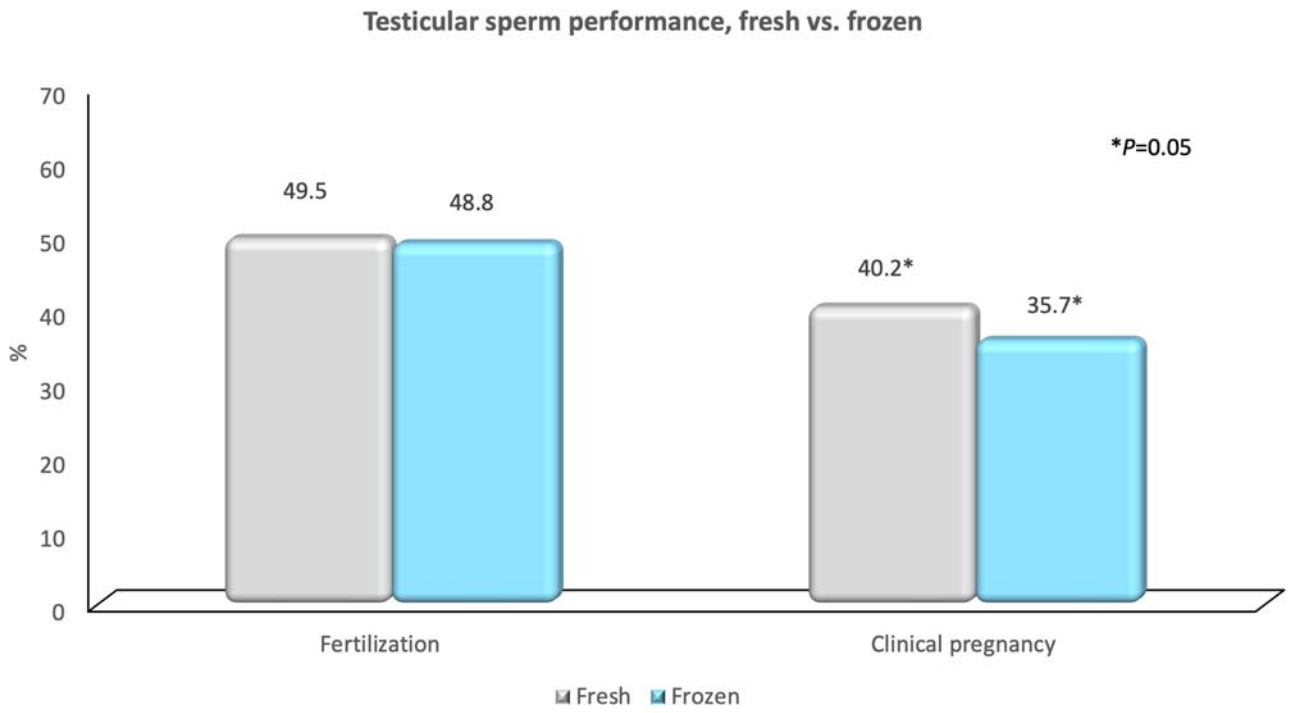

FIGURE 26.4 Fertilization and CPR from ICSI cycles that utilized fresh or frozen testicular spermatozoa. Fertilization is consistent between fresh and frozen testicular sperm. However, similarly to epididymal spermatozoa, fresh testicular spermatozoa perform better in producing a pregnancy ($P = 0.05$).

There were also 949 cycles serving as a control for testicular spermatozoa and 231 requiring extended searching for spermatozoa. A similar reduction of fertilization was seen, falling from 58.7% in the control to 49.9%, 45.5%, 27.8%, and 26.7% for each search interval group, respectively ($P = 0.0001$). Also in these cases, there was no effect on the search time in terms of embryo cleavage and implantation. A trend was noted in the decline of the delivery rate as the search time lengthened, but without reaching significance.

This study proved the efficiency and benefit of ICSI in the most desperate cases. Once a skilled embryologist is able to locate spermatozoa for injection, those spermatozoa were able to generate normal fertilization and implantable conceptuses independently from the morphokinetic characteristics of the male gamete and time spent to find it [16,17].

Elevated DNA fragmentation

Standard semen analysis can be supplemented with a sperm chromatin fragmentation (SCF) assessment, which assesses the integrity of the sperm DNA considered capable of impairing embryo quality and implantation in couples in unexplained infertility and subtle male factor [16,17,32]. The effect of SCF is clear in cycles of programmed intercourse, intrauterine insemination (IUI), and often standard in vitro insemination, but almost never with ICSI [33]. This difference has been attributed to the absence of exposure of the spermatozoa to their own medium and concurrently of the oocytes to the sperm suspension rich in reactive oxygen species during insemination [34]. Moreover, a clear inverse correlation between the spermatozoa with chromatin fragmentation and their motility has been established. Thus, selecting a properly motile spermatozoon, as it occurs during ICSI, renders it the most suitable technique for these couples [16,17].

It has been estimated that around 30% of normozoospermic men have abnormal sperm chromatin integrity [35] resulting in repeated IUI failure despite normal semen parameters and a young female partner with a negative infertility workup [35]. In these couples, ART, particularly ICSI, has invariably yielded better clinical outcome.

However, if even ICSI fails for these couples, to minimize exposure of the spermatozoa to the offending factors causing oxidative stress and potential DNA damage present in the male genital tract, the surgical retrieval (SR) of gametes from the seminiferous tubules has been suggested. In a study at our center, consenting men with high SCF (32.9 ± 20%) carried out by terminal deoxynucleotidyl dUTP transferase nick-end labeling (TUNEL), higher than our normal threshold of <15%, in their ejaculate underwent a topographic surgical sperm retrieval. We found that SCF decreased as the

spermatozoa were retrieved proximally in the male genital tract. In these men, the average SCF was 20.4 ± 10% in the gametes isolated from the vas deferens ($P < 0.05$), 15.8 ± 8% in those isolated from the epididymis ($P < 0.00001$), and it became normal in gametes from the testis, at a level of 11.4 ± 6% ($P < 0.00001$). This triggered a pilot study at our center on 25 couples that failed to achieve a pregnancy with ICSI utilizing ejaculated spermatozoa where SCF was 36.9 ± 12%. Therefore, in subsequent cycles, we performed ICSI with SR spermatozoa that increased implantation over the ejaculated counterpart from 3.0% to 12.8% ($P < 0.05$), CPR from 6.1% to 29.3% ($P < 0.01$), and delivery rates from 4.1% to 22.0% ($P < 0.01$). Emboldened by these findings, in 45 couples where the male partner had high DNA fragmentation (36.2 ± 15%) and a history of pregnancy failure with ejaculated spermatozoa elsewhere, they were treated directly with SR spermatozoa at our center. Despite achieving lowering fertilization, from 70.4% to 65.1% ($P < 0.05$), SR gametes were superior in terms of implantation that increased from 7.5% to 19.1%, CPR rose from 13.3% to 40.0%, and delivery rates from 12.0% to 34.3% ($P < 0.01$) [36].

This approach, while effective, is drastic and requires an invasive procedure that some couples may not find appealing. Moreover, couples that fail to achieve a pregnancy even after the utilization of SR spermatozoa may seek a more conservative approach. Based on the explicit inverse correlation between SCF and sperm motility [16,17], we proposed an alternative for couples plagued by elevated SCF in their ejaculate, microfluidic sperm selection (MFSS). This is a technique that we have tested at our center even for the most severe SCF cases. A pilot study of 23 men demonstrated that MFSS decreased SCF from 20.7 ± 10% in the raw semen to just 1.8 ± 1%. We then treated 16 consenting couples with elevated SCF in the ejaculate by ICSI using specimens processed by density gradient centrifugation versus MFSS. We were able to significantly improve CPRs, from 0% (0/7) to 50% (6/12; $P < 0.05$), confirming the efficacy of this selection method over surgical sperm retrieval [37].

The understanding that certain components of SCF, such as double-stranded DNA (dsDNA), may induce structural chromosomal abnormalities [38] brought to the assessment of MFSS for those peculiar infertile cases, often plagued by a large cohort of aneuploid embryos.

We have utilized this novel technique in 35 ICSI cycles of 29 couples who generated an unexpected high number of aneuploid embryos tested by preimplantation genetic testing for aneuploidy (PGT-A). For these couples, in their previous cycles, the spermatozoa, processed by density gradient centrifugation, yielded 23.8% (26/109) euploid embryos euploid, an implantation rate of 4.3%, and a CPR of 8.3%, all resulting in pregnancy loss [37]. However, following MFSS, the incidence of euploid embryos rose to 48.9% (90/184; $P < 0.0001$) that once transferred, achieved an implantation rate of 65.5%, CPR of 73.0%, and an ongoing/delivery rate of 69.2% [38a].

Persistent fertilization failure

Although ICSI was conceived to obviate complete and unexpected fertilization failure that plagued standard in vitro insemination, fertilization failure can nevertheless occur in 2%–3% of all ICSI cycles [39,40]. In this scenario, it is important to discern the eventual contribution from the spermatozoon and/or the oocyte. The reasons can be due to an inability of the spermatozoon to activate an oocyte, or to an ooplasmic dysmaturity rendering the oocyte incapable of being activated once inseminated by a spermatozoon.

In a recent study, we identified 114 couples with extremely poor fertilization, ranging from 0% to 10%, despite a young female partner with a negative infertility workup, at least three mature oocytes injected, and spermatozoa concentrations at or above 1×10^6/mL. In an attempt to identify the gamete responsible for the fertilization failure, the male partner in 76 of these couples underwent a phospholipase C zeta (PLCζ) assay to determine whether there was an adequate presence of cytosolic factor in the sperm head. The sperm-bound labile protein identified as PLCζ, once released into the oocyte following insemination, causes several Ca^{2+} oscillation spikes. This phenomenon releases calcium from the endoplasmic reticulum of the oocyte, triggering oocyte activation [41,42].

In couples where the male partner had a confirmed presence of PLCζ ($n = 52$), fertilization failure was clearly attributed to the oocyte. In those cases, we counseled couples to repeat their ICSI attempt with a tailored superovulation protocol aimed at enhancing ooplasmic maturity. This is realized by increasing the time interval between the administration of the hCG trigger to oocyte retrieval, denudation, and eventually ICSI [43]. The tweaking of the superovulation protocol and lengthening of the crucial timings increased fertilization significantly from 2.1% to 59.0% ($P < 0.0001$), and subsequent CPR rose from 0% to 28.6% for the couples included in this study ($P < 0.0001$). These beneficial effects on the timing post-hCG have been supported by other authors [43].

In another cohort of couples, the men failed to carry PLCζ in their gametes, therefore attributing the couple's fertilization failure to the spermatozoon ($n = 24$). To confirm the absence of PLCζ, these specimens underwent a confirmatory mouse oocyte activation test [44], and four of them also consented to genetic and epigenetic testing to identify possible mutations and gene function. Nucleic acid sequencing supported that these

men indeed had a deletion on the *PLCZ1* gene, corroborating the findings of our assay.

These couples were then counseled to undergo ICSI with AGT. The AGT protocol involved exposing spermatozoa to calcium ionophore for 10 minutes prior to injection, which was carried out with ~0.4 pL of calcium ionophore aspirated within the pipette. Following injection, oocytes were also treated by exposure to 50 μM calcium ionophore for 10 minutes at 37°C, prior to being rinsed and then reallocated into fresh culture medium.

These couples had a history of 27 ICSI cycles with a fertilization rate of 9.1% (18/197) and only four of them received an embryo transfer with no resulting pregnancies. They subsequently underwent 43 ICSI cycles with AGT, which yielded a 42.1% fertilization ($P < 0.05$) and 36.0% CPR ($P < 0.05$) leading to the delivery of six healthy children. Reassuringly, neonatal follow-up of the children did not evidence any developmental delays at 3 years of age, confirming the safety of the AGT protocol [45].

Considerations and future perspective

ICSI arose from the need to treat infertile couples suffering from complete and unexplained fertilization failure due to male factor and has since become the most popular insemination technique worldwide due to its ability to grant consistent fertilization to all couples [18]. ICSI allows the utilization of emerging techniques such as oocyte cryopreservation and in vitro maturation and supports sophisticated genetic tests of the embryo. It also allows a more direct identification of oocyte maturity and serves as a tool to learn specific timing of insemination, syngamy, and to study the effects of cytoplasmic maturity. The information gained through this technique, together with the ability to allow a dysfunctional sperm cell to fertilize an oocyte, appears as an evolution of ART and IVF itself—being able to indiscriminately treat all couples, provided there is an individual parental gamete.

The advancements in ICSI allow the ART laboratory itself to become more sophisticated. Inquiries into the genetic and epigenetic qualities of the spermatozoon are being performed to better understand the embryo developmental competence of the male gamete, including the ability of the resulting conceptus to implant [46]. To overcome the obvious limitations of the current ART technique, bold experiments are being performed, such as aiming at creating a niche with spermatogonial stem cells to coax differentiation in vitro, creating gametes to induce or restore fertility to men [47–49] or to women. Indeed, functional female gametes are being created through the fusion of a somatic cell and a donor ooplast to later be inseminated by ICSI [50–52]. Lastly, progress is being made on ICSI-on-a-chip technology, which would perform the sperm selection, oocyte denudation, injection, and allow embryo development in a single microfluidic cartridge to streamline the IVF process, reducing cost and enhancing accessibility. Through all of these endeavors, ICSI will be used to maximize the potential for reproductive success of infertile couples.

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CHAPTER

27

Embryo transfer

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Introduction

A great deal of effort has been made in recent years to improve the success rate of assisted reproductive technologies. Embryo transfer (ET) remains a critical rate-limiting step of the whole *in vitro* fertilization (IVF) procedure. In fact, a poor ET technique can represent up to 30% of all failures in assisted reproduction [1]. Actually, embryos can move toward the cervical canal or toward the fallopian tube [2] probably due to the presence of endometrial waves [3]. Recent studies on simulators have shown that the movement of the air bubbles accompanying the embryos at the time of injection into the uterus does not always follow that of the embryo itself. The embryos can move to the salpinges, to the cervical canal, or remain at the injection site, giving rise to extrauterine pregnancies in the various implantation sites or to intrauterine pregnancy. 15%–45% of the transferred embryos are found outside the cavity after a transfer [4–6], and methylene blue was visualized in the external os in 42% of cases [7]. Similarly, the radiopaque dye remained in the uterine cavity in only 58% of cases [8]. Much effort has been made to study the possible impact of the various steps of the ET procedure on embryo implantation and pregnancy outcomes. Uterine contractions, expulsion of embryos, blood, mucus, bacterial contamination, or retained embryos have been associated with unsuccessful ET. Furthermore, several studies show that the technique of loading the embryo into the catheter, the speed of its injection into the uterus, the intrauterine position in which the embryo is deposited, together with the operator's experience in performing ET can affect the overall success of IVF-ET [5,8–10].

Learning curves

The importance of training for ET technique [9,11] must be seriously taken in account. Actually, a significant variability was demonstrated among individual providers [8–16]. The success rate among the various operators can vary between 13% and 54% [13,14] and tends to stabilize after at least 50 transfers [15,16]. The importance of training is also underlined by the lack of significant differences in the results obtained from transfers carried out by clinicians and midwives [15]. Also, simulation of ET seems to allow to improve the quality of the transfer already in the first 10 transfers carried out by the fellows, leading to a more rapid acquisition of the technique. These data suggest potential value in adopting ET simulation, even in programs of live ET in fellowship training [17]. It is clear that many variables can positively or negatively influence the possibility of ET and implantation; among these, the operator's ability to carry out the transfer of embryos to the uterus appears to be absolutely decisive for the result. The training for operators is often performed during intrauterine insemination (IUI) and mock transfers, but not during live ET. On the other hand, the ultrasound-guided method reduces the validity of the training carried out using a transfer catheter for IUI; indeed, ultrasound guidance in IUI is not useful [18,19], and the IUI itself only partially reproduces that of the ET. Recent data show that the clinical pregnancy rate and live birth rate after ET performed by attending staffs or fellows are comparable [9].

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Biofluidic dynamics of embryo transfer

Recent studies on simulators have made it possible to explore some biophysical parameters of ET and have highlighted how the position of the uterine fundus, the tip of the catheter, and the embryo ejection speed are crucial. The suggestion is to perform the low-speed transfer using an ejection time of not less than 10 seconds, while high speed might favor ectopic pregnancy [6,20,21]. Regarding the embryo injection into the uterus, the results of a survey obtained by evaluating 161,300 cycles performed in 265 centers in 71 countries highlight the importance of transferring embryos to the uterus at a very low speed in 61% of cycles [22]. Nevertheless, the remaining 39% is divided between those who consider such attentions irrelevant (11%) and those who consider it important to inject the embryos into the uterine cavity at high speed (28%) to avoid the embryos remaining in the catheter. Regarding the type of catheter, it should be kept in mind that a very small internal diameter, on the one hand, offers the possibility of loading the embryos in a small volume of transfer medium, and on the other hand, it produces an excessive increase in the expulsion speed up to 80%. A high speed of expulsion of embryos can cause damage, differently for an embryo positioned close to the catheter wall compared to another located in the center of the lumen of the catheter [23], or it can favor the projection of the embryos in the tubes [6]. The transfer should therefore be carried out smoothly and with minimal speed, eliminating any narrowing of the lumen of the catheter itself, which would lead to a further increase in the shear stress [23].

The role of the catheter and loading embryos

Type of catheter has a positive or negative impact on the ET procedures. Two types of catheters are to be considered: soft and hard. The soft catheter should reduce the risk of damage to the endometrium, avoiding the risk of possible negative impact on the embryo implantation. Blood is more often found on the rigid catheter rather than on the soft one, which prevails as a whole [24], even if the comparison of the results between the two is not always in its favor. Many factors can be held responsible for the presence of blood on the catheter transfer: endometrial disruption, endocervical pathologies, or coagulopathies. To get better quality, the soft catheter is often used with an introducer, with the main advantage of protecting it from bacterial contamination from the external ostium to the uterine cavity. The downside is that the loading of embryos into the soft catheter is sometimes more elaborate, and the transfer time can be lengthened.

The volume of culture medium used for transfer is another variable that has been hypothesized to influence the outcome of IVF. Some authors have suggested that a large volume of fluid can cause the embryo to be ejected from the uterus, while very low volumes (<10 μL) can cause implantation failure. Others have reported that larger volumes of culture medium (35–40 vs. 15–20 μL) may favor the implantation of the embryo [12]. In most studies the volume of culture medium used to load embryos is between 20 and 30 μL [10,25], although there is no consensus on the volume of medium to be used during transfer.

Different techniques are used to load the embryos into the catheter. A recent result shows that the majority of embryologists load the medium-air-embryo-air-medium sequence and that the permanence of embryos in the catheter is extremely short, even in cases of low or very low embryo injection speed [22].

Preparation of the uterus for transfer

The techniques and technologies developed allow today to obtain embryos of excellent quality and with high possibilities of implantation and subsequent development of pregnancy. However, the clinical-biological work carried out becomes useless if a careful preparation of the uterine cavity that will host the embryo is not properly done. To this end, the awareness and scientific documentation underlining the importance to correct some congenital and acquired uterine malformations, which can affect the implantation of the embryo and the progression of pregnancy, have considerably increased. The improvement of ultrasound, hysteroscopic, and laparoscopic diagnostic technologies have made it possible to have detailed pictures of the size and position of myomas, the presence, size, and number of endometrial polyps, the presence and size of the uterine septa, the adhesion syndromes, the T-shape of the uterine cavity, and evidence of chronic endometritis. Many of these pathologies are correlated with the state of infertility, and surgical correction or preventive medical treatment is now accepted in any pregnancy-seeking procedure. In fact, the surgical correction of most of the mentioned pathologies can allow the ease of ET, the embryo implantation, the development of pregnancy, and improve the take-home baby rate [26–28]. On the other hand, the debate is still open on the usefulness of diagnostic hysteroscopy as a first-line examination, carried out for the search for chronic endometritis [29], possibly in association with the search for plasma cells in endometrial biopsies. Important published works would seem to lead to the usefulness of the examination as a first-line diagnostic [30], also due to the

current simplicity and low cost of the examination itself, capable of providing valuable information before a cycle of homologous or heterologous ET. From the cost/benefits point of view, the impact of a diagnostic hysteroscopy on the total cost of an egg or embryo donation or on a regular IVF/ICSI cycle is almost negligible, while the discovering of an endometrial cavity abnormality can improve the success while medically or surgically treated.

Mock transfer and transvaginal ultrasound for the measurement of the endometrial cavity length and position

The mock transfer and ultrasound for the measurement of the endometrial cavity length and direction can be useful in helping physician to shorten the transfer time, in particular when the transfer is performed by a fellow or by a less skillful operator. It is important to correctly evaluate the uterine cavity length and direction to discover any unexpected difficulties when performing a proper ET and to choose the most suitable catheter [2]. This evaluation can be performed with a mock transfer in the cycle preceding the real one or by transvaginal ultrasound while monitoring ovarian stimulation. Mock transfer was introduced to minimize the possible difficulties encountered at live ET and then to improve the success rate [5–25,31–33]. Actually, mock transfer and ultrasound can be used for the assessment of the uterine cavity angle [19] and external ostium-fundus length [34] to facilitate ET and not touch the uterine fundus, thus avoiding bleeding, uterine contractions, and offering the possibility to deposit the embryos in the uterus at the desired distance from the fundus. However, there is still no agreement on routine mock transfer performance except in patients at high risk of difficult transfer, versus those without this risk [33].

Is the ultrasound support effective?

Studies have shown that ultrasound-guided transfers are better in terms of clinical pregnancy outcomes than the clinical touch method [35].

Performing the transfer under ultrasound guidance with a full bladder allows the operator to have a good view of the ultrasound tip of the catheter to be sure to leave the embryos in the cavity. This procedure, visible on the monitor also by the patient, allows the control during the transfer process carried out by the fellows during the learning period of the technique with probable reduction of the learning curve.

The ultrasound-guided procedure is the one usually chosen for ET, also supported by the evidences as reported in the NICE guidelines. The use of ultrasound has been the subject of a number of studies. In a randomized study [36] the pregnancy rate was significantly higher in the ultrasound-guided ET group (50%) than in the clinical touch method (33.7%) ($P < .002$), but in another randomized study, this advantage has not been reported [37].

The ultrasound support can reduce difficulties and times in carrying out the transfer [37] as well as facilitating the path to the catheter with consequent slight trauma to the endometrium and related bleeding [37]. The possibility of not touching the fundus allows one to not induce contractions and therefore reduces the risk of expelling the embryos outside the cavity.

Another positive aspect is that the transabdominal method allows, by filling the bladder, to improve visibility and to reduce the angle of flexion in the anteverted uterus, therefore shortening the execution time of the procedure [13]. More recent studies have highlighted the advantages of the transvaginal ultrasound approach, still not very widespread, which does not require the complete filling of the bladder and the consequent discomfort, with greater relaxation of the woman. Discomfort due to a full bladder can affect up to 63% of women undergoing ET [38]. Several studies have described the advantages and disadvantages of transfer methods with or without ultrasound guidance. The major advantage of the ultrasound-guided transfer is to be able to follow the path of the catheter tip through the cervical canal and into the uterine cavity without reaching the uterine fundus, thus being able to leave the embryos in the chosen place. This goal can be also reached by a previous measurement of cervical and total uterine cavity length. The catheter can be then introduced at calculated depth, and the ET can be performed with very good accuracy.

Placement of embryos in the uterine cavity

The place of release of the embryos into the uterine cavity appears to have an impact on the chances of implantation. Several studies have highlighted higher pregnancy rates when embryos are released into the uterus at a distance between 1.5 and 2.0 cm from the fundus or in the middle portion of the cavity [39–42]. Though, there is no full agreement on the impact that the position of the embryos released in the uterus should have on the outcome in terms of implantation and pregnancy and why the different positioning of the embryos into the cavity should determine greater or lesser success [43,44].

Not touching the fundus of the uterus at the time of the transfer, in order to not induce contractions and possible bleeding, is a generally accepted fact. Strong contractions could be induced from the uterine fundus by the catheter. Actually, it should be taken in account that the peristaltic movements of the endometrium, consequent to the muscular contractions of the uterus and well documented ultrasonographically, represent a physiological activity of the uterus. It is likely that to go above the physiological threshold of contractions, a negative impact on embryo implantation might occur. Further conditions can have a negative impact on embryo implantation: recent evaluations on simulators in the laboratory have shown that the position of the uterus, anteverted or retroverted, and the speed of ejection of the embryos by the catheter can determine the displacement of the embryos in the cavity.

Certainly, a transfer carried out in an easy, atraumatic, delicate way for the uterus and embryos, deposited and not shot in the uterine cavity, represents the best choice to obtain the maximum possibility of implantation and physiological development of the pregnancy.

Conclusions

Numerous aspects of the ET procedure have been evaluated to determine their impact on pregnancy outcome. Consistent evidence does appear to support the use of soft catheters and ultrasound guidance, optimizing the "ease" of the whole transfer procedure. Limited evidence supports removal of cervical mucus, presence of blood on the catheter, avoiding uterine contractions, and bed rest after the transfer, while increasing evidence shows the importance of the learning curve, the skill of the physician, biofluidic aspects, optimizing the uterine conditions, and transvaginal ultrasound support. There is no consensus for an optimal ET procedure, but certain approaches, with comparable embryo quality, are associated to improved outcomes [45].

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CHAPTER

28

Luteal phase support

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Introduction

Reproduction is a fundamental phenomenon for the preservation of the species, which requires a sequence of events necessary for a successful pregnancy. One of the most critical moments in the steps to establish a pregnancy is endometrial decidualization and embryo implantation. For this to occur, the development of an adequate luteal phase is required [1].

The luteal phase is the period between ovulation and pregnancy or the onset of the next menstrual period. In a natural cycle, it lasts approximately 2 weeks, where after ovulation, the corpus luteum is formed and generates changes in the secretion of steroid hormones such as estradiol and progesterone, with progesterone being the one that predominates in the second half of the cycle [2,3].

The existence of luteal phase defects is well known. In the 1970s the first studies on luteal phase defects were conducted. Currently, luteal phase deficiency is defined as luteal phases shorter than 11 days, a 2-day delay in endometrial histological development, or progesterone values < 10 ng/mL in the mid-luteal phase [4]. In the context of assisted reproductive treatments (ART), there is always a deficit of the luteal phase. Luteal phase support (LPS) is the term used to define the administration of exogenous medication intended to support the implantation process. Therefore, the purpose of LPS in ART is to fill the gap in progesterone secretion because of the absence of the corpus luteum [2].

Physiology of the luteal phase

The cycle is divided into two phases: follicular and luteal phases. The duration of a woman's cycle is from 21 to 35 days, with the follicular phase varying from 14 to 21 days and the luteal phase lasting exactly 14 days [5,6], although luteal phases of 11–17 days in length are considered normal. Its length depends on the survival of its fundamental functional unit, the corpus luteum. A variety of endocrine, paracrine, and autocrine factors contribute to this process.

In a natural cycle, the dominant follicle generates an increase in serum estradiol concentration. This increase represents a shift from negative feedback control of luteinizing hormone (LH) secretion to a sudden positive feedback effect, resulting in a 10-fold increase in serum LH concentrations and a minor increase in serum follicle-stimulating hormone concentrations [7]. The LH surge results in the restart of oocyte meiosis, luteinization of the granulosa cells, ovulation, and the onset of corpus luteum development [8]. It is well known that the corpus luteum, when conception is not generated, is a transient gland, which develops and reaches its structural and functional maturity during the mid-luteal phase (MLP) and is followed by its regression and luteolysis. The regression of the corpus luteum is necessary for the cyclicity of the reproductive process and is determined by apoptosis or programmed cell death [9]. However, if the oocyte is fertilized and the embryo implants in the endometrium, the trophoblast cells begin to produce human chorionic gonadotropin (hCG), which will rescue the corpus luteum from atresia and maintain progesterone production.

During the early luteal phase (ELP) the secretion of progesterone, which is essential, among other things for proper endometrial transformation, is maintained in a stable secretion pattern with no direct relation to the pulsatile release of LH [10,11]. In contrast, as the luteal phase progresses, increases of progesterone concentration in plasma are directly related to episodes of LH release, with a time difference between the LH surge and the progesterone surge of 25–55 min [11]. The secretion of progesterone in the granulosa cells of the corpus luteum results in a gradual increase in progesterone concentrations in the mid- or late luteal phase leading to a progressive deceleration of LH pulses and consequently progesterone. As a result, there can be significant

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variations in serum progesterone concentrations during the luteal phase. Mean progesterone levels in ELP increase from 2.6 ± 1.8 to 19.4 ± 6.4 in MLP and decline to 7.0 ± 4.8 in late luteal phase [11].

There is clear evidence that women have a definite period of endometrial receptivity, dependent on the action of ovarian steroids in the uterus [12], particularly progesterone, which will allow the transformation to a receptive endometrium for proper embryo implantation [1]. This endometrial period is known as the "implantation window" [13,14]. In order for progesterone to act, it is required to not only have an endometrium prepared with estrogens, but also to reach correct levels and a determined period of exposure, which is the reason it is important to study the LPS in ART [12].

Pharmacodynamics and types of presentation of progesterone

While LPS is widely accepted following ART treatments, where a significantly higher pregnancy rate has been demonstrated in patients receiving progesterone versus patients not receiving it [15], there is no clear consensus on which formulation to use or route of administration [16].

The bioavailability of progesterone varies according to the pharmaceutical preparation. Natural progesterone after oral administration is rapidly degraded by hepatic and gastrointestinal metabolism, having a low bioavailability [17]. Meanwhile, since progesterone is a liposoluble hormone and its formulation for the muscular route is prepared in oil, it has the highest levels of absorption and bioavailability. This is because it avoids the first hepatic step and also accumulates in adipose tissue. All this has led it to be considered the gold standard route of administration for a long time [9,16]. However, other formulations gradually replaced it due to its side effects, the most popular being the vaginal route. Nevertheless, in recent years, in addition to the vaginal route, other forms of presentation have been introduced, such as nasal, sublingual, rectal, and subcutaneous [9,18].

Routes of administration

Oral progesterone

Orally administered progesterone has a high level of degradation in the digestive system as it is subject to a first prehepatic step and to hepatic metabolism itself. This ends in the degradation of progesterone to its 5α and 5β reduced metabolite [19]. Because of this, it was sought to improve the bioavailability levels of the pathway leading to a process of micronization of progesterone [9,16]. Nonetheless, bioavailability remains low (<10%), requiring high doses of progesterone to be given in an attempt to produce adequate endometrial secretory transformation, which generates systemic side effects that are poorly tolerable for the patient [20]. The most frequent side effects of this route are neuropsychological effects such as sedation, dizziness, and nausea [21]. For all the aforementioned, its use is not recommended for LPS in ART [22].

It should be noted that dydrogesterone has recently appeared on the scene for this route. It is an optical isomer of progesterone, biologically active, with good oral bioavailability, structurally and pharmacologically similar to natural progesterone, and with the advantage of few side effects [16,23,24]. It has a high oral bioavailability, suggesting that it is as effective as the micronized progesterone, with a dose 10 to 20 times lower [25]. In addition to its oral form, it can be administered vaginally, with higher uterine level concentrations, but it is frequently associated with the presence of vaginal bleeding with a washout if bleeding is severe [24].

Several trials have shown that oral dydrogesterone is as effective as micronized vaginal progesterone for LPS with similar side effects and teratogenic profile [24,26,27].

In addition, a recent systematic review indicated that higher pregnancy and live birth rates are obtained in women with oral dydrogesterone compared with micronized vaginal progesterone [28]. Therefore, dydrogesterone would be recommended as LPS with a moderate level of evidence and a dose of 30 mg/day [22].

Intramuscular progesterone

Intramuscular progesterone is rapidly absorbed, avoiding the first hepatic step, reaching a high bioavailability [9]. It was the first route used, in doses of 50–100 mg/day, and has been considered the gold standard of administration routes. Its advantages are that it avoids the risk of inappropriate application since it must be administered by a health care professional and doses can be modified by monitoring serum progesterone levels; however, its side effects have forced looking for other alternatives. It frequently causes pain at the injection site, and due to its oily base (sesame or peanut oil), it could generate allergic reactions and has a small risk of sterile abscess [16,26]. Similar results have been compared and obtained between the vaginal and intramuscular routes in terms of clinical pregnancy rate, ongoing pregnancy rate, miscarriages, and live birth rates [29,30]. Hence, the vaginal route is preferred because of its better adherence. However, it is still a recommended route that can be used. The recommended dose is 50 mg/day [22].

Vaginal progesterone

The support of this route is the epithelium of the vaginal mucosa, and the lymphatic route allows the direct diffusion of progesterone from the vagina to the endometrium. This is called the "first uterine pass effect." It has allowed improving bioavailability at the uterine cavity with low systemic side effects [9]. Therefore, vaginal administration arises as a better alternative to the previously described progesterones and seems to be the best remaining option to administer progesterone by the nonoral route and at the same time avoid the inconvenience of injections [31]. The benefits of this route are the absence of pain, absence of hepatic metabolism, rapid absorption, absence of neurological side effects, relatively high availability, the positive effect of the vagina as a reservoir for the drug, and the local endometrial effect: first uterine passage. There are different forms of presentation such as tablets, suppositories, creams, oil-based solutions, or gels, and their absorption depends on the type of formulation [32]. It should be noted that all presentations are equally effective and safe with similar side effects [29]. As mentioned earlier, the vaginal application avoids the first step of metabolism in the gastrointestinal tract and at the hepatic level, reaching its maximum concentrations in plasma between 3 and 8 h post administration and gradually decreasing in the following 8 h, depending on the vehicle used [9,32]. Although the relatively low circulating levels of progesterone cause concern [31], this route is as effective as intramuscular in clinical and ongoing pregnancy, miscarriage, and live birth rates, with fewer side effects and better patient adherence [19], [30]. A recent survey of 303 *in vitro* fertilization (IVF) units reported that the majority (74.1%) of respondents prefer the vaginal route as the route of administration of progesterone [16]. For formulation or product preference in the aforementioned survey, 46.7% preferred vaginal tablets, 25.9% vaginal gel, 13.8% vaginal suppositories, 10% vaginal pessaries, 2% other routes, and 1.6% never used the vaginal route [16]. Adverse effects of this route are infrequent [9] and include vaginal discharge, local warmth, and irritation [33]. Recent studies have reported that this pathway may also alter the vaginal microbiota [34].

Subcutaneous progesterone

Cyclodextrins have allowed the solubilization of progesterone. Particularly, hydroxypropyl-b-cyclodextrin is a cyclodextrin that has a high water solubility that allows the solubilization of high quantities of progesterone [35]. Once absorbed after injection, progesterone immediately dissociates from its cyclodextrins, remaining free in the circulation as if it were produced endogenously by the ovaries, while the cyclodextrins are metabolized [36].

Doses of 25 mg/d mimics the physiological amount produced daily by the ovary during the MLP and results in a complete predecidual transformation of the endometrium [37].

The use of this route allows self-administration with fewer side effects for the patient than the vaginal route, with comparable results in terms of implantation rate, pregnancy rate, live birth rate, and miscarriage rate [38,39].

Transdermal progesterone

There are two important reasons why it is not recommended. The first one is that very high doses must be administered to mimic physiological values. The second one is that the skin has high levels of 5a-reductase, an enzyme that metabolizes progesterone. Therefore, a significant fraction of the progesterone will be inactivated before reaching circulation. For all these reasons, it does not prove to be a valid option [36].

LPS in assisted reproductive treatments

Intrauterine insemination

Intrauterine insemination (IUI) involves the delivery of sperm through the vagina into the uterine cavity and aims to increase the conception rate by maximizing the number of healthy sperm at the fertilization site [40]. The outcome of this treatment option depends on many factors, one of the most uncertain of which is the quality of the luteal phase [4]. The effects of LPS in IUI cycles are unclear and remain controversial [41].

It appears that the need for LPS, in this type of treatment, depends on the type of drug for the ovulation trigger used. There is evidence that clomiphene citrate treatments enhance corpus luteum function [42]. Related with this evidence, LPS did not benefit those women who underwent induction with clomiphene citrate. Conversely, those patients who had received gonadotrophins for ovulation triggering increased their probability of clinical pregnancy and live birth with LPS administration [43]. In the latter, the clinical pregnancy rate and live birth rates were similar for oral dydrogesterone, micronized vaginal progesterone, vaginal progesterone gel, and intramuscular hydroxyprogesterone [44].

In addition to the type of ovulation trigger used in IUI, the age of the patient would also be an important factor. LPS would be beneficial for older women [45].

At present, large multicenter randomized clinical trials are still needed to confirm the information described

before to establish the true cost benefit of LPS in IUI and to determine the length of administration and type of treatment to see a clinical benefit [43].

In vitro fertilization

It is widely demonstrated that LPS is crucial to support the gap between the disappearance of exogenously administered hCG for ovulation triggering and the onset of hCG production by the implanted embryo [16]. IVF cycles are unfailingly associated with a defective luteal phase, with an imminent need for LPS [2,46]. This contrasts with an inadequate luteal phase of only 8.1% in natural cycles [46–48]. A Cochrane meta-analysis reported higher ongoing pregnancy and live birth rates with luteal phase supplementation with progesterone versus no treatment (5 RCTs, OR 1.77, 95% CI 1.09–2.86, 642 women) [49]. The average length of luteal phase varies with the type of drug used for ovulation triggering. With hCG triggering, there is a length of approximately 13 days and with GnRHa unloading of 9 days [50].

Initial theories postulated that the disruption of the luteal phase in IVF cycles was a consequence of the removal of a high concentration of granulosa cells at the time of the oocyte pick-up, but this was dismissed when oocyte retrieval of natural cycles was performed, and it was seen that there was no decrease in either steroid concentration or luteal phase length [48]. Secondly, with the prolonged use of gonadotrophin-releasing hormone (GnRH) agonists to avoid the LH peak at controlled ovarian stimulation (COH), it was theorized that the pituitary's desensitization, by prolonged exposure to GnRHa, resulted in very low LH levels and consequent defective luteal phase. Nevertheless, this was dismissed when premature luteolysis and luteal phase deficit continued to be observed in cycles where GnRH antagonists were used to avoid the LH surge [48]. It is currently postulated that one of the main causes of the luteal phase deficiency would be associated with a dysfunction of the corpus luteum due to the supraphysiological steroid levels found in controlled ovarian hyperstimulation, generating an alteration of the hypothalamus-pituitary complex. All these endocrinological alterations compromise the support of the corpus luteum due to a disturbance in LH pulsatility [36,48]. Therefore controlled ovarian hyperstimulation in itself constitutes an indication for LPS [36].

Progesterone represents the preferred product for LPS and is recommended after IVF [22]. Nonetheless, there is still debate, and specialists do not always base their decisions on scientific evidence, as to when to initiate it, which is the best route, dosage, and duration, and when to use other agents for the LPS [50].

Progesterone's dosages and routes

There is limited evidence as to the best route and dose of administration. Any of the routes mentioned earlier can be used. Empirically, the recommended doses are 50 mg once daily for intramuscular progesterone, 25 mg once daily for subcutaneous progesterone, 90 mg once daily for vaginal progesterone gel, 200 mg three times daily for micronized vaginal progesterone-in-oil capsules, 100 mg two or three times daily for micronized vaginal progesterone in starch suppositories, or 400 mg two times daily for vaginal pessary [22].

However, the reported emerging use of oral dydrogesterone suggests a possible change in clinical practice as a result of recent evidence showing a reassuring safety score for oral progestins [50]. A 2020 survey of 148 clinicians in 34 countries showed that the most common route of administration currently used is vaginal (80% of respondents) [50]. Nevertheless, in another survey conducted in 2019, clinicians were asked, "If all progesterone formulations had the same results, which one would you prefer?" And, 62.2% would prefer the oral route, and 85.9% thought that this route would be the most comfortable and with the best adherence in patients [51].

Personalized luteal phase

Luteal phase insufficiency in natural cycles was described as early as 1949 [52]. Classically has been defined as a luteal phase of 10 days or less in length, but alternative biochemical definitions have also been proposed [53]. Suboptimal progesterone values have been defined in natural cycles in the range of less than 5–10 ng/ml [54,55]. Correlating with these findings in natural cycles, in the last decade, numerous authors have focused on a new factor related to the luteal phase in artifiacially prepared cycles, the progesterone value at the time of embryo transfer. The vast majority of studies agree that serum progesterone levels below 10 ng/ml could lead to impairment in early pregnancy [56]. Labarta et al. have conducted extensive studies on the minimum cut-off value required on the day of embryo transfer. In cases of progesterone deficiency detected on the day of transfer, they propose a protocol with a daily injection of 25 mg of progesterone subcutaneously from the day of embryo transfer plus 400 mg twice daily of vaginal micronized progesterone [57]. They have initially defined that progesterone values below 9.2 ng/ml on the day of transfer determined a lower ongoing pregnancy rate, which is therefore why these patients should be supplemented with higher doses of exogenous progesterone [18]. Subsequent studies defined a serum progesterone threshold of 8.8 ng/ml on the day of embryo transfer for the artificial

endometrial preparation cycles needed to maximise the results, in cycles with own or donated oocytes. In this same study, they identify that the subgroup of patients supplemented with vaginal micronized progesterone should have their mean luteal phase values monitored to adjust the dose required by each patient in a personalized manner [58]. In this way, they have been able to obtain similar live birth rates in patients with adequate progesterone levels (≥9.2 ng/mL) as in those with lower values but with individualisation of the luteal phase, demonstrating the importance of tailoring the luteal phase to the individual patient [57].

The onset of progesterone supplementation

The onset of LPS support has not been adequately studied to date [22]. While LPS is extremely important, premature administration of progesterone can cause advanced endometrial with embryo-endometrial asynchrony and premature closure of the implantation window [59]. Conversely, late administration may be insufficient to develop an adequate endometrium, interfering with its endometrial receptivity [16]. In correlation with the above, a study comparing the initiation of LPS in the 24 h prior to oocyte retrieval with the initiation on the day of follicular pick-up and with the initiation on the day of embryo transfer showed that there were lower pregnancy rates in those patients who initiated LPS 24 h prior to oocyte retrieval [60]. Likewise, when the onset of LPS was evaluated beyond the third day post oocyte pick-up, there were also lower pregnancy rates [61]. In a systematic review conducted in 2015, where five papers comparing different onset of LPS were included, it was suggested that the ideal time to initiate progesterone is between the evening of oocyte retrieval and the third day after it [62].

Recent guidelines published by the European Society of Human Reproduction and Embryology suggest that while more studies are needed to investigate the correct timing of LPS initiation, it should be initiated in the window between the night of oocyte retrieval and the third day post oocyte retrieval [22].

Nowadays, in daily practice, between 71% and 85% of clinicians answered they prescribe progesterone to their patients from the day of egg retrieval or the next day [16,50].

Ending of LPS

For many years, clinicians have considered the placental luteal shift described in the 1970s to maintain the LPS until that time or slightly longer. Between 6 and 7 weeks of gestation, corpus luteum function begins to naturally decline. During this period of luteal-placental transition, progesterone production shifts to the developing placenta, but this transition appears to be subject to some degrees of individual variation [63]. Over time, supported by the suggestion of potential teratogenic effects of prolonged fetal progestin exposure in pregnancy and the undesirable side effects, some authors have proposed to stop progesterone after a positive pregnancy test, based on the fact that trophoblast-derived hCG can sustain the corpus luteum with adequate progesterone production [64]. In addition to the discomfort and side effects of LPS, there is also the debate about the increase in treatment costs [65]. Therefore, many groups have now questioned the use of progesterone beyond a positive pregnancy test or an early pregnancy ultrasound [16]. In fact, some studies have shown that early discontinuation of progesterone (around week four of pregnancy) has no detrimental effect on fresh IVF cycles on the hypothesis that trophoblastic-derived hCG should be sufficient to rescue the corpus luteum [26,64]. The recent European Society of Human Reproduction and Embryology (ESHRE) guidelines suggest that progesterone for LPS should be administered at least until the day of the pregnancy test (low level of evidence) [22].

A recent systematic review and meta-analysis suggests that prolonged progesterone supplementation is not necessary and that early discontinuation would not have a detrimental effect on clinical outcomes (ongoing pregnancy, live birth, and miscarriage rates) [64].

Regarding serum progesterone dosing, during LPS, the data suggest that routine monitoring would not be necessary [64]. It should be mentioned that different serum progesterone levels have been reported depending on the route of administration, with plasma levels being low when the vaginal route is used with adequate endometrial maturation [19,30]. However, there are doubts for cycles where there is concern about the possibility of severe corpus luteum deficiency or threatened miscarriage [64].

Despite these points, several recent surveys have highlighted that more than half of clinicians continue LPS until 10–12 weeks of gestation [16,50]. This shows physicians' perception that the evidence for early cessation of LPS is weak and insufficient to generate a change in daily practice [65].

LPS and egg donation/frozen-thawed embryo transfer (FET) cycles

Fresh cycles and frozen-thawed cycles are completely different in hormonal dynamics and luteal phase. In fresh cycles there are multiple corpus luteum, and in a frozen-thawed transfer, there might be at most one corpus luteum, and generally there is not [26]. The lack of corpus luteum in these patients makes it essential to prepare the endometrium for adequate receptivity [9]. On the other hand, this type of treatment makes it

possible to achieve a more physiological environment without excess steroid hormones [26]. The great efficacy of regimens designed for endometrial priming has allowed using them not only in oocyte recipients but also for FET [66]. However, the optimal LPS is still under study [26].

In natural and modified-natural FET cycles, whether luteal support should be supported following FET is still debatable. Some authors report higher live birth rates in patients who received between 200 and 400 mg/day of vaginal progesterone, while others do not support the benefit of LPS in these cases based on the long luteotropic effect of hCG administered for ovulation triggering [26].

In the case of programmed cycles where the corpus luteum is absent, LPS is mandatory without consensus on its duration. In these cases it would be suggested to measure serum progesterone levels during MLP, achieving better clinical results with levels greater than 9–10 ng/mL [26].

The vast majority of studies show the same effectiveness for both the intramuscular and vaginal routes for endometrial priming [66]. Nevertheless, recent studies suggested an increased risk of miscarriage in the group of patients with vaginal progesterone [67]. There is not yet enough information to recommend dydrogesterone [26].

Use of estradiol in LPS

The corpus luteum produces progesterone and estrogens, which is the rationale behind the proposal to coadminister estrogens and progesterone [66]. There is however a great deal of controversy on the value of including estradiol in LPS with authors in favor [68] and others against [2]. In a survey conducted in 2018, when asked about the use of estrogens in LPS, 16.6% answered "always," 45.3% "in selected cases," and 38.1% "never," showing this disparity, so the lack of consensus exists [16].

The meta-analysis conducted by Cochrane found no benefit in adding estrogens in LPS [29].

Recent ESHRE guidelines do not recommend the use of estrogens for LPS, albeit with a low quality of evidence [22].

Use of hCG in LPS

Because of hCG's ability to rescue the corpus luteum, hCG has been used as the gold standard for LPS in the early days of ART treatment [15]. Also, the use of hCG or progesterone as LPS has been shown to have significantly higher pregnancy rates compared to placebo [19]. Despite the available evidence suggesting similar efficacy between progesterone and hCG, the latter has been associated with significantly greater risk of ovarian hyperstimulation syndrome (OHSS) with consequent lower usage due to serious safety concerns [26,29]. Therefore, in ovarian stimulation cycles triggered with hCG, hCG as LPS is not recommended [22].

Currently, ovulation triggering with GnRH agonists (GnRHa) is widely used, generating good oocyte maturation with luteolytic properties that favor the prevention of OHSS, but this also leads to a higher probability of pregnancy loss compared to cycles discharged with hCG [69].

In view of the luteolytic effects of GnRHa, a customized LPS with "hCG rescue" is suggested in cycles where ovulation triggering with GnRH analogs is performed and fresh transfer is desired [70]. In these cases, the application of hCG (1500 IU) in a single dose, 48 h after oocyte retrieval, without the need of any other support, has been recommended [71]. Other protocols recommend the use of daily microdoses of hCG (100–150 IU), generating safe levels of progesterone in the MLP, like those obtained with protocols with 6500 IU hCG and progesterone supplementation without OHSS risk. However, this type of protocol is limited by the lack of microdose hCG in the market [66].

Use of LH in LPS

Recombinant LH for LPS is not routinely recommended given the high costs associated with the doses required. In a study in patients triggered with GnRH analogs, supplementation of 300 IU/day of recombinant LH from oocyte pick-up together with 600 mg vaginal progesterone had similar reproductive outcomes to those receiving hCG [72]. However, recent ESHRE guidelines suggest that the addition of LH for LPS can only be used in the context of clinical trials [22].

Use of GnRH agonists in LPS

In 2004, the first prospective study was conducted to evaluate the potential benefits of GnRHa as a LPS agent by asking whether the luteal administration of a GnRHa can be considered a therapeutic action aimed at promoting implantation [73,74]. In this study, two recipients of sibling oocytes were administered placebo or GnRHa on day 6 post retrieval and showed better pregnancy and live birth rates, with similar miscarriage rates for the group that received the single dose of GnRHa. On the one hand, it is believed that GnRHa with an appropriate dose may retain its stimulatory effect to preserve LH production to support the luteal phase [75]. In addition, a single dose may directly influence early embryo quality for recipients without corpus luteum, although a direct effect on the endometrium cannot be excluded [76].

Most of the proposed protocols suggest a dose of triptorelin 0.1 mg or leuprolide 1 mg on the sixth day after oocyte retrieval, either in cycles with own or recipient oocytes [22,73,77]. However, there are some protocols that propose multiple doses (between 5 and 14 days of

administration), and there is discussion as to whether or not this would generate better results [78].

Nonetheless, as the evidence remains scarce, recent ESHRE guidelines propose a GnRHa bolus, in addition to progesterone for LPS or repeated GnRHa injections, alone or in addition to progesterone in hCG triggered cycles only be used in the context of a clinical trial [22].

Disorders of endometrial receptivity and Personalized Embryo Transfer

Embryo implantation is a complex and multifactorial process. The concept of hostile versus receptive endometrium has evolved over the years leading to a great deal of basic and clinical research. Trying to understand the basis of implantation provides a greater understanding of infertility of unknown cause and recurrent embryo implantation failure.

The endometrium is a dynamic tissue, and the window of implantation is known to be present between days 19–24 of a spontaneous cycle [79]. The difficulty arises when trying to diagnose the receptivity of the endometrium, due to the absence of a single efficient marker capable of ensuring that the endometrium is receptive in the same cycle in which the embryo transfer is to be performed. Multiple histological, biochemical, and ultrasonographic markers have been investigated, but no useful conclusions have been reached in clinical practice, because many of them are invasive and have no predictive value. Nowadays the ultrasonographic marker is the most used in clinical practice, although it has a limited value; the ultrasonographic pattern of the endometrium and its thickness are the parameters that the clinician considers before performing an embryo transfer.

Endometrial receptivity describes a phenotype in which embryo attachment and placentation are allowed. It was the pioneering work of Wilcox that first wrote about these events in which the embryo implants between 8 and 10 days post ovulation [80].

Endometrial receptivity is the result of the synchronization and joint action of ovarian hormones, growth factors, lipid mediators, transcription factors, cytokines, paracrine signals, among other events. The clinical diagnosis of the window of implantation remains somewhat uncertain and subjective and, in most cases, is considered a constant in patients who undergo ART, so it is not a study that is routinely requested at the beginning of the study of the infertile couple.

Molecular markers are the ones that are having a research boom. These markers are known collectively as OMICS, among them are genomics, epigenomics, transcriptomics, proteomics, metabolomics, and lipidomics. Transcriptomics is considered the most established marker for the study of endometrial factor. The DNA microarray technique allows the detection of multiple transcripts of multiple genes simultaneously, a fact that has revolutionized medicine today. The transcriptome reflects the activity of certain genes that are being expressed in each cell in each tissue. The set of gene expression detected at the mRNA level represents the transcriptomic signature of that tissue at that moment. Transcriptomics attempts to analyze gene expression patterns and correlate with their underlying biology.

This fits within personalized medicine understood as medicine that uses genetics or any other biomarker such as a molecular profile, together with diagnostic, prognostic, and therapeutic strategies precisely tailored to the requirements of each patient, including the therapies and doses necessary for an optimal outcome. The terms genetic, personalized, stratified, or precision medicine, pharmacogenetics, and pharmacogenomics have been used interchangeably to refer to "the study of genetic variations and their influence on how people respond to drugs." The endometrial transcriptome has already been characterized at different stages of the menstrual cycle [81].

Using the array technique, a customized panel has been developed to evaluate and date endometrial receptivity by studying gene expression at different times of the menstrual cycle. In their original work, Diaz Gimeno and colleagues designed a panel of 238 genes, called the ERA test (endometrial receptivity array), and to demonstrate its translational efficacy, they also designed a bioinformatics test with predictive power to classify the gene expression profile of the human endometrium compatible with LH + 7, which would also allow the detection of endometrial disorders related to the same [82]. In further work, the group of researchers was able to demonstrate that the accuracy of the test was superior to the histological study and that it could be reproduced in the same patient even 19–40 months later [82,83].

The objective is to be able to personalize embryo transfer at the most receptive moment for the embryo, especially in those patients with recurrent embryo implantation failures in oocyte donation cycles or in IVF cycles in patients under 40 years of age and even in cases of PGT-A with negative results. This was the objective of the work published by Ruiz Alonso et al. in which they demonstrated the clinical value of the endometrial receptivity test in those patients with recurrent embryo implantation failure (RIF), defined as a patient who had had three embryo transfers of embryos morphologically classified as of good quality. This group of patients represents a sector for which reproductive medicine does not yet have an effective treatment. The different causes of RIF can be grouped into anatomical defects of the uterine cavity, hydrosalpinx, acquired thrombophilia, and embryonic chromosomal anomalies, all of

them solvable, but when none of these pathologies is the origin of RIF, a big question mark remains as to the next step to be taken. In Ruiz Alonso's work, they found that 25.9% of the patients in the RIF group had a displaced window of implantation compared with 12% of the control group. They were able to repeat the test in 18 of 22 patients with endometrial preparation performed as indicated by the test and found that 15 patients were now receptive, and in three cases remained nonreceptive, requiring further analysis [84]. The test has a sensitivity of 0.99758 and a specificity of 0.8857, respectively, and it has a high reproducibility. The synchronization between the endometrium and the embryo is fundamental for implantation; the ERA study came to demonstrate that the implantation window is not "fixed" as always believed. It is known that controlled ovarian hyperstimulation treatments advance the implantation window, and it is believed that it could be closed by the time of transfer. Works published by Schapiro show higher pregnancy rate in delayed transfers with frozen embryos [85].

Having the transcriptomic signature of the window of implantation of each patient would allow to identify causes of treatment failure, and it is also important to know the genetic status of the embryo by PGT-A to have a better understanding of the causes that can lead to RIF.

Endometrial receptivity testing is a step forward in trying to improve ART outcomes. The question Mahajan asks in his paper published in 2016 is "What is the place of endometrial receptivity testing in infertile patients?" For the authors it has a place in RIF where a quarter of the cases could be due to alterations in the implantation window, and it could take place after two egg donation transfers. Knowing if there is a shift in the window of implantation would generate less stress, physical, psychological, emotional, and lower costs. It is useful in cases of endometriosis, endometritis, and adenomatosis. Among the limitations are the cost, the invasiveness of endometrial biopsy, and the need to cryopreserve the embryos. The author considers that embryonic PGT-A is of utmost importance but also recognizes that having the receptivity test and PGT-A, there are still no reports of 100% pregnancy rates, which leaves the window open to think that there is still much to be understood about maternal immunity in the process of embryo implantation [86].

Conclusions

One of the most critical moments in the steps to establish a pregnancy is endometrial decidualization and embryo implantation.

Nowadays, there are multiple proposals to customize a patient's LPS. There is still debate, and specialists do not always base their decisions on scientific evidence, as to when to initiate onset, which is the best route, dosage, and duration, and when to use other agents for the LPS. Nevertheless, there are different schemes that have proven to be useful, and there are also innovative proposals that could become useful in those patients who have not responded to more classical LPS schemes.

The development of new techniques to know in greater depth the implantation window will also allow improving and personalizing a patient's LPS.

It will be the task of the specialist in reproductive medicine to personalize the LPS in terms of the patient's clinical history, the type of treatment used, and the patient's preferences.

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CHAPTER

29

Preimplantation genetic testing

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Principle and history of preimplantation genetic testing

Principle

The aim of preimplantation genetic testing (PGT) is to have a healthy child from a pregnancy started with an embryo tested for specifics genetic disease and/or chromosomal disorders, in couples with a high transmission risk.

The couple requesting a PGT undergoes assisted reproductive techniques (ART) to produce embryos to biopsy and analyze for genetic and/or chromosomal defects. Embryos affected by genetic diseases or chromosomal abnormality are deselected for clinical use; both disease-unaffected and euploid/balanced karyotype embryos can be used for embryo transfer and potential future pregnancy.

PGT is an invasive embryo procedure. To be clinically applicable, the entire procedure must guarantee an accurate diagnosis without affecting embryo survival and live birth chances.

The first candidate couples for PGT are the fertile and infertile ones at risk of transmission of genetic diseases (PGT-M) as well as those in which one of the partners has an altered karyotype (PGT-SR). Both categories find in the application of PGT a possibility to avoid pregnancy termination after positive prenatal diagnosis [1,2].

PGT for aneuploidies (PGT-A) finds an area of application in infertile couples having a normal karyotype and undergoing *in vitro* ART treatment. The main indications for PGT-A are the following ones: advanced maternal age (AMA), defined as over 37–38 years old [3] given that the aneuploidy rate in the oocytes and produced embryos increases with maternal age [4], repeated implantation failure (RIF), defined as three and more failed embryo implantations after the transfer of high-morphologic-quality embryos, and repeated miscarriage (RM), defined as two or more pregnancy losses before 24 weeks of gestation, including chemical pregnancy [5]. The severe male factor (SMF) has often been considered an indication for PGT-A. The aim of PGT-A in infertile couples is to avoid miscarriage due to aneuploid embryo/fetus and increase live birth rate.

PGT-A can be added to PGT-M.

The first steps of clinical preimplantation genetic testing

The first PGT experience occurred in 1968 thanks to the pioneers Robert Edwards and Richard Gardner who selected rabbit blastocysts according to sex [6]. In 1990, and thanks to the invention of the polymerase chain reaction (PCR), Handyside's team [7] obtained the first pregnancies selecting embryos based on Y-specific regions amplification for couples at risk of X-linked diseases transmission. The same group performed the first diagnosis for a recessive disease, the cystic fibrosis [8]. At that time, PGT was called preimplantation genetic diagnosis. Embryo biopsy was performed removing one to two blastomeres on embryos at six to eight cell stage, and the diagnosis had to be completed in a very short time because unaffected embryos were transferred on the same ovarian cycle. The two molecular diagnostic methods were PCR for the diagnosis of genetic disease and fluorescent *in situ* hybridization (FISH) for chromosomal defects [9,10].

In the meantime, Verlinsky's group from Chicago was working on the preconception genetic diagnosis in which the aim was to deduce the content of metaphase (MII) oocyte from the genetic/chromosomal results of the first and the second polar bodies (IPB and IIPB) [11].

Then, the first misdiagnoses occurred! The groups basing their diagnosis strategy on PCR amplification discovered the phenomenon of "allele drop-out" (ADO), which is the preferential amplification (and detection) of one allele in a diploid cell. ADO resulted, depending

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on cell lysis method, in spatial DNA access to the PCR reagents and annealing temperature in the first cycles of PCR reaction [12,13]. ADO causes a genotyping error with different error gravity according to the diagnosed genetic disease. In case of recessive autosomal disease, the gravest error is the nontransfer of a heterozygous embryo wrongly diagnosed as homozygous mutated; in case of dominant autosomal disease, a heterozygous affected embryo could be misdiagnosed as wild-type and transferred. The risk of ADO was the reason why, in a first long period, dominant autosomal diseases were not diagnosed at preimplantation stage. Furthermore, it was established that the absence of signal in a molecular diagnosis could not be interpreted as a diagnosis per se because it could lead to a misdiagnosis [7,14].

Worldwide applications of PGT and the first questions on its efficacy

At the beginning, only a few groups offered PGT. Then, this number increased and the first consortium group from the European Society of Human Reproduction and Embryology and Reproduction (ESHRE) was created in 1997 to collect centers' data [15]. From then on, PGT was commonly applied for the most common genetic diseases (cystic fibrosis, beta-thalassemia, spinal muscular atrophy, Tay-Sachs disease), and the PGT-A application (called at that time "preimplantation genetic screening") started to spread among infertile couples undergoing *in vitro* fertilization (IVF) treatments. But at that time and using the available protocols, what was the real efficacy of PGT-A in increasing the chance of having a healthy pregnancy? Was it sure that PGT-A was not decreasing the *intrinsic* probability of pregnancy compared to a regular IVF? The team of Mastenbroek performed a randomized controlled trial and demonstrated that PGT-A was significantly decreasing ongoing pregnancy rate compared to treatments without PGT-A (37%−25%) [16]. The scientific community realized that several aspects of the procedure had to be improved.

First of all, cleavage-stage embryo biopsy results were inappropriate because the biopsy of one blastomere decreased the implantation rate [17] and live birth rate [18] by 39%. Embryos at the cleavage stage have the highest rate of aneuploidy [19] and chromosome instability [20]. Mosaicism reaches 91% of the overall blastomeres, making it clear that a single blastomere cannot be representative of the embryonic chromosomal content. Finally, FISH was insufficient to investigate aneuploidy because it tested a limited number of chromosomes. The cell could be normal for the investigated chromosomes and aneuploid for others.

Fortunately, important signs of progress occurred in the IVF and molecular laboratories. In the IVF lab, the procedure of keeping embryo culture until the blastocyst stage became a routine thanks to new culture medium [21] and better embryo culture conditions [22,23]. Vitrification protocols reaching nearly 100% of blastocyst survival were developed [24−26]. So, it became possible to use comprehensive chromosome screening methods, such as a-CGH (array-comparative genomic hybridization) that needed a longer processing time and was incompatible with fresh embryo transfer [27]. With the new massive parallel sequencing method "next-generation sequencing" (NGS), it became possible to perform both PGT-M and a comprehensive PGT-A from the same biopsied sample. In 2017, the terms "preimplantation genetic diagnosis" and "preimplantation genetic screening" were changed in PGT [28].

Yet, the clinical efficacy of PGT-A in randomized controlled trials (RCTs) still had to be clarified (Table 29.1) [29].

Protocol of PGT

The candidate couples for PGT-M and PGT-SR undergo IVF cycles to produce as many embryos as possible to test. Couples undergoing IVF for infertility can require PGT-A.

ICSI and embryo culture

The female patient undergoes an ovarian stimulation [30] to retrieve as many MII oocytes as possible to microinject with the partner's sperm by intracytoplasmic sperm injection (ICSI), or to vitrify and accumulate for postponed ICSI [31]. ICSI is the recommended fertilization method to avoid the biopsied sample contamination by paternal DNA from the spermatozoa attached to the zona pellucida (ZP) or maternal DNA through the cumulus cells.

Once ICSI is performed, the (fresh and/or thawed) micro-injected MII oocytes are cultured in dedicated incubators and the *in vitro* embryo culture starts. Time-lapse incubation systems help identify the best timing for embryo biopsy without altering *in vitro* culture conditions [32,33].

If the biopsy occurs at an early stage and the remaining time before fresh embryo transfer is sufficient to complete a genetic/chromosomal test, the embryo can be maintained *in vitro* up to the blastocyst stage, between the fifth and the seventh day. As an alternative, the embryo can be maintained in culture until the appropriate stage for freezing. The common protocol is to keep the embryo in culture until the blastocyst stage [34] for its biopsy and vitrification [26] until PGT will be completed.

TABLE 29.1 Reports of actual PGT applications.

PGT	Genetic/chromosomal defect	Molecular technique					Examples
		Array		Next-generation sequencing			
		a-CGH	SNP array	Direct mutation	SNP	Copy number	
PGT-M	Point mutation		X	X	X		Beta-thalassemia, sickle cell disease, hemophilia A, Tay-Sachs disease, Stickler syndrome type 1, retinitis pigmentosa 4, Marfan disease
	Microindel mutation		X	X	X		F508del cystic fibrosis, Crouzon disease
	Large deletion/ insertion (longer than read length)		X	X	X	X	Alpha-thalassemia, BMD, DMD, Charcot–Marie–Tooth disease, retinoblastoma 1, Roberts syndrome
	Dynamic mutation		X		X		Fragile X mental retardation 1, Huntington disease, myotonic dystrophy, Kennedy disease
	de novo disease with unknown locus		X	X	X		Achondroplasia in a sibling, Olmsted syndrome 1 in a sibling
PGT-SR and PGT-A	Balanced translocation	X	X			X	Reciprocal translocation Robertsonian translocation, insertional translocation, complex chromosomal rearrangement
	Unbalanced translocation	X	X			X	
	Whole chromosome aneuploidy	X	X			X	For all chromosomes
	Segmental chromosome aneuploidy	X	X			X	According to platform resolution

Continued

TABLE 29.1 Reports of actual PGT applications.—cont'd

PGT	Genetic/chromosomal defect	Molecular technique					Examples
		Array		Next-generation sequencing			
		a-CGH	SNP array	Direct mutation	SNP	Copy number	
	Inversion						Not observable
	Ring chromosome	X	X			X	Y ring chromosome
	Presence of sSMC						Not yet studied
	Uniparental disomy		X		X		Prader–Willi syndrome, Angelman syndrome
	Mosaicism (whole or segmental chromosomes)	X				X	According to platform resolution and sensibility
	Polyploidy	X	X			X	Not for all variants

In case of vitrified/warmed embryo transfer, the warmed blastocyst should be cultured until re-expansion is observed before transfer.

Embryo biopsy

The biopsy can be performed at MII oocyte/zygote stage removing the IPB and IIPB, at cleavage or morula stages removing one to two blastomeres, or at the blastocyst stage removing 5–10 trophectoderm (TE) cells. The embryo biopsy at cleavage and blastocyst stages are the most applied.

The IPB and IIPB can be removed simultaneously (between the sixth and the ninth hour post-ICSI) or sequentially (within the fourth hour for the IPB and as soon as the IIPB is expelled) from fresh or frozen/thawed oocytes. During the biopsy, the ZP is opened by a diode laser or mechanically, and the two polar bodies are analyzed together or separately. From the IPB and IIPB analyses, the genetic/chromosomal contents of MII oocyte are deduced. No data are available on the paternal contribution. The strategy of the IPB and IIPB biopsies does not substantially increase the live birth rate in women aged 36–40 years [35], and its clinical application is rarely reported in the updated scientific literature.

For biopsies at the cleavage stage, the embryo must have reached the six to eight cell stage [36]. In case of total or partial cell-compaction, the embryo is preincubated in a Ca^{2+}/Mg^{2+}-free medium to dissociate the blastomeres. During micromanipulation, the embryo is immobilized on a holding pipette and the ZP is opened using a diode laser or mechanically. Then one to two (nucleated) blastomeres are removed and tubed together or separately for testing and under microscopy check.

The biopsy at the morula stage requires Ca^{2+}/Mg^{2+}-free medium for embryo decompaction. The procedure is similar to the biopsy at cleavage stage (see above). Only a few PGT data from morula biopsy have been reported [37,38]. The consequences of decompaction at the morula stage on further embryo development remain for study as it is not possible to distinguish between the cells that will form inner cell mass (ICM) from TE.

The blastocyst biopsy can be performed on fresh or frozen-thawed embryos. Blastocyst stage is reached between the fifth and the seventh day of *in vitro* culture. The blastocyst is graduated according to ICM and TE cells morphology and the degree of embryo expansion [34]. While the blastocyst is expanded (or re-expanded if post warming) and the ICM cells are distinguishable from the TE cells, embryo biopsy is performed. The embryo is immobilized on a holding pipette and the ZP is opened using a diode laser. A few cells (5–10) are removed from the TE cells. A biopsy can also be performed on few external cells of a (spontaneously) hatching blastocyst. The zona opening can be anticipated on days 3–4 to facilitate the release of a few TE cells. It is recommended to perform biopsy before the completed blastocyst hatching.

After biopsy, the blastocyst is usually frozen because the time to complete genetic/chromosomal analysis is not compatible with embryo culture. In case of inconclusive genetic/chromosomal analysis, a frozen embryo can be thawed, biopsied again, and refrozen [39]. The biopsied cells are washed carefully and then tubed.

All procedures (material preparation, embryo biopsy, tubing) must be performed in a dedicated DNA-free environment to avoid exogenous DNA contamination [40]. Tubing pipettes are changed after each embryo. Biopsy pipettes can be used for several embryos only if carefully rinsed between two biopsies.

The biopsied cells are processed in the same laboratory or clinic or sent to an external genetic laboratory. The cells are prepared on sterile conditions and maintained at the lowest temperature as possible (from room temperature to −78°C with dry ice). Transport must be done as soon as possible to maintain temperature conditions in a hermetic package to avoid temperature variation and DNA contaminations.

Molecular analysis

After the biopsy, the cells are processed for genetic disease and/or chromosomal content. The methodologies used for PGT are PCR-based except for FISH, which is a molecular cytogenetic technique.

FISH is based on specific DNA sequence localization. In PGT, FISH is used to detect aneuploidy, balanced/unbalanced translocation, and sex determination from single blastomeres [41]. The cell is fixed on a slide and sequence-specific DNA probes are labeled with different fluorochromes that hybridize to target sequences in the interphase nucleus. After treatment and hybridization, the signals are evaluated by a fluorescent microscope. The main advantages of FISH are the short delivery times at competitive costs. But the limited reliability of the technique and the limited number of chromosomes to analyze made FISH abandoned for routine PGT-SR and PGT-A. Furthermore, it is not applicable for multicell samples such as biopsied TE cells.

For all PCR-based protocols, the biopsied cell (or group of biopsied cells) is tubed after biopsy. Several protocols of cell lysis are available. Proteinase K/sodium dodecyl sulfate [42] can be applied but alkaline lysis is more commonly used [43] because it leads to higher allele amplifications.

In the first protocols of PGT-M, nested-PCR was used to increase the quantity of DNA observable on agarose

gels. The presence or absence of pathogenetic variants was researched by methods such as restriction enzyme digestion, double amplification refractory mutation system, or Sanger sequencing. These methods were rapid and compatible with a fresh embryo transfer. Nevertheless, the number of detectable mutations was limited, the contamination could not always be detected, and the linkage analysis was not possible. Low allele amplification could be undetectable, leading to misdiagnosis or no diagnosis.

The mini-sequencing method was applied to diagnose a single gene defect. This method consists of a multiplex PCR followed by a mini-sequencing reaction performed by primers annealing a base before the mutation site; the extension step involves the incorporation of a single fluorescent dNTP complementary to the mutated base. The primer extension reaction is followed by automatic sequencing and analysis of the peak signals. Thanks to multiplex PCR, it is possible to simultaneously analyze single nucleotide polymorphisms (SNP) markers to perform segregation analysis. Different mutations in the same gene can be detected [44].

Quantitative real-time PCR (qPCR) was used to detect whole chromosome aneuploidy. This technique is based on a multiplex amplification using 96 probes, four for each chromosome. The resulting amplicons are quantified by qPCR using the delta delta threshold cycle (ΔΔCt) method [45]. The advantages are the low costs and a turnaround time of only 4 h, making it suitable for fresh embryo transfers. PGT-A ad PGT-M can be performed simultaneously. However, the low number of available probes has a negative impact on the resolution (20 Mb). qPCR highlights unbalanced translocations only if a probe is present in the translocated region. Mosaicisms, uniparental disomy (UPD), segmental mutations, and normal or balanced translocations cannot be detected [46].

Important changes in the PGT protocols occurred with whole-genome amplification (WGA) and the possibility to perform comprehensive chromosomal analysis on platforms based on arrays (a-CGH and SNP array) [47] or massive parallel sequencing such as NGS [48].

With WGA it is possible to obtain a suitable quantity of template starting from a few picograms of DNA in biopsied embryonic cells. The categories of WGA are temperature cycled (PCR-based) methods [49] and isothermal amplification methods [50]. PCR-based methods rely on ligation of a common primer sequence to sheared DNA or the use of degenerate oligonucleotides for priming. NEB-WGA and multiple annealing and looping based amplification cycles (MALBAC) are based on multiple annealing and looping amplification cycles chemistry. The constant region of the primers used in MALBAC is designed so the products of the initial reaction can form loops, thereby potentially excluding these products as templates for further DNA synthesis. Isothermal WGA methods, including multiple displacement amplification, utilize polymerases with high processivity and strand-displacement activity that extend from randomly primed sites.

A-CGH is based on the labeling of biopsied DNA samples and DNA references with different fluorochromes (usually green and red) that are mixed in equal parts and hybridized on a microarray slide covered with probes representing specific regions of the human genome. After the incubation and subsequent washing, the microarray slide is scanned, and a specific software processes the fluorescence intensities of the DNA sample and DNA reference. According to the fluorescent signal, the diagnosis of the entire or part of the chromosome is monosomic, euploid, or triploid. A-CGH is highly reliable and can detect translocated segments with a resolution of about 5–10 Mb [46] and mosaicism. It remains less sensitive than the NGS platform. The main limitations are the impossibility of detecting uniparental disomy (UDP), the distinction between normal to balanced rearrangements, and the high costs.

SNP array identifies variations of a single nucleotide in a specific locus, SNPs. SNPs have a high density throughout the human genome and are mostly biallelic. The first use of the SNP array for PGT-A was reported in 2010 [51]. SNP array detects unbalanced translocations, UDP, polyploidies, and mosaicisms thanks to its high resolution, but it has high costs [52].

NGS is the most powerful platform for PGT, and its power of genetic investigation seems to have no limits. It is possible to create universal protocols to both diagnose monogenic diseases and follow the allele transmission, to prevent the transmission of *de novo* diseases in which the precise chromosome locus is unknown, to perform PGT-SR and PGT-A for whole chromosomes, or segment and to quantify mosaicism for each (segmental) chromosome. PGT-M and PGT-A can be performed from the same biopsied cells.

For each sample to analyze, libraries of several hundred base-pair nucleotide fragments are created and barcoded with specific nucleotide sequences. All libraries are run together. The sequences are compared to human genome hg19 through cloud-based software. The variant analysis is processed using a dedicated workflow for the identification, filtering, and annotation of variant(s) for genetic analysis. To validate the PGT-M, polymorphisms with a high degree of heterozygosis are selected (STR, SNPs, CNV). Their minor allele frequency values should be superior to 0.3–0.5 and a distance inferior of 1 Mb to the gene defect to be highly informative and prevent crossing over. To determine the DNA sequence of mutated and wild-type alleles, cell samples from the patients and affected or unaffected relatives or arrested embryos from the same cycle are necessary.

Through informative SNPs uniformly distributed along each chromosome, karyomapping allows the diagnosis of aneuploidy and the gamete in which the aneuploidy occurred by linkage analysis [53,54]. UDPs and *de novo* mutations in which the precise DNA locus is unknown can be detected too.

For each sample in NGS, at least 100,000 reads are required. Sequencing data is reliable if the uniformity of base coverage is at least 99%, and the target base coverage is 500X. For monogenic diseases, a high end-to-end coverage of each amplicon is required with at least 20,000 reads per sample. A lower average coverage of 0.1X is sufficient for chromosomal analysis [55].

Embryo vitrification/warming and embryo transfer

After the biopsy, the oocyte/zygote/embryo at cleavage or morula stage/blastocyst can be frozen [26,56]. Vitrification warming is used worldwide. Once PGT is completed, the cells can be thawed for clinical use. In the case of biopsied blastocyst warming, the blastocyst is cultured until re-expansion, and transferred afterward [31]. It is recommended to perform a single frozen tested embryo transfer.

The embryo transfer is performed on a natural cycle and 7 days after luteinizing hormone (LH) surge, or on day 5 of progesterone administration after estradiol priming in a hormonal replacement therapy cycle. Other protocols of ovarian stimulation are reported in the literature [57].

Genetic counseling

As for any genetic analysis, PGT treatment must be preceded and completed by a genetic counseling in which all aspects of the protocol, such as accuracy and limits of the genetic or chromosomal test, are clearly explained. The prospective and limits of alternative testing strategies such as noninvasive prenatal testing and invasive prenatal diagnosis are explained in the pre-PGT phase.

All possible cellular and genetic results must be anticipated and discussed. The couple must be aware that all scenarios are possible, e.g., no embryo reaching the biopsy embryo-stage, or none of the embryos being transferrable according to PGT. For chromosomal testing such as PGT-A and PGT-SR, all the possible results including mosaicism must be discussed too. The policy of embryo transfer is established. An indicative percentage of success and failure in obtaining transferrable embryo(s) on similar clinical cases to the couple can be given if available.

After PGT and before the embryo transfer, *in vitro* and genetic results are discussed with the couple.

In case of pregnancy, it is reminded that prenatal diagnosis will help to confirm PGT (Fig. 29.1).

Quality and risk assessment

PGT is a complex process in which the processes can be divided into *in vitro* fertilization, embryo culture and biopsy, cell tubing and transportation, embryo vitrification and warming, post warming culture and transfer, and molecular diagnosis.

PGT fails when (1) no or few embryos are available to biopsy or transfer, (2) no pregnancy starts after embryo transfer, or (3) misdiagnosis occurs, and an affected embryo diagnosed as nonaffected is transferred with or without pregnancy.

In a risk analysis assessment, the successes and failures depend on four variables: (1) the patients and their biologic material (including embryos), (2) the operators (clinician, IVF lab biologist, molecular biology), (3) the procedures, and (4) the material (laboratory structure, equipment, and consumables). The risks related to external cell carriers must be analyzed too.

All along the PGT process from the oocyte retrieval to the transfer of the tested embryo, the traceability and matching of biopsied cells, embryos, DNA, or genetic report must be ensured to avoid mismatch, cell loss, or PGT error.

Along with the biopsy procedure, the traceability and the matching between the biopsied cell(s) (polar bodies, blastomeres, TE cells) and the cell they belonged to (oocyte or zygote, cleavage-stage embryo, morula, or blastocyst) must be ensured and perfect. The same ID code is used for the biopsied cell(s) and the oocyte, zygote, or embryo on each support or recipient (test tube, dish, straw). The traceability and matching during genetic or chromosomal analysis, vitrification and warming, postwarming culture, and embryo transfer must also be fully maintained. Each oocyte, zygote, or embryo must be frozen on a separate straw. A second operator supervises the embryo-biopsied cells matching during biopsy, vitrification, and warming, and the biopsied cells' DNA matching during molecular analysis.

Each area (clinician, IVF, molecular biology) must optimize their procedure efficacies through standardized and proper key performance indicators (e.g., postbiopsy and warming survival rates in IVF) [58] (in molecular biology: both allele amplification efficacy and allele recognition, detection of mosaicism percentage).

A route cause analysis performed by the "failure mode and effects analysis" method of each PGT step is a tool to evaluate how adequate the proper protocols are to each couple's request and what could be improved.

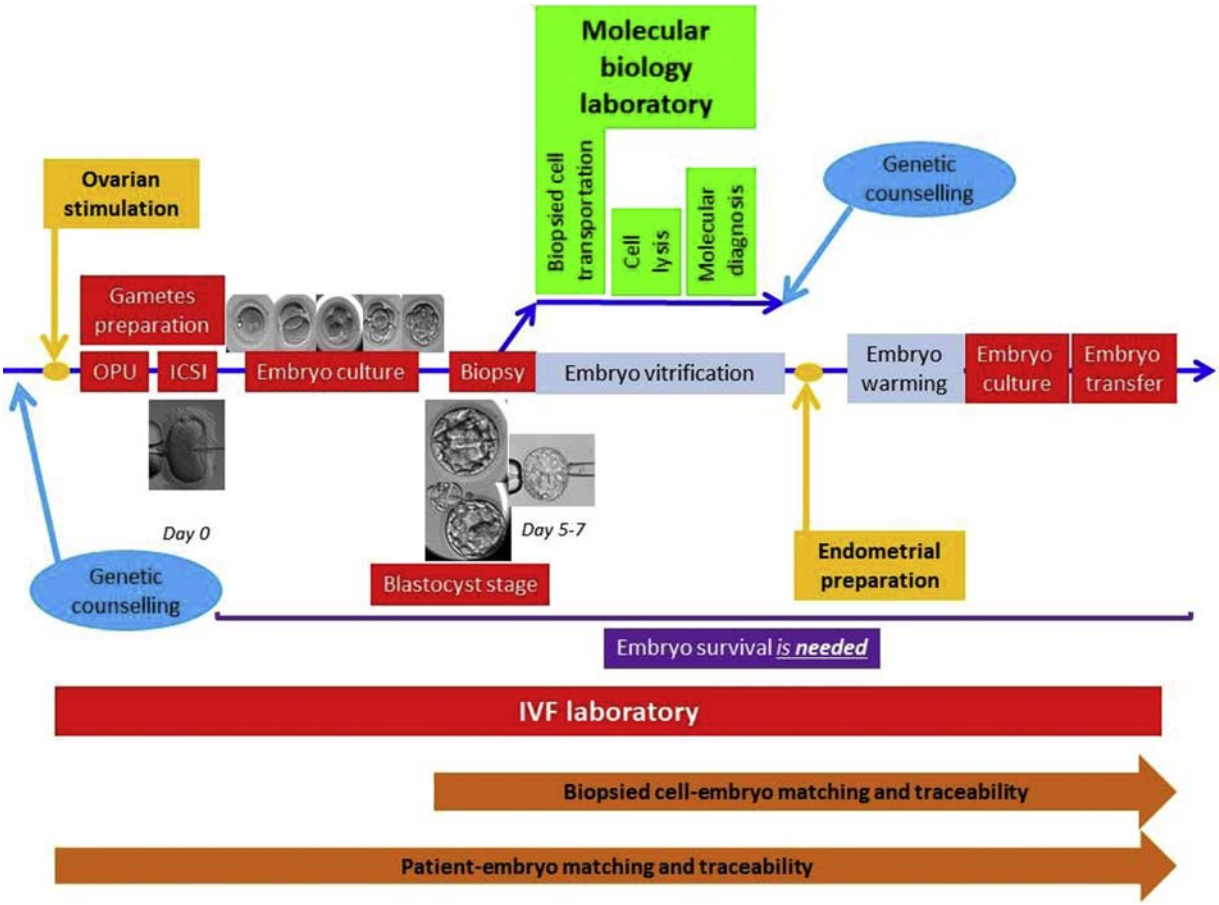

FIGURE 29.1 Summary of the steps of PGT protocol.

Table 29.2 summarizes the causes of PGT failure in a protocol based on embryo biopsy at the blastocyst stage, followed by embryo freezing and NGS analysis.

Results and efficacy of PGT

Efficacy of embryo culture, embryo biopsy, and consequences on clinical outcomes

The higher the number of oocytes to use for ICSI are, the higher are the chances to obtain embryos for clinical use [31,59,60]. The *in vitro* culture conditions in the IVF laboratory must be suboptimal to obtain as many possible embryos to biopsy and transfer. Morphokinetic parameters from time-lapse equipped incubators increase the blastocyst rate.

As previously described, embryo biopsy at the cleavage stage has a detrimental effect on embryo capacity to implant and give a pregnancy [18,61]. Blastomere removal on the third day delays embryo compaction, cavitation, and blastocyst expansion [62,63]. The first studies reported no increased risk to the health of singleton children after blastomere biopsy was observed [64].

TE biopsy seems to affect the implantation and live birth rates of frozen-thawed euploid embryos [65,66]. The reduction of TE cells due to biopsy reduces the level of serum β-human chorionic gonadotrophin (beta-hCG) in early pregnancy [67] and is associated with an increased risk of preeclampsia [68].

Database results

Data of PGT related to the years 2013–15 in Europe [69] and 2014–16 in the United States and United Kingdom [70] were recently published.

In Europe, more than 29,000 oocyte retrievals were performed with a prevalence of PGT-A (63.5%), followed by PGT-M (32.9%) and PGT-SR (11.9%). PGT for sexing due to X-linked diseases represented 0.7% of the cycles. ICSI was the fertilization method for 95.6% of the cycles. Biopsy was applied at all stages (polar body 7.5%, cleavage stage 63.8%, morula 4 3.4%, blastocyst 25.3%) with an increasing trend for blastocyst-stage biopsies. All molecular diagnostic methods were used (FISH, PCR only, qPCR, methods, a-CGH, NGS, SNP array). The number of WGA based methods was

TABLE 29.2 Causes of PGT failure.

Origin of PGT failure		No embryo to biopsy	No embryo to transfer	No pregnancy	No PGT result	Wrong PGT result
Patient or embryo		Response to ovarian stimulation	Gamete quality	Endometrium receptivity		Cell origin (mosaicism)
		Gamete and embryo quality	Embryo quality and survival	Embryo quality and survival		
Operator	**Clinician**			Embryo transfer		
	IVF lab	Gametes and embryo handling	Biopsy handling	Biopsy handling	Nuclear integrity of biopsied cells	DNA contamination
			Number of biopsied cells	Number of biopsied cells	Cell lysis, cell at biopsy	
					Cell tubing	
	Cryopreservation		Embryo survival (handling)	Embryo survival (handling)		Wrong embryo
						Wrong straw
	Mol. Biol. Lab				Genetic analysis process	Wrong straw
Protocol	**Clinician**	Ovarian stimulation protocol	Ovarian stimulation protocol	Endometrium preparation		
	IVF lab	ICSI procedure	Day of embryo biopsy	Day of embryo biopsy		
						Cell mosaicism (to be determined)
		Embryo handling		Postthawing culture		
	Cryopreservation		Embryo survival	Embryo survival		
	Mol. Biol. Lab				Cellular lysis protocol	Molecular strategy for PGT
					Molecular protocol	
Material	**IVF lab**		Culture medium	Culture medium		DNA contamination (IVF lab, culture medium)
		Culture conditions (lab and incubators)	Culture conditions (lab and incubators)	Culture conditions (lab and incubators)		
	Mol. Biol. Lab				DNA analysis platform	NGS platform sensibility
Outsourcing					Cell packaging	
					Cell transport conditions	
					Cell loss	
Traceability and matching		Patient, embryo, biopsied cell, DNA				

increasing through the years. A diagnosis was completed in 91.1% of the successfully biopsied samples.

Several data were not accessible such as the embryo survival rate for each biopsy method, the successful diagnosis rate for each molecular diagnosis method, and the clinical outcomes for each PGT indication according to the stage of embryo biopsy.

PGT data from the Human Fertilization and Embryology Authority (HFEA) in the United Kingdom and the American Society of Assisted Reproductive Technology in the United States were analyzed in the same period [70]. In the United Kingdom, PGT is only justified for monogenic diseases and structural abnormalities and is applied in 2% of the IVF cycles. From the HFEA data, live birth rates per embryo transferred and treatment cycles are superior from frozen cycles compared to fresh for all female ages. In the United States, PGT reached 21% of the overall IVF treatments.

Results and efficacy of PGT-M

According to the ESHRE PGT consortium data, half of the PGT-Ms are performed for autosome dominant diseases, a quarter for autosome recessive diseases, then for X-linked diseases (15%) and others [69]. The most diagnosed diseases are Huntington disease, myotonic dystrophy type I, neurofibromatosis type I or Marfan syndrome for dominant diseases, cystic fibrosis, beta-thalassemia, spinal muscular atrophy for recessive diseases and X-fragile, Duchenne and Busker muscular dystrophies, hemophilia A/B and incontinentia pigmenti for the X-linked diseases.

In the early period of PGT-M, the diagnosis was based on the mutation locus only [44,71]. The actual NGS platforms have enlarged the area of sequencing and make possible the direct sequencing of the mutated gene and the traceability of wild-type and mutated alleles through the sequencing of linked upstream, downstream, and intragenic informative polymorphism [72–74].

Nevertheless, NGS has the limitation of fragment length to read (e.g., 400 bp maximum). It suits for genetic diseases due to point mutation or short deletion or insertion (e.g., codon 39 in beta-thalassemia or F508del mutation in cystic fibrosis) but not for large deletion (e.g., alpha-thalassemia), dynamic mutations due to triplet extension (Huntington disease) or undetermined *de novo* mutations. In these cases, the karyomapping with specific SNP is performed [75].

For X-linked disease, it can be decided to eliminate male embryo transfer or to investigate for the mutated allele together with the sex determination in a view to deselect for transferring only the affected male embryos.

PGT-M can be performed in combination with PGT-A [74,76].

During PGT-M, a couple can ask for the selection of an unaffected embryo based on its human leukocyte antigens (HLA) compatibility to an affected sibling. After birth, stem cells of the double selected embryo are used to treat the affected infant. Since the first application of PGT-M with HLA compatibility for Fanconi anemia [77], different cycles have been performed in regard to the local legislation.

Recently, cycles of PGT-M for late-onset diseases such as breast cancer increased in number. These applications go beyond the original concept of PGT for a single gene disease that was to anticipate a prenatal diagnosis decision.

The legislation on embryo selection varies according to country. The accessibility to PGT-M for specific couples is a balance between the ethical and social principles and the individual freedom led by sensitivity and painfulness acceptability [78].

Very few data on misdiagnosis are reported in the scientific literature. The risk of misdiagnosis for a single gene disorder without linkage analysis and based on PCR was estimated at 0.4% [79]. Due to the increased resolution level of NGS platforms and the use of upstream, downstream, and intragenic informative linked polymorphisms, this percentage should be much lower even if it has not been calculated yet. Special care must be taken for those cases in which few or no informative linked polymorphisms are available.

Results and efficacy of PGT-SR

PGT-SR is applied for structural and numerical chromosome abnormalities transmitted by one member of the couple. The structural chromosome abnormalities are translocations (reciprocal, Robertsonian, and insertional), inversions, deletions, duplications, and ring chromosomes.

When the translocation is balanced and there is no breakpoint inside a gene, the patient is not aware of being a carrier excepted from a karyotype analysis. The two most common translocations are the reciprocal and the Robertsonian. The reciprocal translocation is an exchange of segments between two nonhomologous chromosomes. The exchange can occur between two autosomes or one autosome and one sex chromosome (X or Y). The Robertsonian translocation is a centromere-fusion of two homologous or nonhomologous acrocentric chromosomes (chromosomes 13, 14, 15, 21, and 22). The translocation der(13; 14) (q10; q10) is observed in 75% of the Robertsonian translocation [80]. The translocation incidence is 0.2% for the reciprocal and 1.1% for the Robertsonian in the general population.

During gametogenesis, the gametes produced by a (reciprocal or Robertsonian) translocation carrier are of four types: (1) nullosomic, (2) disomic for chromosomal segments involved in the translocation (the entire acrocentric chromosome for Robertsonian translocation) leading to monosomic or trisomic zygote, (3) monosomic carrying the balanced translocation and leading to a patient-like balanced translocated zygote, and (4) monosomic normal leading to a normal diploid zygote after fertilization. Consequently, the couple with a translocation carrier is at risk of having an affected child, suffering infertility, and miscarriage. The reciprocal and Robertsonian translocation nearly represent 62% of the indications for PGT-SR [69]. The percentage of transferrable embryos is different according to whether the translocation is carried by the male (23.2% for reciprocal and 36.6% for Robertsonian) or the female (20.2% for reciprocal and 30.1% for Robertsonian). The female carriers have a poor response to the gonadotrophin, and the imbalanced embryos rate is higher [81]. It is not rare that couples request the substitution of the carrier patient's gamete with a donated one. A systematic review on reproductive outcomes concluded that PGT-SR should not be offered as first-line method to fertile couples due to unproven benefits [82].

Initially, FISH was the diagnostic method for PGT due to reciprocal or Robertsonian translocations. Nowadays, array-CGH and NGS are mainly used [83,84]. A comprehensive chromosomal analysis is performed testing both the chromosomes involved in the translocation and the others. Normal and balanced translocated embryos can be distinguished only through an SNP array strategy [85].

Particular attention must be given to the autosome-allosome reciprocal translocations and especially to autosome-X chromosome. The embryo transfer of female embryos carrying the balanced X-autosome translocation should be avoided or carefully discussed with the patients as the unpredictable phenotype varies from normal to pathologic [86].

The insertional translocation is the insertion of a segment from one chromosome into another nonhomologous chromosome. Theoretically, 50% of the produced gametes of the carrier patient are abnormal because of nullosomic or disomic for the chromosomal segment involved in the translocation, and 50% of the gametes are balanced or normal. Two cases of PGT-SR for ins(14; 2) (q21; q31q35) [87] and ins(3; 2) (p23; q23q14.2) [88] have been reported.

The inversion on one chromosome is due to the breaking and reinsertion of a fragment in the same chromosome. The inversion can be pericentric (involving the p- and q-arm) or paracentric (on the same arm). The unbalanced parametric inversions result in gametes with acentric (no centromere) or dicentric (two centromeres) chromosomes and nonviable gametes [89]. On the opposite and in case of unbalanced pericentric translocation, the embryo can have a segmental chromosome monosomy or trisomy. Few studies reported PGT-SR application via FISH [90] and NGS [91]. The percentage of transferrable embryo is nearly 35% [69].

Complex chromosomal rearrangements involve more than two breakpoints and often more than two chromosomes. PGT-SR for complex chromosomal rearrangement has been reported using FISH, a-CGH, or NGS [88,92–94].

A ring chromosome is an aberrant chromosome whose ends have fused together to form a ring. We recently reported a couple that underwent PGT-SR for 46,X,r(Y). Four blastocysts were obtained from seven oocytes. After NGS, they were diagnosed as 46,XX (1 transferred embryo that gave the birth of a healthy girl), 45,X0 (1 embryo), 46,X,r(Y) (1 embryo) and 46,XX,50/46,XXdel(2) (q23.1qter)50 (1 embryo) (Personal data).

The numerical chromosome abnormalities carried by one member of the couple and to test by PGT are the mosaic Turner Syndrome [95] and sex chromosome aneuploidy such as Klinefelter syndrome or 47,XYY male [96,97].

The small supernumerary marker chromosomes (sSMC) are additional centric chromosome fragments too small to be identified or characterized unambiguously by banding cytogenetics alone. They are present in 0.04% of newborn children. To date, one study reported PGT-SR for sSMC using FISH [98].

In all structural and numerical chromosome abnormalities, the length of chromosomal segment to detect must be defined. The resolution varies according to the technique and is defined for CGH (10–20 MB and 25–100 Mb), array-CGH (2.5 and 2.8 Mb), SNP array (2.4 and 5 Mb), and NGS (5 Mb) [89,99]. For shorter chromosomal segments, specific sequencing strategies such as SNP-Seq or CNV-Seq should be applied [100]. Mosaicism at 20% and more should be detectable.

Results and efficacy of PGT-A to indications

Even if PGT was first invented for couples with a specific genetic or chromosomal indication, since the beginning of clinical applications, the proportion of PGT-A cycles irresistibly grew and reached 63% of the overall cycles in Europe after only 10 years of clinical application [101]. In the first period, the chromosomal analysis was performed on a limited number of chromosomes (X, Y, 13, 16, 18, 21, and 22) using FISH. Nowadays, PGT-A is given to perform a comprehensive chromosomal analysis. Between 2013 and 2015, 18,453 cycles of PGT-A were performed in nearly 60 European centers [69]. The main indications for PGT-A were AMA alone (47.4%) or combined with RIF (10.4%) or RM (8.8%).

In the United States, even if the true number of PGT-A is not precisely known, it increased since PGT-A is considered a benefit for both clinicians and patients [70]. The clinicians mainly based their opinion on four small RCTs [62,102–104], and the patients asked and paid for the "adds-on" promoted on the IVF clinic websites [105].

Unfortunately, objective data analysis tends to demonstrate that PGT-A efficacy is not what it was wished to be.

On one side, PGT-A is discussed as an opinion debate between experts [106,107] that is the lowest grade of evidence-based "medicine." On the other side, cumulative data analyses fail to show the PGT-A benefit. According to HFEA and 10 other professional and patient bodies, there is no evidence that PGT-A improves the chances of having a baby for most fertile patients. For specific infertile patient groups, the benefit of PGT-A remains to demonstrate, and it gives no further information on couple infertility [108].

A recent Cochrane study on "PGT-A in *in vitro* fertilization," reviewing 13 RCTs from 2008 to 2019, concluded that there is insufficient good-quality evidence of IVF with PGT-A on normal IVF in improving cumulative live birth rate, live birth rate after the first embryo transfer, and decreasing miscarriage rate. The effects of PGT-A on the clinical pregnancy rate are uncertain. The comprehensive chromosomal analysis of TE cells does not reduce miscarriage. There is insufficient evidence to support PGT-A in the routine clinical practice [109].

The benefit of PGT-A remains unclear analyzing the results according to indications (AMA, RIF, RM, or SMF).

While maternal age increases, the aneuploidy rate of produced embryos increases [4], reaching 34.5% at 35 years old and 58.2% at 40 years old. This is the reason why AMA is an indication to PGT-A application. In the last RCT, PGT-A increased the pregnancy rate for patients over 35–40 years old who had at least two blastocysts to biopsy [110]. However, this evidence was low [111], and there was no improvement when analysis was made per intention to treat and regarding miscarriage rates. Therefore, the low implantation rate of euploid embryos in patients with AMA seems to be due to factors other than aneuploidy contribution [112].

Two systematic reviews and meta-analyses found low evidence of PGT-A in improving clinical pregnancy, implantation, and live birth rates in patients with RIF [111,113]. PGT-A does not solve the problem of RM [114] as there is insufficient evidence that it decreases early pregnancy loss and the time to pregnancy [111]. The patient's miscarriage history is not associated with embryo aneuploidy [115], but it is essential to understand influencing factors such as the uterine environment, immunological and endocrine causes, uninvestigated genetic causes [116,117], or embryo damage due to PGT-A procedure.

The male factor remains a limited indication for PGT-A as the euploidy rate and implantation potential of tested embryos are independent of sperm quality [118,119].

The reasons for failed PGT-A efficacy are various:

- Once it was stated that embryo biopsy at the cleavage stage is detrimental to embryo vitality and gives an inaccurate chromosomal result, it was admitted that biopsy must be performed at the blastocyst stage. However, prolonged embryo culture implies embryo selection. Despite that the conditions of *in vitro* culture have greatly improved in the last decade, it is still unclear whether or not the embryos reaching the blastocyst stage in *in vitro* conditions would be competent if transferred at an earlier stage. This point is particularly sensitive for women with a reduced ovarian reserve such as AMA category [120].
- Embryo biopsy and cryopreservation could damage the embryo. Even if high standards have been reached, 100% of embryo recovery success cannot be ensured.
- The biopsied TE cells can be not representative of the ICM karyotype. Even if the TE and ICM originate from the same fertilization event, abnormal cellular lineages can appear in euploid embryos. According to the number of aneuploid cells in the biopsied cells and the sensitivity of the analysis platform, the result of PGT-A is aneuploid, euploid, or mosaic (see next paragraph). One euploid (ICM) embryo can be diagnosed as aneuploidy due to a "false positive" diagnosis and eliminated for transfer. The cells of the embryo under biopsy can be in a stage of DNA replication phase (S-phase), and the chromosome would result not readable. In this case, a second biopsy is needed.
- Uninvestigated genetic and nongenetic factors can be the cause of PGT cycle failure such as mitochondrial content [121].
- The consequences of embryo biopsy on implantation capacity remain for study as the biopsy of few TE cells reduces the levels of beta-hCG and increases preeclampsia events.

PGT-M or PGT-SR should be completed by comprehensive PGT-A to avoid the transfer of an embryo unaffected by genetic or chromosomal trait(s) under first investigation, but that would result in a pregnancy termination because of aneuploidy [122].

Whole chromosome mosaicisms and segmental chromosomal abnormalities

In 2015, the first pregnancies reporting healthy euploid live births from transferred mosaic aneuploid

blastocysts were reported [123]. Six pregnancies gave live births from 18 embryo transfers. This work highlighted the sensitivity of the analysis platform in determining the different karyotypes of a sample of a few cells and the limits of PGT-A reliability from TE cells. It appeared clearly that a healthy baby could be born from a noneuploid PGT-A result. In other words, the TE cells can be not fully representative of ICM.

Whole chromosome mosaicisms and segmental chromosomal aneuploidies are two limitations of PGT-A reliability. Chromosomal mosaicism is defined as the presence of more than 1 cell lineage in an individual. All cells originate from the same fertilization event, but during successive mitosis, failure in sister chromatid segregation can happen, leading to a gain or a loss of chromosomes in a group of cells. The mitotic mechanisms responsible for chromosomal loss of are the nondisjunction, anaphase lagging, and endoreplication of a chromosome. Endoreplication and anaphase laggings can occur during the embryonic stage [124].

Mosaicism is known as being responsible for genetic diseases, chromosomal syndromes, congenital malformation, mental retardation, and disorders such as autism and schizophrenia, cancer, embryo development arrest, and miscarriage [125,126]. Its rate increases with aging. At the embryonic level, the earlier a mitotic error occurs in the development, the more abnormal cells will be present in the organism. Consequently, aneuploid cells can be present in the entire organism, in specific tissues, in only one tissue such as the gonads, or a group of cells. Due to the mosaicism cellular territory and the chromosomal abnormality, consequences on development and health are different.

One can distinguish the diploid-aneuploid mosaicism with the presence of both diploid and euploid cells, the polyploidy mosaicism with the presence of any combination of haploid, diploid, and polyploid cells, and the chaotic mosaicism with random chromosome complements in each cell [127].

A study of 36 good-quality day 2 embryos from young women found 16.7% of the embryos normal in all their blastomeres and 83.3% mosaic [128]. It appears, once again, that PGT-A is not applicable at the cleavage stage. The ICM is the result of three cells from the eight-cell embryo [129].

While the embryo develops to the blastocyst stage, the rate of aneuploid cells decreases as the percentage of diploid cells increases [130]. From mouse experiments, it was demonstrated that aneuploid cells located in the ICM tend to be eliminated, while those in the TE have a slow-down proliferation [131]. An euploid/aneuploidy mosaic embryo is able to rescue in a fully euploid embryo.

In humans, confined placental mosaicism affects approximately 2% of the viable pregnancies [132]. In particular, mosaicism diagnosed by chorionic villi samples is confirmed by amniocentesis as being a true fetal mosaicism in only 13%, and 2.1% are due to uniparental disomy [133]. Chorionic villi samples have limits in representing the true fetal karyotype. As the chorionic villi originates from TE, the probability of nonmatching between a few biopsied TE cells and the ICM must not be underestimated.

UDP is the presence of two chromosomes from the same parental origin. It may be the result of a trisomy rescue or an entire chromosome endoreplication after a nondisjunction with anaphase lagging. Being a double copy of the same chromosome, the recessive traits are expressed. The chromosomes involved in UDP are the chromosomes 15 with Angelman syndrome due to double paternal chromosome copy and Prader–Willi syndrome due to double maternal chromosome copy, and the chromosomes 6, 7, 11, and 16. In PGT-A, UDP can be detected in an euploid sample using specific SNPs in NGS or karyomapping.

Segmental chromosomal abnormalities are the presence of a gain or loss of chromosomal fragments in a chromosome arm. They can be generated during meiosis or at the postzygotic stage due to a mitosis default. All along the human chromosomes, hotspots are specific fragile sites on the DNA where chromosome breakages are known to occur, generating segmental aneuploidy [134]. *De novo* segmental aneuploidies have also been reported on embryos [135]. The segmental aneuploidies are frequent in the cleavage embryo (24.3%), and less in the blastocyst (15.6%), suggesting that the abnormal cells are eliminated during the development [134].

Chromosomal concordance between inner cell mass and trophectoderm cells

At the blastocyst stage, the concordance between ICM and TE for whole chromosomal aneuploidies due to meiotic default for one or more chromosomes is 96.8% [136]. This result was confirmed on day 8–12 human embryos from extended embryo culture. The concordance is 100% for aneuploidy and 61.9% for complete euploidy [137]. It can be concluded that the aneuploidy generated by meiotic error and involving the entire embryo is detected by NGS.

Whole or partial chromosome mosaicisms have been reported in nearly 17% of the blastocysts on day 5–6 [110], involving between 2% and 13% of the cells [138] with a nonuniform distribution of aneuploid cells between ICM and TE [139]. The causes of mosaicism remain to be established and could be intrinsic to the patient or depending on laboratory procedures [138,140,141].

In case of whole chromosome mosaicism, the PGT-A result changes with the biopsy spot and consequent concordance with ICM [138,142]. In case of segmental chromosome aneuploidy, the concordance between TE cells and ICM drops to 42.9% [139] making the TE cells not representative of ICM.

To assess mosaicism, 5 to 10 cells should be biopsied for PGT-A. Comprehensive chromosomal analysis must be performed on an analysis platform with high sensitivity in a view to detect at least 20% of mosaicism and a resolution noninferior to 10 Mb. Shorter segmental abnormalities can also occur [116,117]. Specific protocols of NGS platform validation must be performed in each laboratory.

COGEN and Preimplantation Genetics Diagnosis International Society (PGDIS) stated the priority of the embryo to transfer according to the chromosome(s) involved in the mosaicism [143,144] and the percentage of aneuploid cells [136]. It is recommended to not transfer the embryos with viable aneuploidies. The transfer of mosaic embryos with trisomy 2, 7, 13, 14, 15, 16, 18, or 21 is to be avoided as the child could be affected by the trisomy. When mosaic embryos are transferred, amniocentesis should be performed to know the true fetal karyotype.

The transfer of mosaic embryos is associated with reduced clinical outcomes and higher miscarriage rates [141]. The best results for clinical outcomes are obtained for mosaicism inferior to 50% [136,141]. After euploid embryo transfer, the highest clinical outcomes are obtained for segmental mosaicism (low percentage then increasing), followed by whole chromosome mosaicism (low percentage first then increasing) involving an increasing number of chromosome (one chromosome first, then two, and so on). As in the mouse embryo, a process of mosaicism rescue in the human embryo would eliminate abnormal cells and make the embryo become fully euploid. This process does not exist for aneuploidies due to meiotic error [137]. Nevertheless, mosaicism can persist through the development and a case report has been reported [145]. Long follow-up of children from mosaic embryos should be performed.

The use of platforms such as a-CGH and NGS based on copy number methods and distinguishing between complete aneuploidy or mosaicism affecting whole or partial chromosomes is recommended. Due to the variability of false positive, embryos should be re-biopsied when segmental abnormalities are found. The false-negative diagnostic rate was estimated inferior to 4% [138]. Consequently, the proportion of euploid embryos eliminated because diagnosed as aneuploid for whole or segmental chromosome would be superior to the number of true aneuploid embryos diagnosed as euploid.

Noninvasive PGT

The common PGT protocols described here are invasive and demanding in regard to the embryo and may impact implantation rate. On the opposite, the advantage of noninvasive PGT is that it is performable from released material not essential to embryo development.

The two noninvasive PGT methods are blastocentesis and the analysis of spent culture medium (SCM). They both have been tested for PGT-M and PGT-A.

The blastocoel is a fluid-filled cavity formed in the blastocyst that contains metabolites, proteins, and DNA. The first experiment of blastocoel DNA amplification was the sex determination of embryos amplifying both Y-chromosome genes and an autosomal control gene on chromosome 17. The DNA detection rate was 90% [146]. Other groups tried to test the applicability of PGT-M from blastocoel fluid, but their amplification rates were too low to be reliable and ADO reached 44.4% [147]. To date, the DNA concordance between blastocoel fluid and TE cells needed for PGT-M remains to established [148].

The first PGT-A experiments showed that blastocentesis has a high chromosomal concordance with polar bodies, blastomeres, and TE biopsies [149]. But once again, 82% of reported DNA detection [150] has not been reproduced by others [151] that found a high chromosomal discordance. As the origin of blastocoel DNA remains to be established, its use for embryonic chromosomal status determination cannot be reliable. It could originate from aneuploid cells discarded in a process of euploidization in a mosaic embryo. The quantity of aspired DNA being inferior to 10 pg, it is easily degraded, limiting the potentiality of blastocoel DNA for PGT.

The SCM from embryo *in vitro* culture is another source of embryonic DNA [152]. Both mitochondrial DNA and genomic DNA have been reported since the second and third day [148,153]. Nucleotide molecules pass through the ZP due to the high degree of permeability of the glycoprotein membrane, and the quantity of DNA collected from SCM at cleavage or blastocyst stage is superior to the blastocoel DNA.

The first PGT for beta-thalassemia from SCM performed from 88 donated embryos showed a concordance of 64.5% with TE cells, reaching 100% with euploid TE cells [154]. The quantity and integrity of SCM DNA is superior to blastocoel DNA, with a coverage comparable to TE cells [148]. Nevertheless, the main limitation of SCM for PGT-M is the DNA contamination from polar bodies, cumulus cells, and DNA from protein-supplemented culture medium. As for invasive PGT protocols, ICSI is recommended to avoid paternal DNA contamination. Specific strategies

of DNA linkage are required to improve genetic analysis and detect exogenous DNA contamination.

Different groups amplified SCM DNA for PGT-A through different protocols of DNA amplification [148]. Even if a recent multicenter study showed encouraging results [155], the chromosomal concordance with the whole embryo remains low and variable [148]. As for blastocentesis, the origin of SCM DNA is to clarify. If this DNA originates from discarded cells and organelles in a mosaic embryo in an euploidization process, the lack of SCM amplification could indicate a top-quality euploid embryo that does not need to repair. On the opposite, the presence of DNA would indicate a low embryo quality [153].

In conclusion, even if PGT performed on blastocoel fluid or SCM would eliminate the invasiveness of traditional PGT, the grade of reliability and the genetic concordance with the whole embryo or the ICM remain to be established prior to clinical use.

Conclusions and the future of PGT

PGT was thought and designed as an alternative to prenatal diagnosis, testing embryos in couples at risk of genetic disease and/or chromosomal abnormalities transmission, aiming to avoid pregnancy termination for affected fetus diagnosis. Since the first applications in the early '90s, the PGT protocol has deeply changed due to important technical improvements in each step of the process. The embryo biopsy is now currently performed at the blastocyst stage. The biopsied embryo is vitrified (and warmed) with a high survival rate. The biopsied cells can both be analyzed for genetic defect(s) and chromosomal structural and numerical abnormalities by current massive parallel sequencing platforms such as NGS. Nearly all genetic diseases in which the DNA sequence is known or that have been located on the chromosomal map together with numerical and structural chromosomal abnormalities can be detected. With the third generation of sequencing that reads long DNA molecules, it becomes possible to identify the precise breakpoints in case chromosomal rearrangements such as translocation, gene fusion or deletion and insertions, offering the opportunity to differentiate carrier from noncarrier embryos [156].

NGS has definitively changed the accessibility of genetic data for each embryo created in the IVF lab. The limitation of genetic investigations from a single cell sample is only correlated to the understanding of DNA sequence, the cost of genetic investigation, and the local law. It is now possible to choose the sex of the transferred embryo (not for X-linked disease) and to make an embryo selection from genes that would not be analyzed in a prenatal diagnosis context but for diseases that the embryo *could* be expressed at late adult age. These diseases tested in PGT for polygenic disorders (PGT-P) are diabetes, cancer, heart disease, genetic cancer (such as breast, prostate, testicular, malignant melanoma, basal cell carcinoma), heart attack, etc. [157,158]. Numerous ethical questions on designed babies are arising and need to be clearly discussed. The fate of embryos carrying variants with uncertain significance or aneuploidy giving birth to individuals with normal mental development (e.g., Turner syndrome, Klinefelter syndrome) needs to be defined as well.

PGT protocol still is not perfect and some limitations remain. NGS platform's accuracy and sensitivity have shown that TE may not be always perfectly representative of ICM. Postzygotic mitotic events leading to few cells aneuploidy (mosaicism) involving whole or segmental chromosome(s) in TE cells limit the concordance with the rest of the embryo. Some scientists found in blastocoel fluid or spent culture medium (SCM) the possibility to perform noninvasive PGT for all embryos. The validity and feasibility of these strategies compared to the present invasive PGT have to be done and confirmed by large RCTs.

Infertile couples undergoing IVF have the possibility to test embryos for aneuploidies due to (mainly oogenesis) meiotic errors. PGT-A is intended to increase the live birth rate per embryo transfer and to decrease miscarriage and time to pregnancy. Among the candidate couples, the patients with AMA are the ones with the highest rate of aneuploidy transmission risk. Nevertheless, after years and a multitude of studies, the benefit of PGT-A remains limited. PGT is a demanding procedure in regard to the embryo due to embryo culture carried out until the blastocyst stage and the invasiveness of biopsy. Furthermore, both RIF and RM seem to not be done solely to embryonic aneuploidy but to other factors such as endometrial receptivity. The altered levels of mitochondrial DNA in an euploid embryo compromise the potential of an embryo to become a baby [121].

PGT is a complex process whose success depends on a tight multidisciplinary collaboration and monitoring. The applicability to the wider number of couples is strictly correlated to the efficacy of every single step from ovarian stimulation to tested embryo transfer. The couples undergoing a PGT treatment must be aware of the opportunities and the limits of PGT according to their own clinical contest. The couples at risk of genetic defect transmission, usually solved through prenatal diagnosis, are still the best candidates for PGT(-M). These fertile and infertile couples should be widely

informed on PGT as an opportunity to avoid pregnancy termination due to affecting the fetus.

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CHAPTER

30

Embryo quality evaluation and cryopreservation

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Embryo quality evaluation

Embryo quality evaluation is one of the most important tools used to improve the successful outcome of in vitro fertilization (IVF) programs. Thus, careful morphology assessment before embryo transfer (ET) may increase the chance of implantation, although the genetic assessment of the embryo remains a key point in embryo selection.

All embryo classification systems are subjective, regardless of classification methods. The most common tool or system for describing embryo quality is the microscopic static evaluation of morphologic features. However, microscopic evaluation presents a high inter-observer variability [1].

In recent years, with the introduction of time-lapse technology, embryologists have recognized the importance of morphokinetic characteristics in embryo quality selection. Kinetics appraisal involving synchrony of cell divisions has become a reliable tool for this important aim. However, the micro-environment of each laboratory, such as culture media and temperature, has shown to influence even the kinetics of in vitro development (Fig. 30.1).

Several scoring strategies have been developed to standardize and optimize embryo selection during the culture.

Traditional embryo assessment is based on time-point evaluations using light microscopy. Static observations during embryo development have improved significantly the evolutive physiological steps. The most widely used grading system, by Gardner and Schoolcraft [2], is based on the assessment of three parameters: blastocoele expansion and hatching status, size, and compactness of the inner cell mass (ICM), and the cohesiveness and number of trophectoderm (TE) cells. Several studies confirmed the rapidity of embryo evolution in terms of morphologic changes. It has been demonstrated that embryo status can markedly change within a few hours. Time-lapse technology revolutionized this evaluation system, improving embryo selection and providing a more stable embryo culture condition. This technology, which was introduced by Payne in 1997 for the first time [3], has been modified over the years. The development of the embryos may allow thorough morphokinetics evaluation combining an assessment of the morphologic features and of the timing in which cellular events occur. This tool provides a more accurate and unbiased embryo selection in IVF laboratories worldwide.

Widespread use of time-lapse technology may change many of the morphologic parameters currently in use in IVF laboratories. Morphokinetics evaluation is considered a powerful tool for embryologists [4], potentially increasing the rate of success of an IVF treatment.

In this chapter, we discuss the criteria developed by Alpha Executive and ESHRE Special Interest Group of Embryology in Istanbul in 2010 due to international consensus in the morphologic assessment of embryos and daily application in IVF laboratory [5].

FIGURE 30.1 Schematic representation of embryo development (from zygote to blastocyst stage).

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After a sperm–oocyte interaction, a series of dynamic processes with specific timing leads to fertilization and the formation of a blastocyst (Table 30.1). These events include sperm penetration, sperm–oocyte fusion and oocyte activation, male and female pronuclear (PN) development, and their gradual migration to a central position in the oocyte.

Fertilization check is a critical time point, but like all biological processes, there is a wide range of variability in timing. Asynchrony in the timing of any of the events associated with fertilization could compromise embryo development.

As already mentioned, morphologic parameters of the zygote are important. Its appearance is accepted to be a reliable indicator of gamete quality and embryo implantation potential. Many studies have underlined the predictive value of zygote morphologic assessment through correlations with chromosomal makeup and the incidence of zygotic arrest [6,7]. Recent strategies in embryo selection include sequential morphology assessment based on PN scoring. This feature has shown to play an important and promising role as an indicator of gamete constitution as well as a possible prognostic tool for embryo competence. However, other reports questioned the predictive value of PN scoring systems for IVF outcome [8,9].

During PN formation, nuclear precursor bodies (NPBs) are visualized and migrated in the nucleoli. This process is highly time dependent. Regular fertilization is defined by the presence of two centrally positioned, juxtaposed PNs with clearly defined membranes and two polar bodies. Continuous monitoring through a time-lapse incubator allows a deeper clarification of the cascade of events occurring during the zygote stage compared to the traditionally isolated observations using light microscopy [10]. An abnormal PN number (whether 1, 3, or more) is observed to be related to low chances of pregnancy [11]. Similarly, aberrant PN size and position have been correlated with developmental arrest and aneuploidy. PNs anomalies encompass unequal size, localization far apart or peripherally, or the presence of fragmented or additional micronuclei [12].

Correct alignment of PNs on the polar axis is considered a fundamental feature for the success of the first cleavage division and normal sequential development [13,14].

Three categories for PN scoring are established based on the morphology of NPBs and PNs. They are zygotes that exhibit the following characteristics:

1. symmetrical equal numbers and size of NPBs, either aligned at the junction between PNs or scattered in both PNs;
2. nonsymmetrical comprises all other patterns including peripherally localized PNs;
3. abnormal includes single NPB (“bull’s eye”) or total absence of NPBs.

In addition to the number and morphology of NPBs and PN, other characteristics, such as the morphology of the cytoplasm (normal or granular) and the presence of small or large vacuoles can be assessed to achieve a comprehensive evaluation of the zygote.

Cleavage-stage embryos range from the two-cell stage to the compacted morula composed of 8–16 cells. Many scoring systems based on the morphologic evaluation of cleavage-stage embryos have been developed [15]. These embryo classification systems are based on the evaluation of the number of blastomeres, the fragmentation degree, the symmetry of the blastomeres, the presence of multinucleation, and the compaction status.

Early cleavage checks are a beneficial tool in selecting embryos with high implantation potential and decreasing chromosomal anomalies [16,17]. Early cleavage checks should be observed 26 ± 1 and 28 ± 1 hours postinsemination for intracytoplasmic sperm injection (ICSI) and IVF embryos, respectively (Table 30.1).

The number of blastomeres is considered the main relevant characteristic with the highest predictive value [18]. In addition to the morphologic features, good quality embryos must also exhibit appropriate kinetics and synchrony of division. In normal developing embryos, cell division occurs regularly every 18–20 hours. Embryos presenting an abnormal timing of development, dividing either too slow or too fast, may present metabolic and/or chromosomal defects [19,20].

The mitosis of embryos very frequently results in the externalization of the cytoplasm’s cell, producing anucleate fragments. The number of such fragments has been used to predict the potential implantation of the subsequently transferred embryos. This parameter can

TABLE 30.1 Expected timing of fertilization check and embryo development [4].

Stage	Timing (postinsemination)	Development stage
Fertilization check	17 ± 1 h	Pronuclear stage
Syngamy check	23 ± 1 h	Up to 20% may be at the two-cell stage
Early cleavage check	26 ± 1 h post-ICSI 28 ± 1 h post-IVF	Two-cell stage
Day-2 embryo	44 ± 1 h	Four-cell stage
Day-3 embryo	68 ± 1 h	Eight-cell stage
Day-4 embryo	92 ± 2 h	Morula
Day-5 embryo	116 ± 2 h	Blastocyst

be associated with adverse outcomes, such as aneuploidy [19]. Based on the ratio of total embryo volume, the relative degree of fragmentation is defined as mild (<10%), moderate (10%–25%), or severe (>25%). A degree of fragmentation lower than 10% of the total embryo volume (defined as "mild") does not have a significant impact on the development potential [21,22].

The number of nuclei is a parameter of normal cell division. Healthy cells in eukaryotic organisms usually have only one nucleus, and this is especially true for developing embryos. Mitosis involves the duplication of the chromosomes before cellular division. The presence of one nucleus is a good indicator of normal development. Error in embryo cell division produces more than one nucleus. This condition is known as multinucleated blastomeres, which are associated with genetic embryo disorders [23]. This condition impairs cleavage rates and the implantation potential of human embryos [24]. It has been associated with an increased miscarriage rate [25]. Multinucleation can be evaluated on day 1, 2, and 3 of development.

The nucleus is not the only organelle containing genetic inheritance; even the external part of the cell may give information about the quality of the embryo. A clear homogeneous cytoplasm, for example, is acknowledged as a predictor of normality for cleavage-stage embryos. The presence of a high number of vacuoles or the aggregation of organelles resulting in granular cytoplasmic regions should be considered in embryo quality assessment [26–28].

The grading scheme for cell size should be binary, noting whether all cell sizes are appropriate or not to the relative stage of development. It is important to notice that such parameters can vary; differences are present within a single patient's embryos and between different patients.

The consensus scoring system for cleavage-stage embryos is reported in Table 30.2 [4]. Day 2 embryos (44 + 1 h postinsemination) should present four equally sized mononucleated blastomeres in a three-dimensional tetrahedral arrangement, with ≤10% of fragmentation. Subsequently, an optimal day 3 embryo (68 + 1 h postinsemination) is recognized by the presence of eight equally sized mononucleated blastomeres, with a fragmentation ≤10%.

The embryo in the morula stage (92 ± 2 h; Table 30.3) should be already compacted or in ongoing compaction, by the fourth round of cleavage. The consensus scoring system for day 4 embryos is presented in Table 30.3.

Finally, an optimal blastocyst (116 + 2 h; Table 30.1) is described as an expanded and hatched blastocyst with a prominent ICM composed of many compacted and adhered cells, with a TE forming a homogenous epithelium. ICM has a well-known high prognostic value for implantation and fetal development, as well as a functional TE.

TABLE 30.2 Consensus scoring system for cleavage-stage embryos.

Grade	Rating	Description
1	Good	<10% fragmentation Stage-specific cell size No multinucleation
2	Fair	10%–25% fragmentation The stage-specific cell size for the majority of cells No evidence of multinucleation
3	Poor	Severe fragmentation (>25%) Cell size non-stage-specific Evidence of multinucleation

TABLE 30.3 Consensus scoring system for morula.

Grade	Rating	Description
1	Good	Entered into the fourth round of cleavage Evidence of compaction that involves virtually all the embryo volume
2	Fair	Entered into the fourth round of cleavage Compaction involves most of the volume of the embryo
3	Poor	Disproportionate compaction involving less than half of the embryo, with two or three cells remaining as discrete blastomeres

For each of the developmental stages, the ICM and TE should be graded relative to the Gardner A–C scale, but a grade of 1–3 (rather than A–C) should be used as suggested by the Istanbul consensus.

Essentially, the difference between the "Istanbul consensus" suggested grading system and the Gardner and Schoolcraft is in the coding: the score is expressed using numeric grades (for the latter) instead of letters.

A blastocyst collapsed at the time of assessment, the consensus reads, cannot be graded. These blastocysts should be reevaluated 1 or 2 hours later, as regular cycles of collapse and re-expansion of blastocysts are expected as normal. Nonviable embryos are defined by an arrest in development for at least 24 h, or in case of visible degenerated or lysed cells.

The primary goal of blastocyst culture must be to increase the success rate of IVF. Blastocyst culture has been used as a tool to select the most viable embryos, reducing the number of embryos transferred in a row

with a consequent decrease in the incidence of multiple gestations. The blastocyst grading system introduced by Gardner and Schoolcraft in 1999 [2] was useful in the classification of the blastocyst expansion degree and of the morphologic appearance of ICM and TE cells.

The Istanbul consensus document follows, in broad terms, the Gardner and Schoolcraft system with some exceptions. The degree of expansion reflects the number of cells and the blastocyst's ability to create a cohesive barrier of cells. According to the number and cohesiveness of the cell populations in the ICM and the TE, cells are assigned three grades (A, B, C).

In addition, other morphologic features of human blastocysts are described: cellular degeneration in blastocysts, cytoplasmic strings/bridges between ICM and TE, vacuoles/vacuolation, and more than one point of natural hatching.

During blastocyst development, the process of cell death can occur by necrosis or apoptosis. Necrosis involves swelling of cells and membrane rupture, to which follows irreversible damage [29]. Cell death generally occurs by apoptosis, characterized by cellular shrinkage, and involves the aggregation of nuclear chromatin, condensation of the cytoplasm, and indention of nuclear and cytoplasmic membranes. Also, the fragmentation in the nuclei is responsible for blebs and apoptotic bodies [30,31]. Occasionally, these apoptotic cells, or more likely, cells that have been arrested at a later development stage, are present internally during blastocyst formation. Thus, rather than being sequestered in perivitelline space, they are incorporated by the blastocoel cavity and take no further part in blastocyst development.

Extending both from the ICM and the mural TE, an abundant quantity of short filopodia is found in the blastocoel cavity during the initial stages. These extensions are still present during expansion, which could be an indicator of poor embryo development, breakdown of polarization, or poor media conditions [14].

Furthermore, the presence of two or more sites of hatching is a rare occurrence in blastocyst assessment [32]. It has been suggested that this might arise in ICSI-generated blastocysts due to the incomplete closure of the zona breach created by the micro-injection pipette [33]. Hatching at more than one point in the zona pellucida (ZP), particularly when one of the holes is very small, could result in trapping of the blastocyst within the ZP, as the pressure within the blastocoel cavity would be dissipated and not concentrated on one hatching site (Table 30.4).

Scoring of embryos has been used since the beginning of IVF application, primarily to study and define embryo development rather than as a tool for selecting the best embryos with the highest implantation potential to transfer. A highly detailed description of the embryo features and identification of embryos with the best score are crucial to increase the probability of a successful and healthy pregnancy after either fresh or frozen ET. Hence, a common language for embryo evaluation is pivotal to compare, share, and improve results in IVF laboratories.

TABLE 30.4 Consensus scoring system for blastocysts. The scoring system for blastocysts is based on the stage of development and the grade of the ICM and the TE.

	Grade	Rating	Description
Stage of development	1		Early
	2		Blastocyst
	3		Expanded
	4		Hatched/hatching
ICM	1	Good	Prominent, easily discernible, with many cells that are compacted and tightly adhered together
	2	Fair	Easily discernible, with many cells that are loosely grouped together
	3	Poor	Difficult to discern, with few cells
TE	1	Good	Many cells producing a cohesive epithelium
	2	Fair	Few cells producing a loose epithelium
	3	Poor	Very few cells

Cryopreservation

Oocyte cryopreservation (OC) is an established method in assisted reproduction technology (ART). It has substantially changed many procedures in the IVF lab and has provided the opportunity to manage different types of patients for their benefit.

Cryopreservation of oocytes and embryos is an essential part of most IVF cycles and has revolutionized the world of ART. Cryopreservation consists in storing embryos at very low temperatures, keeping them unaltered and ready to be thawed in case of ET.

Initially, the standard slow-freezing method provided adequate results in terms of embryo storage and transfer outcomes. However, around 2008, IVF laboratories started to adopt the "vitrification" system, following a series of publications confirming the efficiency of this new technology, in particular on OC [4–6]. This technology spread rapidly worldwide as a reliable method, not only to preserve oocytes but also for embryos cryopreservation, changing the daily IVF practices.

Vitrification significantly improved ART outcomes including, but not limited to, embryos survival rates,

cumulative pregnancy rates, and efficiency of ET and IVF treatment. Moreover, vitrification reduced the risk of multiple gestations favoring a single-embryo transfer.

The process consists in solidification at low temperatures. The use of high cooling rates increases viscosity and prevents formation of ice crystals. The rapid cooling process can minimize chilling injury and dangerous osmotic shock to the sample.

In fact, cryopreserved samples are stored at extremely low temperatures (liquid nitrogen is −196°C), suspending all biological and physiological processes [34].

The most dangerous event in cryopreservation is the extra/intracellular ice crystal formation. To reduce the risk, a mix of permeable and nonpermeable cryoprotectants is used. However, such compounds may also induce cellular damage, either directly or indirectly (like osmotic injury).

The vitrification process implies a higher starting concentration of cryoprotectants compared to other techniques. Moreover, it requires fast cooling of the liquid medium (liquid nitrogen [LN_2] most of the time), achieved by using minimal volumes of cryosolutions. The absence of ice crystal formation is an important condition for correct vitrification.

This process is currently performed manually, but many research centers are working on automation [35]. High-level technical expertise and skills are necessary for embryologists and insiders to manipulate cells and tissues for cryopreservation and subsequent warming.

In an attempt to standardize outcomes, a semiautomated protocol [36] that allows automatic fluid exchange and loading has been developed, controlling the variables involved in manual vitrification. Nonetheless, the warming procedure still needs to be performed manually. Preliminary data using this automated system for oocyte vitrification have shown post-warming survival rates comparable to manual vitrification [37]. This is also the case of other equipment available in the market. Undoubtedly, the time when equipment is capable of providing a fully automatic process of vitrification and warming will come, thus ensuring the consistency of results.

On the market, several devices to vitrify oocytes and embryos are available. However, these tools have similar shape and utilization, and a size that minimizes the amount of vitrification solution required (Fig. 30.2).

There are different vitrification techniques. Vitrification can be categorized into an "open" and "closed" system depending on the contact with the liquid medium (LN_2). The first method allows reaching extremely high cooling rates due to direct contact with LN_2, presenting relatively high risks for potential cross-contamination and disease transmission through the medium (especially in the case of long-term storage). On the other hand, closed vitrification avoids direct contact with LN_2 using a high concentration of cryoprotectant, thus influencing the efficiency of cooling. Even in this case, studies have reported a decrease in cryosurvival rate, attributed to a cryoprotective compound used in closed vitrification [38].

FIGURE 30.2 Graphic representation of how an embryo is loaded into the vitrification device.

Embryos can be cryopreserved at different developmental stages: zygote stage (day 1); cleavage stage (day 2−3); morula stage (day 4); and blastocyst stage (day 5, day 6, and occasionally day 7).

Vitrification ensures a very high rate of survival of embryos (around 95% or above), independently of the stage at which they are frozen. This preservation technique seems to allow embryos to maintain high implantation rates, comparable to the results after fresh embryos [39,40].

Early-stage zygote cryopreservation is considered in case a patient presents very few fertilized oocytes or a generally poor embryo development is expected.

Blastocyst remains the stage to cryopreserve with the highest rates of positive outcomes. However, even at this point, there are morphologic variables that can affect the results of the preservation process. For example, recent evidence shows that the quality of the blastocysts cryopreserved impacts the performance results. In fact, expansion of the blastocoel and TE grade before freezing were indicated as the most reliable morphologic predictors of good pregnancy outcome, in terms of live birth. Similarly, the degree of re-expansion postthaw was selected as the most predictive parameter of live birth rate [41].

Frozen embryos have shown a very good resistance: authors proved a survival of decades, or even centuries, in safe cryo-storage with intact viability [42,43]. Despite the relatively limited literature, initial studies do not demonstrate good potential results of vitrification in terms of the embryo survival under storage [44,45].

The selection of carriers and cryo-storage containers should be based on their efficiency and ease of use.

There is a widespread suspicion that the risk of cross-contamination may be influenced by the selected cryopreservation device, or the storage method chosen. In fact, there are two different ways to store the vitrified cells: using nitrogen vapor or submerging the cells in liquid nitrogen. However, as of today, no evidence of

cross-contamination during storing cryopreserved oocytes or embryos has been reported.

In the last 15 years, several vitrification protocols differentiated by the type of cryoprotectant used have been described. For example, ethylene glycol (EG), dimethyl sulfoxide (DMSO), 1,2-propanediol (PROH), sucrose, Ficoll, and/or Trehalose [46].

The most used method involves preequilibrating embryos in 7.5% ethylene glycol (EG):7.5% DMSO for 12 minutes followed by a quickly transfer of embryos into the vitrification solution (15% EG, 15% DMSO, 0.5M sucrose) twice for 30 seconds each, loading them into the vitrification device, and rapidly putting into LN_2 (Fig. 30.3).

At present, most embryos and oocytes are vitrified by exposing the sample to direct contact with liquid nitrogen (open system) to increase the cooling/warming rates, and thus, the efficiency of the procedure [47].

Vitrified embryos are thawed by immersing them in 1 M sucrose in thawing solution for 1 minute. Then, they are transferred to 0.5 M sucrose in dilution medium for 3 minutes, followed by two incubations in the washing solution, 5 and 1 minute each (Fig. 30.4). After that embryos can be placed into a culture or transferred to patients.

The obvious reason to cryopreserve is to maintain viable and stable supernumerary embryos which are not used or useable for fresh ET, waiting for a future transfer. In the last years, the "freeze-all" strategy has emerged as an alternative to fresh ET during IVF cycles. The storage of all embryos derived from an ART cycle gives the advantage to control and delay ET. For example, during a natural cycle or a programmed hormone stimulation to prepare the endometrium. Such strategy can be appropriate if a patient presents ovarian hyperstimulation syndrome (OHSS) in correspondence with the scheduled ET. Another common practice is the use of gonadotrophin-releasing hormone agonists to trigger ovulation because they alter endometrial receptivity. Freezing all the embryos may be suitable for women with poor ovarian response, in case of prolonged stimulation, for patients with elevated progesterone levels at the end of the ovarian stimulation phase, low oocyte/embryo number, and other fertility conditions such as endometriosis [48–50]. These scenarios require a freeze-all approach in most cases.

Patients who undergo preimplantation genetic testing (PGT) also typically freeze their embryos. This is because the time required to report a genetic diagnosis exceeds the survival of the maximal embryo in culture. Even when it is possible to obtain a PGT testing result rapidly enough to allow a fresh embryo transfer, implantation and pregnancy outcomes appear to be superior after frozen ET compared to fresh ones [51]. Although cryopreservation of embryos is now a well-established procedure, long-term follow-up studies on possible effects on offspring are still few. Data from systematic reviews and individual cohort studies are mostly reassuring, suggesting that pregnancies obtained from a cryopreserved embryo (or oocyte) do not show an increased perinatal risk compared with those resulting from fresh ET. Interestingly, obstetric complications and perinatal negative outcomes (e.g., antepartum hemorrhage, preterm birth, small for gestational age, low birth weight, and perinatal mortality) are even lower in case of frozen ET. It is suggested that this can be

FIGURE 30.3 Schematic embryo vitrification. *ES*, equilibration solution (7.5% EG, 7.5% DMSO); *VS*, vitrification solution (15% EG, 15% DMSO, 0.5M sucrose); LN_2, liquid nitrogen (−196°C).

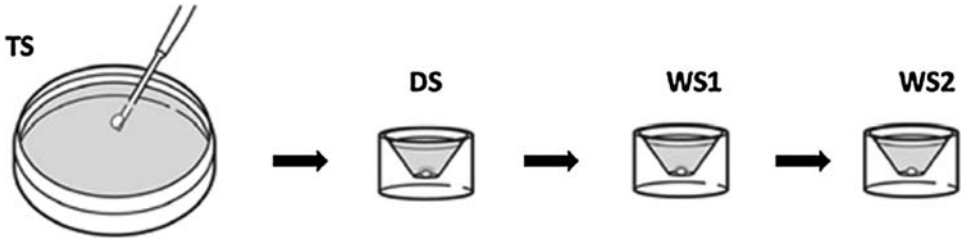

FIGURE 30.4 Schematic embryo thawing. *TS*, thawing solution (1M sucrose); *DS*, dilution solution (0.5M sucrose); *WS*, washing solution.

related to the improved uterine environment in case of delayed transfer, which may give greater support to the early mechanisms of placentation and embryo development [52].

Fertility preservation is increasing in popularity and frequency worldwide. Some patients may choose to freeze all embryos as part of a fertility preservation strategy, either for medical or for social reasons.

In conclusion, the efficiency of oocyte vitrification for safeguarding fertility is currently a consolidated option that can be offered as a way of forestalling age-related fertility decline to women at risk of losing their ovarian function for medical reasons. These include patients with cancer or women diagnosed with endometriosis, and women who wish to delay motherhood. Embryo cryopreservation with freeze-all strategy has been used with patients at high risk of OHSS, polycystic ovary syndrome or ovarian hyperresponsiveness, the requirement for preimplantation genetic diagnosis or screening (PGD/PGS), late-follicular phase elevated serum progesterone levels, endometriosis or adenomyosis, and recurrent implantation failure due to defective endometrial receptivity. Live birth has been reported after the transfer of frozen-thawed embryos that have been cryopreserved for up to 20 years.

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CHAPTER

31

Frozen embryo transfer

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Introduction

Infertility, defined as failure to conceive after 1 year of regular intercourse, remains a global burden, as it is estimated that it can affect up to one in every six couples worldwide during their lifetime. This condition is now treated primarily through assisted reproductive technology (ART), namely in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI). Since the birth of the first "test-tube baby," Louise Brown, in 1978, ART has been subject to exponential development, and huge advances have been made, counting today more than nine million babies born. One integral step in IVF is controlled ovarian stimulation (COS), which, through the use of exogenous gonadotropins, aims to stimulate the growth of multiple follicles, with the intention of obtaining multiple oocytes. The number of oocytes is a crucial prognostic indicator of both quality embryos and cumulative live birth rates, which is the final outcome measure of ART treatments [1,2]. Over time, the improvements in COS, culture conditions, and cryobiology techniques have led to higher numbers of good quality and transferable embryos, allowing for surplus embryos to be cryopreserved for subsequent use [3,4]. The first live birth after transferring a frozen-thawed embryo occurred in 1984 utilizing the slow-freeze technology, followed in 1990 utilizing vitrification [5]. Since then, elective freezing of embryos, followed by transfer in a subsequent cycle, also called frozen embryo transfer (FET), deferred embryo transfer, "freeze-all" strategy, or cycle segmentation, has significantly increased and can indeed result in high live birth rates [6]. Key players contributing to this trend include major improvements in extended culture conditions and the implementation of more efficient cryopreservation techniques, such as vitrification, leading to better embryo survival rates [7,8]. In the beginning, this strategy was intended to overcome the risk of developing ovarian hyperstimulation syndrome (OHSS), particularly in high responders [9,10]. However, over the years, indications for FET have gradually expanded, eased inevitably by the continuously reassuring safety data [11,12] and the elective single embryo policies, aiming to reduce multiple fetal pregnancies [13]. Increase of FET is intuitively strictly related to increase in freeze-all policies. Indeed, in addition to cases with a surplus amount of good quality embryos, FET is nowadays also extended to cycles implementing preimplantation genetic testing for aneuploidy (PGT-A) or for monogenic/single gene disorders (PGT-M), where biopsy results are required before transfer [14]. Freeze-all strategies, followed by FET, are moreover used in cycles with late-follicular progesterone elevation [6,15–18]. Likewise, FET is indicated in all cases lacking embryo-endometrial synchrony ranging from inadequate endometrial development to benign endometrial pathology [19]. Furthermore, the recent concerns regarding the possibly deleterious effect of ovarian stimulation (OS) on the endometrium in fresh cycles, which dictates poorer obstetric and perinatal outcomes, have also paved the way for more elective FET to circumvent the nonphysiologic endocrine milieu affecting embryo-endometrial interaction that is believed to be at the root of maternal and neonatal morbidity [20]. Additionally, FET inevitably follows all freeze-all policies necessary in fertility preservation, be it for social or medical reasons, or some oocyte donation cycles, as well as OS cycles for surrogacy, and it remains mandatory in nonconventional OS protocols, such as "random-start" and "double-stimulation" (Duo-Stim) [14]. Finally, FET is easier to plan and simpler than fresh embryo transfer (fresh ET). Inevitably, in this setting, the number of FET cycles was subject to continuous increase, finally surpassing fresh transfer, initially in the United States, where the proportion of FET among all

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embryo transfers was 77%, as reported in the most recent update from the US nationwide database [21–23]. Similarly in Europe, the proportion of FET cycles increased from 28% in 2010 to 34% in 2016 [8]. However, while the overall number of performed FET grows, the search for the better endometrial preparation protocol continues.

Background

It is generally accepted that for a healthy baby to be born, a genetically and morphologically normal embryo needs to implant into a receptive endometrium [24]. Successful implantation incorporates a complex series of events occurring during a specific and precise period of time that requires pedantic synchrony between an embryo with implantation competency and an endometrium in receptive state [24]. The period during the menstrual cycle that combines these prerequisite factors is defined as the "window of implantation" (WOI) and is largely intended as the days during the luteal phase of the cycle, where the endometrium is receptive to the embryo.

While the implantation competency of an embryo is relative primarily to its blastulation, the receptivity of the endometrium is strictly related to progesterone exposure. In natural conception, a progesterone rise occurs following the surge of luteinizing hormone, thus inducing a well-timed and systematic secretory transformation of the endometrium that results several days later in a receptive state [25]. Oocytes are exposed to spermatozoa roughly at the same time that secretory transformation begins in the endometrium, and if both zygote development and secretory transformation of the endometrium are normal, despite them being independent processes, then development will be synchronous and implantation is possible. This meticulous coordination can be lost in IVF cycles, not only due to altered embryonic ploidy or development, but also because progesterone rise may be both robust and untimely, thus shifting the endometrial window of receptivity.

Until now, research has failed in providing clear answers regarding the exact timing, duration, and molecular basis for the WOI; however it is thought to last from 2 to 4 days, opening and closing in the midluteal phase [24,26,27]. In the last decade, techniques to assess endometrial receptivity have notably evolved, and endometrial gene-expression profiling has gained a prominent position. The endometrial receptivity array, requiring an endometrial biopsy, aims to determine a personalized WOI through the examination of the expression of 238 genes thought to be involved in implantation, thus allowing for individualized, customized FET [28,29]. Endometrial biopsy is performed in a mock cycle on specific days based on luteinizing hormone surge or hormone replacement, and results are expressed as prereceptive, receptive, or postreceptive endometrium. The knowledge of the state of endometrial receptivity in relation to the day of endometrial biopsy allows for adjustments in replacement timing of the embryo in subsequent FET cycles, thereby enabling an embryo transfer that is customized to the patient's personal endometrial WOI [29].

Intuitively, in this setting, the determination of an optimal protocol for endometrial priming in FET cycles has become crucial to maximize ART success.

How to prepare the endometrium for FET

The two main methods for endometrial preparation for FET can be generally categorized into artificial and natural cycles (NCs) [20,22]. In NCs, there is no pharmacological intervention, despite a variation where ovulation is triggered, the so-called modified natural cycle (mNC). Conversely, in the artificial cycle, also referred to as a hormone replacement treatment (HRT) cycle, estrogen supplementation (E2) is used to achieve endometrial proliferation and follicular growth suppression, followed by progesterone (P) to induce secretory transformation of the endometrium. Less commonly, mild OS is employed for endometrial priming [30] (Table 31.1).

TABLE 31.1 Endometrium preparation protocols for frozen embryo transfer (FET).

Natural cycle (NC)		Hormone replacement treatment (HRT)	Mild ovarian stimulation (mild OS)
True natural (tNC) cycle • With luteal phase support • Without luteal phase support	Modified natural (mNC) cycle • With luteal phase support • Without luteal phase support	• With GnRH-a suppression • Without GnRH-a suppression	• Clomiphene citrate (CC) + FSH • Aromatase inhibitor (Letrozole) + (FSH)

Natural cycle and modified natural cycle

Performing NC FET indispensably requires the presence of a regular menstrual cycle. Indeed, in NC FET, presence and timing of spontaneous ovulation is crucial. Given the lack of medical intervention, meticulous endocrine and ultrasound monitoring is vital during the proliferative phase, giving the necessity to monitor the development of the dominant follicle and subsequent ovulation. In NC FET, it is the endogenous E2 secreted by the dominant follicle and P secreted after ovulation by the corpus luteum that prepare the uterus to schedule the transfer when the endometrium is synchronized to the developmental stage of the embryo.

The first transvaginal ultrasound, eventually aided by endocrine evaluation, is performed on day 2 or 3 of menses, aiming to rule out cysts or corpus luteum prevailing from the previous cycle. Serum P4 $>$ 1.5 ng/mL usually results in cycle cancellation [22]; however, this common practice is based on data extrapolated from fresh embryo transfer cycles, rather than scientific evidence. Proliferative phase monitoring, beginning on day 8−10, serum E2, luteinizing hormone (LH), and P are assessed on alternate days or daily, in addition to ultrasonographic monitoring that aims to precisely document ovulation to schedule FET accordingly. In NC FET, ovulation is pinpointed through serial blood (or, albeit less accurate, urine) sampling until an LH peak is observed. However, there is no unanimous definition of what to consider LH surge in the literature today, and although it has been historically described as an increase of the level of LH beyond 180% of the mean level observed in the previous 24 hours [31], in clinical practice a variety of definitions are used, including a level of 10 IU/L or a level of 17 IU/L more, leaving an open discussion regarding the place of endocrine evaluation in NC [20,22,32]. Heterogeneity in LH surge definition highlights the usefulness of detecting other signs to confirm ovulation, like ultrasonographic findings, the light drop in serum E2, and the rise in serum P (>1.5 ng/mL) the day after the LH surge. There is lack of scientific data regarding the optimal endometrial thickness in NC FET, and clinical practice is dictated by extrapolation of data from fresh and HRT cycles [20,22], generally considered adequate if $\geq$ 7 mm. The evident advantage of NC FET relies on the absence of estrogen supplementation and all its related possible complications. Nonetheless, this protocol necessitates a higher number of visits to the clinic, has less control, and holds a risk of cycle cancellation estimated up to 6% [33].

On the other hand, in mNC FET, once the dominant follicle is between 16 and 20 mm in diameter, ovulation is triggered with human chorionic gonadotropin (hCG) that serves also as mild luteal support. To date, there are no studies comparing different doses of hCG for triggering [22]. Modified FET is considered more patient friendly, as it requires less endocrine and ultrasonographic monitoring.

In an NC, WOI ranges between LH + 7 and LH + 11 [34], while after hCG administration, ovulation occurs after 36−48 hours [35]. These physiologic changes need to be inevitably considered when deciding the timing of FET in an NC versus mNC. It is a commonly accepted practice to perform NC FET on day (embryonic age + 1) after LH surge (e.g., a day 5 embryo on LH + 6) and mNC FET on day (embryonic age + 2) after hCG injection (e.g., a day 5 embryo on hCG + 7) [20,22].

Comparison of NC FET with mNC FET in randomized clinical trials has provided with conflicting results. While reports from Weissman et al. did not find significant differences in clinical outcomes between truly natural and modified cycles, the study from Fatemi et al. had to be interrupted after interim analysis revealed remarkably lower pregnancy rates in women who were administered hCG (14.3% versus 31.4%, respectively) [20,36,37]. In 2016, a large retrospective analysis demonstrated superior clinical pregnancy rates in an NC FET when compared to a modified one (46.9% versus 29.7%, $P < .001$) [38]. Conversely, the ANTARCTICA trial demonstrated that when it comes to ongoing/clinical pregnancy rates and live birth rates, HRT FET is noninferior to mNC FET, despite providing with higher cancellation rates [39]. Consequently, the issue of whether "to trigger or not to trigger" ovulation in an NC FET will remain unresolved until further prospective randomized trials settle the argument.

Whether to use luteal phase support (LPS) in an NC FET and the correct timing to start it is also a matter of controversy. Our clinical practice is based mainly on the results of one RCT where micronized vaginal progesterone initiated on the evening after FET led to better clinical outcomes [40], while two other retrospective analyses have failed to demonstrate any differences [38,41]. Given its long life, hCG can sustain a luteotropic effect for up to 7 days following administration, so it comes as no biological surprise that two different retrospective studies have reported no difference in reproductive outcomes with or without LPS in mFET [42,43]. The timing of LPS is another hot topic as untimely administration could induce embryo-endometrium asynchrony. Available evidence suggests that LPS is not to be started earlier than LH surge + 3 days [22]. The lack of evidence-based data on the optimal moment to start LPS in NC FET guaranties the heterogeneity in daily practice [20].

Hormone replacement treatment

HRT was initially developed as a priming protocol in donation cycles, but over time, its minimally required

monitoring and easy scheduling proved applicable and successful in the entire ART population. Despite the disadvantages of elevated cost and possibly estrogen—related inconveniences, HRT FET is widely used in IVF clinics worldwide [20,44].

In the HRT cycle, proliferation of the endometrium and follicular growth suppression are ensured through administration of E2, while the added P guarantees the necessary, subsequent, receptive transformation that the endometrium needs to undergo to allow for implantation. Estradiol is typically initiated on day 2−3 of the menstrual cycle either at a fixed, constant dose (6 mg daily) or in a step-up protocol, typically 2 mg/day during days 1−7, 4 mg/day during days 8−12, and 6 mg/day during days 13 until embryo transfer [45]. No RCT has compared these two regimens. However one retrospective study found no difference in terms of reproductive outcomes [45]. The first ultrasonographic evaluation of endometrial thickness and aspect is typically performed after 10−12 days of continuous E2 exposure, and if thickness is greater than 7 mm, P supplementation is commenced and FET programmed accordingly. While several studies confirm the achievement of adequate endometrial priming in as few as 5−7 days, E2 exposure of less than 10 days has been associated with higher miscarriage rates [46−48]. Conversely, E2 can be administered for up to 28−36 days without altering reproductive outcomes, thereby offering great flexibility in the timing of FET [49,50]. Both natural and synthetic estradiol can be used, as well as different administration routes (oral, transdermal, and vaginal), appearing to provide comparable clinical outcomes, as confirmed by a recent meta-analysis by Glujovsky et al. [51]. Nonetheless, in an international survey analyzing 39,152 FET cycles, the oral E2 route was the most commonly used (84%), followed by transdermal (9%) and vaginal (3%) routes, probably owing to the local discomfort of vaginal administration and lesser absorption [30]. The conversion between the different supplementation routes may calculated as follows: 0.75 mg of micronized estradiol (oral administration) = 1.25 g of estradiol gel (transdermal administration) = 1 mg of estradiol valerate (oral or vaginal administration) [46]. In an attempt to increase circulating estrogen and enhance endometrial receptivity, mild OS has also been applied in FET cycles and, when compared to HRT, gonadotropins, or letrozole OS allowed for a slightly increased chance for live birth [44]. To prevent spontaneous ovulation, suppression of the hypothalamus-pituitary axis can be added to HRT protocols. GnRH agonist are most frequently used for this purpose, but GnRH-antagonist use has been reported as well, reporting similar outcomes in donation cycles [52,53]. Premature ovulation is responsible for FET cancellation in 1.9% −7.4% of cycles. In 2004, El-Toukhy et al. reported higher clinical pregnancy and live birth rates when a GnRH agonist was added to HRT [54]. However, this finding was not confirmed by subsequent systematic reviews and meta-analyses [55]. In 2014, a large retrospective study also failed to show any benefit of the use of a GnRH agonist, while remaining significantly more patient friendly [56].

Once endometrium proliferation is considered adequate, P supplementation is administered aiming to induce secretory transformation as the concluding phase of endometrial preparation prior to embryo transfer. Giving the absence of corpus luteum in an HRT cycle, all the available P is iatrogenic. Possible administration routes include vaginal, intramuscular (im), subcutaneous (sc), oral, and rectal [22], with the vaginal route being favored by a first-pass uterine effect [57,58]. Vaginally, different compositions of P can be used, including bioadhesive gels, micronized tablets, capsules, or suppositories, while typical doses are extrapolated from fresh ET cycles [22]. Indeed, there is little agreement on the ideal route of administration and dose. In one retrospective study, doubling the dose of bioadhesive P gel led to significantly higher implantation and delivery rates [59]. Another retrospective study including 2010 HRT FET cycles resulted in better clinical pregnancy rates when comparing the use of 1200 mg P capsules versus 900 mg [60]. While patients intuitively prefer vaginal P supplementation when compared to im, mainly owing to its quick, easy, and painless administration, there is still an ongoing debate as to which offers better clinical outcomes. Conflicting results have been reported retrospectively with several studies favoring the im route and others showing no differences in terms of outcome [61−63]. In 2018, an RCT where vitrified blastocysts were transferred in HRT cycles was designed to compare ongoing pregnancy rates in three arms consisting of 200 mg vaginal tablet P twice daily, 50 mg daily im P only, and 200 mg vaginal P twice daily supplemented with 50 mg im P every third day [64]. In their interim analysis, they found ongoing pregnancy rates to be significantly lower in the vaginal P-only arm (31% versus 50% versus 47%), leading to prematurely termination of this arm [64]. In 2021, the final results of this RCT were published confirming significantly lower live births in vaginal P-only arm (27%) when compared with im P (44%) or vaginal P supplemented with im P every third day (46%) [65]. The other routes of P administration have been less investigated. However, just recently Vuong et al. published the results from their retrospective study comparing the addition of oral dydrogesterone 10 mg twice daily to vaginal micronized P 400 mg twice daily ($n = 732$) versus vaginal micronized P 400 mg twice daily alone ($n = 632$) as luteal phase support in HRT

FET cycles, evicting significantly higher live birth rates and lower miscarriage rates in the oral dydrogesterone group [66]. Nonetheless, prospective randomized trials are needed to confirm these findings.

It is an overall belief that once P levels reach appropriate thresholds, the endometrial secretory shift is set into motion ultimately leading to receptivity [25]. Up to date, there is inconclusive data on the impact of the length of the P exposure on clinical outcomes. To our knowledge, few RCTs have investigated this matter [67–69]. It would appear, that taken together, exposure length to P is optimal when initiated on the day of the theoretical oocyte retrieval or 1 day later [20,22]. In current practice, most cleavage stage embryos are transferred around the fourth day of P supplementation, whereas blastocysts are usually transferred on the sixth day of P administration [20,22]. Another aspect of HRT FET needing further evaluation regards the measurement of serum P in the mid-luteal and luteal phase. Indeed, there is a lack of decisive evidence on what to consider as optimal P exposure before ET [68]. Most studies reporting on the matter are retrospective [70]. Recently, a prospective cohort study including 1205 patients aimed to investigate serum P levels on day of FET and reproductive outcomes. Results confirmed previous findings from the same group, with women who had serum P levels < 8.8 ng/mL (30th percentile) had significantly lower ongoing pregnancy rates (36.6% versus 54.4%) and live birth rates (35.5% versus 52.0%) than the rest of the patients. This threshold is lower than their previous publication, the difference probably due to the larger population sample [70].

On a separate note, FET can be considered immediately after a failed fresh transfer, rather than being postponed to a later time, as this, in addition to the similar pregnancy rates, reduces the time to pregnancy and the burden associated with waiting [16,71].

Finally, no RCTs have investigated the optimal length of luteal support in HRT FET, but from a physiologic point of view, given the lack of corpus luteum, P ought to be administered until the onset of placental steroidogenesis, the so-called luteo-placental shift, occurring during the fifth gestational week according to Scott et al. [72]. Generally, in everyday practice, P is to continue until the 10th–12th weeks of gestation [22].

Maternal and obstetric outcomes of FET

It has been suggested that pregnancies following ART are characterized by an increased risk of maternal and fetal complications, manifesting a wide range of obstetric complications and adverse neonatal outcomes [73,74]. Notably, a few observational studies have reported higher incidence of hypertensive disorders of pregnancy (HDP) in women who had undergone HRT FET, compared to those who had been transferred using NCs or natural conception [75–80]. It is hypothesized that the responsible mechanism for these findings is related to the corpus luteum in HRT FET, whose absence translates into decreased serum levels of vasoactive substances like relaxin and vascular endothelial growth factor levels, lower reactive hyperemia index, and a lack of drop in mean arterial pressure during pregnancy [75,76,81,82]. All of these factors contribute to impaired arterial compliance in early gestation and consequently an increased risk of HDP. Additionally, it has been observed that compared to fresh ET, babies born from HRT FET are more likely to be large for gestational age (LGA) or macrosomic [83,84]. Indeed, in a large retrospective cohort study comparing the birthweight of babies born to different FET protocols demonstrated that singletons conceived after HRT FET were more likely to be LGA than those born after mNC or mild OS (19.92% versus 16.94% and 19.29% versus 16.12%, respectively), with the mild OS group having lower adjusted odds of being macrosomic than the mNC group [85]. The trend for higher birthweight > 4500 gr in HRT FET has been observed in other studies as well [78,79]. As a matter of fact, the Nordic register-based, retrospective cohort study reported by Terho et al. showed that the mean birth weight of FET pregnancies becomes significantly higher starting from the 33rd gestational week for boys and from the 34th for girls, if compared to natural conception [86]. Moreover, there is an increased risk for developing postpartum hemorrhage and undergoing cesarean section after HRT regimens when compared with NC FET or mNC FET [77–79]. Other obstetric outcomes that seem to have a higher risk of incidence in HRT FET compared to mNC FET include preterm delivery, very preterm delivery, and premature rupture of the membrane, while other complications like small for gestational age, placenta previa, and congenital abnormalities appear to lack in difference [77,78].

Conclusions

The indications for FET have continuously increased in the last decade; however there are still numerous aspects of protocol preparation that need improvement or better definition. It would appear that in terms of endometrial priming, NC results are superior to HRT, although emerging evidence has discovered mild OS to be a promising protocol for FET as well. Nonetheless, it is mandatory and urgent for future research to compare and contrast the different endometrial priming regimes in well-designed, powerful RCTs that explore both live birth rates as well as perinatal outcomes.

Caution is warranted in the use of HRT, given that early pregnancy loss rate has been alarmingly high in some reports [87,88]. From available data, it emerges that timing for blastocyst transfer ought to be the sixth day of P start in HRT FET, LH surge + 6 days in NC FET and hCG + 7 days in mNC. Hopefully, future research will provide effective and affordable diagnostic tools that allow for fine tuning of FET timing based on personalized, targeted individualization of each patient's WOI, ultimately leading to increased FET success rates.

Finally, the correlation of serum P levels in mid-luteal and luteal phases with reproductive outcomes ought to be promptly investigated by extensive research, given that it is an efficient and cost-effective rescue protocol.

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CHAPTER

32

Egg and sperm donation

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Introduction

An increasing number of assisted reproduction processes require the use of donated gametes to complete the reproductive project of the woman/couple [1]. In this chapter, we will review the most important aspects of the process. We will basically reference the egg donation program but will also reserve a specific section to discuss sperm donation, as the latter was the first program to be developed but is relatively simpler to organize.

The egg donation program, of all the assisted reproduction programs, is the one which yields the best results in terms of delivery rates [2]. It is also the solution for women who postpone childbearing, an artificially created social problem we hope to recommend less frequently by providing adequate information and cryopreservation techniques for a woman's own oocytes at an appropriate age.

In addition, the donation program presents a unique field of scientific interest: through it we can separate the effects of drugs and lifestyle on the eggs and endometrium, in turn allowing us to better understand the complex process that takes place between ovarian stimulation and fertilization, implantation, and the subsequent development of pregnancy [3].

Finally, the "healing" effect of a young ovum on low-quality sperm is worth mentioning. Egg donation has excellent results not only due to oocyte quality, but also due to the impact these eggs have on the sperm [4].

Indications for egg donation

If, at a theoretical level, egg donation was conceived as a method to solve a medical problem with the same indications as those that sperm donation would have, at present its use is much more extensive.

Female fertility is age dependent, not only in terms of the likelihood of conceiving but also throughout the entire process that leads to having a healthy baby at home. The chances of getting pregnant decrease drastically after 40 years of age, while the chances of experiencing a miscarriage or having a child with genetic abnormalities increase exponentially.

Although no standardized criteria exist, we consider it reasonable that, after 42 years of age, the indication for a first treatment should be donation.

In addition to the age factor, there are other indications in women with primary ovarian failure (Swyer syndrome, Turner syndrome, Savage syndrome, or autoimmune oophoritis), or secondary ovarian failure due to iatrogenesis (radiation, surgery), enzyme problems (galactosemia, 17 alpha hydroxylated deficiency), autoimmune diseases (Addison disease, thyroiditis, adrenal insufficiency, pernicious anemia, diabetes mellitus, myasthenia gravis), or genetic problems (fragile X, congenital cataracts, hereditary diseases, or chromosome structure abnormalities).

The other indication that has grown most rapidly in recent years, along with maternal age, is the recommendation to use egg donation to solve previous failures in assisted reproductive techniques (ART) (poor responders, poor oocyte or embryo quality, repeated fertilization or implantation failure, repeated miscarriage).

Indications for sperm donation

The main indication is the absence of sperm, either because there is no male partner, in the case of single or homoparental families, or because the male partner presents azoospermia in the ejaculate and no sperm are found following testicular biopsy (genetic diseases such as Y microdeletions, numeric or structural chromosome abnormalities).

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Donor sperm can also be used in cases of severe abnormalities in sperm count, morphology, or integrity of the number of male chromosomes (abnormal sperm fluorescent *in situ* hybridization) or in DNA integrity (elevated fragmentation) that are not subject to treatment, have not responded to treatment, or have caused repeated failures in ART.

Another indication is the existence of hereditary diseases that cannot be prevented by preimplantation genetic techniques.

Legal aspects

Being a subject of special social relevance in practically all countries, there are laws that regulate the donation process in aspects such as the following:

- age;
- donor anonymity in relation to the recipient woman/partner and the future child;
- payment, or in cases where donation is considered altruistic, the compensation that can be given to donors for their donation;
- the number of times it is possible to donate and the number of descendants that can originate from one donor;
- the mandatory records to keep;
- the required medical studies to be carried out on donors.

As these topics vary according to culture, religion, and politics, we can encounter all possible variants. It can condition our way of working and the results that can be obtained.

In general, to become an egg donor the female must meet the minimum age requirement of between 18 and 21 years of age, and the maximum acceptable age normally ranges from 30 to 35 years of age. The female must have the full capacity to act and to make decisions, must be able to give her informed consent, and must be free from hereditary and infectious diseases that may affect offspring or the mother.

Normally the requirements for sperm donors are similar, though the maximum acceptable age is higher, up to 40–50 years of age.

Likewise, the legislation of many countries also regulates the conditions to be a recipient. In most cases, the maximum acceptable age to be able to undergo assisted reproduction techniques, although not standardized, is usually around 50 years old.

Organizing a gamete donation program

The general rule must be to achieve maximum security with minimal inconveniences for donors.

In a sperm donor program, all the necessary tests and analyses (blood and semen), as well as the medical and psychological consultation, can be organized in a single visit. Once approval is granted to enter into the donation program, the donor simply has to go to each donation appointment to provide the sperm sample, update the necessary analyses, and sign the necessary documents for each donation. The most important criteria to be assessed must be sperm quality.

Everything is a bit more complicated with egg donation, but the process is similar: all necessary tests, analyses, gynecological consultation, and interview with the psychologist can be organized into a single visit. The main criteria to be registered is ovarian reserve (assessed by antral follicle count). During the cycle the donor will have one visit for cycle organization, one for cycle initiation, one for follicle tracking (progestin supplementation), one for egg retrieval, and a final follow-up visit after completing the cycle.

For a donation program to be successful, all staff (doctors, biologists, nurses, and auxiliary team) must be exclusively dedicated to it [2].

Similarly, it is essential that donors have direct contact with the clinic. The clinic must have complete control over the entire process.

Phenotype matching

We must always strive to achieve maximum similarity between the donor and the recipient. The order of preference should always begin with the recipient's race and ethnicity, followed by blood group, height, eye color, and hair color. A photograph of each person, the donor and the recipient, is essential when carrying out the matching process. At present, computer facial-recognition programs are a great help in performing this type of matching process.

Genetic matching

With the development of new genetic diagnosis systems, we have platforms that allow us to determine the carrier status for multiple recessive diseases. Performing this test on both the donor and the recipient's partner makes it possible to rule out donor-partner matches in the event that both individuals are carriers

of the same genetic abnormality that can result in a child affected by an illness. If the carrier panel is not run, it would be mandatory to test for the most prevalent diseases (cystic fibrosis, beta thalassemia, and spinal muscular atrophy), in addition to the fragile X premutation condition.

Donors

Selection of donors

Donors have to meet all the legal requirements of each country in terms of age and other circumstances. Habits such as tobacco and alcohol consumption, weight, and donor lifestyle must also be recorded. We must select donors who are as healthy as possible.

Most of the programs work with pure donors, that is, women who donate all the eggs they produce, but there is the possibility that a patient can donate some of the eggs that she produces in a cycle to a donation program, using the rest for herself in exchange for some type of financial compensation or not (for example, to advance a waiting list). We advise against this model because it can be complicated for the woman on a psychological and/or financial level in the following situations: in the event that she does not get pregnant but the recipient of her eggs does, or in the event that this woman requires assisted reproduction treatment to achieve a pregnancy, except in cases of male factor infertility, as we do not know what implication these oocytes may have [2].

Donor age

Age depends on the legislation of each country. Generally donors up to 35 years of age can be selected. However, we must establish a limit of 30 years of age, whenever possible, for two reasons:

- At a younger age, a slight improvement in the results has been demonstrated.
- In the event that a recipient wants to have a second child, we could contact the donor to repeat the process.

In some programs, younger donors (18–20 years old) are not permitted [2].

Treatment protocol for the donor

In general, there are two ways of carrying out the cycle: with fresh eggs (synchronous), in which we need to synchronize the donor and the recipient, and with vitrified eggs (asynchronous), in which we use an egg bank that is either internal or external to the clinic.

Lately, a third modality is being utilized, also asynchronous, in which fresh eggs are used, but without synchronizing the recipient; in this case, fertilization is performed on the day of egg donation with fresh or frozen semen and the embryo is vitrified after reaching blastocyst stage. This modality subsequently simplifies the recipient's cycle and also allows the blastocysts to be sent anywhere. This procedure avoids vitrification and devitrification of the oocyte, replacing it with that of the blastocyst, which is technically easier and offers better success rates from a results perspective.

Synchronous donation

Synchronization with the recipient

The most practical way to synchronize the recipient is to use contraceptives prior to treatment, but it can also be done using any type of hormonal treatment. Normally, donors are already taking contraceptives and once there is a phenotypically compatible recipient they are programmed to synchronize their menstruations by ending the contraceptive cycle on the same day, both the donor and the recipient.

In this way, the start of stimulation can be programmed for a fixed date, and the same in relation to egg retrieval and transfer. This is important for scheduling purposes in the laboratory and for the recipient, especially in the case of women who live a long distance from the clinic given that other circumstances such as airline tickets or hotel reservations have to be arranged.

We recommend ending the contraceptives on Tuesday or Wednesday to start stimulation on Monday; that way the egg retrieval is usually on Friday and transfer on Wednesday (day +5).

If the schedule allows it, the ideal situation is to have no more than 15 days of contraception, but in cases of necessity, it can be increased to 40 days for synchronization purposes. We normally recommend 6 days without treatment, if you take the pill in the morning, or 7 days if you take it in the afternoon. Fewer than 15 days of contraceptive pills is not recommended as the patient may not have a menstrual period as a result. Using contraceptives usually lengthens the stimulation by 1 day and consumes an additional 150 IU of gonadotropins compared to not using contraceptives.

Ovarian stimulation for the donor and trigger

The recommended dose of follicle-stimulating hormone (FSH) for a donor should not be high, ranging from 150 to 225 IU, but it should be personalized, especially when the donor has undergone a previous stimulation. It does not matter what type of gonadotropins are used for ovarian stimulation [5], although we

recommend the use of recombinant FSH as some more eggs are obtained than with higly purified HMG (HMG hp).

To prevent a premature luteinizing hormone (LH) surge, we can use a gonadotrophin-releasing hormone (GnRH) antagonist (0.25 mg of Cetrorelix or Ganirelix), starting when the follicles reach 14 mm, or progestins. We recommend the use of progesterone, for example, medroxyprogesterone acetate 10 mg, once per day from the start of stimulation until the day before the pick-up, for the convenience of the donor (we avoid injectables, and we can reduce the number of ultrasound scan controls to just one, on day 9 of stimulation), and also because more oocytes are obtained.

Ovulation must be triggered with an agonist bolus (0.2 mg of leuporelin or triptorelin). This way, ovarian hyperstimulation syndrome (OHSS) is prevented. The cases described in the literature of OHSS after triggering ovulation with an agonist bolus have more to do with peritoneal irritation due to post pick-up bleeding than with an actual case of hyperstimulation [6].

We usually wait for most of the follicles to be larger than 17 mm. In donor stimulation, when in doubt, we recommend delaying bolus administration, since more oocytes are obtained and there is no damaging effect on oocyte quality.

If antagonists are used, we must remember that more than 12 h and less than 24 h must pass between the final dose of antagonist and the administration of the agonist bolus. In the case of morning egg retrievals, it is ideal to administer the gonadotropins in the morning and the antagonist in the afternoon. All of that is simplified by using oral progesterone [5].

It is not necessary to perform estradiol analyses for cycle monitoring. Ultrasound controls are sufficient.

We recommend that the donor goes to the clinic to inject all the medication, thus guaranteeing correct administration. In the event that this is not possible, an alternative means of validation should be available to ensure correct medication administration by the donor [5].

Donor pick-up

It is performed 36 h (from 35 to 38 h) after the agonist bolus. Special care must be taken during egg retrieval to prevent bleeding, as donors normally have many follicles. Once the egg retrieval has been completed, any fluid present in the pouch of Douglas must be aspirated as it normally contains blood and can cause peritoneal irritation and pain [5].

Sedation of the donor must be carried out the same way as for all other patients. Antibiotic prophylaxis should be used for safety.

Once the pick-up is finished and we have confirmed that the donor is not bleeding, she can go home as soon as she has recovered. A doctor's telephone number, as well as instructions on what to do in the event of any unexpected situation, must be given to the donor.

No post pick-up medication is necessary and discomfort from the pick-up usually disappears in about 24–48 h. If required, the donor can take analgesics (for example paracetamol 500 mg every 8 h). On the third day post pick-up, the donor is usually pain-free and abdominal distention has decreased; if this is not the case, an ultrasound is recommended.

Menstruation normally arrives 5 days after the pick-up [6]. At that point the donor can resume taking contraceptives.

Asynchronous donation

In this case, the donor begins the ovarian stimulation on day 2–3 of menstruation and the eggs are vitrified, or if the recipient's endometrium is properly prepared, they are fertilized. Except for the synchronization part, the rest of the process is the same as in a synchronized cycle.

This modality gives the advantage that no time is lost due to the use of contraceptives. Ovarian stimulation usually lasts 1 day less with this modality and more oocytes are obtained. The results are somewhat inferior to synchronous donation, mainly due to the vitrification and devitrification of the oocytes, which require experience and specific technical conditions [7].

As we have mentioned before, a variant of this type of donation, which would eliminate the problems mentioned earlier, is to fertilize the donor's eggs on the day of the pick-up and vitrify the embryos.

In both cases, fertilization during the recipient's cycle can be done based on when her endometrial conditions are optimal, even in a natural cycle.

Complications

In donors, risks must be minimized as best as possible. There are no risks with sperm donation, but in egg donation cycles there may be. Complications, except for some degree of abdominal discomfort, are quite infrequent [8]:

- Risks associated with stimulation: The existence of OHSS in a cycle triggered with an agonist bolus is nonexistent.
  Abdominal discomfort: It is frequent but tolerable, especially if the donor expects it ahead of time. Pain relievers can be taken both during stimulation and after the pick-up. Discomfort usually disappears about 24–48 h after the pick-up procedure. A thorough review should be performed in the event of major discomfort [8].
- Risks associated with pick-up:

Post pick-up bleeding: It occurs about 1% of the time and is usually light and self-limited. Carefully examining the vaginal cul-de-sac and applying pressure on the bleeding site can prevent external leakage. A vaginal ultrasound after pick-up is mandatory. When an abnormal amount of fluid in the pouch of Douglas is found, aspiration is recommended to prevent discomfort as the blood can cause peritoneal irritation. In the event of significant intraperitoneal bleeding, the recommended attitude is expectant with only ultrasound and analytical controls, whereas surgery is avoided. Vaginal paracentesis can be done, but usually it is not effective as the blood has already clotted; in any case, it would always be done before considering a laparoscopy [8].
Infections: These are also extremely rare but described. Antibiotic administration during the pick-up is highly recommended to prevent this complication [8].
Extremely infrequent complications, which we must bear in mind, include ovarian torsion, intestinal tears, or abundant bleeding due to the puncture of large vessels [8].

- Risks associated with anesthesia: These include allergies to any of its components and risk of aspiration [8].

Recipients

Recipient selection

They must undergo the usual check-ups that are performed on all women who are going to receive ART (medical evaluation, general analyses, and serology testing) [2]. Since it is a special group, generally in relation to their age, they must be informed about the risks of motherhood at an advanced age and the clinic must be able to offer psychological support [3].

Recipient's age

In each country, we must adapt to the existing legal limitations. From a medical point of view, there is no age limit, so if there is no legal indication, clinics must, according to their internal procedures, establish an age limit [9].

Setting an age limit implies a debate that goes beyond the medical field. What is clear is that as age increases—and long before the age of 50—so do complications. Thus, we must be especially demanding in terms of the preliminary testing done on older women, and we must also keep in mind that they require a unique medical, and sometimes psychological, assessment.

It would be advisable for older women to undergo an independent medical evaluation that reports that there are no medical problems that contraindicate pregnancy. Likewise, it is recommended that women of advanced age first have the acceptance of the gynecologist who will monitor their pregnancy once it is achieved [9].

Synchronization with the donor

At the time of egg donation, the recipient's endometrium must be in optimal condition. To reach that point, the recipient's menstruation needs to be scheduled for the same day or the day before the donor's menstruation.

In the case of women in menopause, synchronization is done by lengthening or shortening the hormonal replacement theraphy (HRT) until it ends on the same day as the donor's contraceptives. Prior to the treatment cycle, women must have had a minimum of three periods.

In the case of women who are not in menopause, synchronization is usually done with contraceptives or with hormone treatment until the day the donor finishes contraceptives. The administration of a GnRH analog can be used, usually as a depot injection, approximately 1 month before the expected date of donation to prevent follicle development during endometrial preparation. This strategy has not been proven more effective in terms of clinical pregnancy or cancelation rates compared to doing nothing [10].

Similarly, in some protocols, in cases where GnRH analogs are used in the recipient, a dose of human chorionic gonadotropin (hCG) is administered to the recipient on the same day that the donor uses it with the intention of taking advantage of the effect that hCG may have on the endometrium. This has also not been proven more effective compared to doing nothing.

Endometrial preparation and growth

It consists of the administration of estrogens to increase endometrial thickness in the embryo transfer cycle. There is no protocol that has been shown to be superior to another in relation to pregnancy rates. There is no difference between starting the endometrial preparation on the first day of menstruation and beginning it on day 3 post menstruation. There is no difference in starting with a fixed dose (3 patches of 50 mcg estradiol or 6 mg of oral estradiol valerate) versus an ascending regimen [10].

We recommend the transdermal route over the oral route because we avoid the first liver passage; similarly, we recommend an ascending regimen with increasing doses of estradiol until a thickness of 7 mm is reached.

The ascending regimen has the advantage that we use the minimum dose of estrogen necessary [11].

Regarding endometrial thickness, a thickness of more than 7 mm and with a trilaminar structure is generally recommended, although acceptable pregnancy rates can be achieved with a thicknesses of only 5 mm.

Hyperechoic endometria may correspond to an ovulatory escape with an increase in endogenous progesterone, so in those cases, a progesterone analysis is recommended: if the result is elevated, the cycle should be canceled.

When an adequate endometrium is not achieved, alternative treatments have been described in different publications [10,12,13], alone or in association:

- increase the estrogens dose
- use the vaginal route
- use vasodilators like sildenafil (Viagra), orally or vaginally
- low-dose aspirin
- pentoxifylline
- platelet growth factor

There is an alternative model of endometrial preparation that can be used in asynchronous donation in which the recipient, once she has her menstruation, begins to take estrogens and continues with them uninterruptedly until the donor that phenotypically corresponds to her makes the donation. Normally there are no bleeding problems until about 3 months of treatment, but if this occurs the woman will stop the medication, and after a few days, she can resume it again, being prepared again after about 10 days of estrogen treatment. It is a type of preparation that was used a few years ago but has now fallen into disuse because the pregnancy rate is lower [14].

In cases where an oocyte donation cycle is carried out using eggs or embryos that were previously frozen, the transfer can be scheduled in a natural cycle by synchronizing the dates according to the endogenous LH peak with or without the use of exogenous progesterone, once we have the right endometrium [15].

Apart from that, in all cases the recipient should be advised to take folic acid and vitamin supplements that would be given to any woman trying to conceive [16].

Endometrial preparation for embryo reception

Prior to the transfer of the embryos, the woman must start a treatment with progesterone to facilitate endometrial receptivity.

Type and route of progesterone to be used

When micronized natural progesterone is used, greater absorption and a decrease in side effects (tiredness, drowsiness) has been observed when using the vaginal route (doses of 400–800 mg) compared with the oral route. The oral route has been shown to be less effective than the vaginal route, except in the case of dydrogesterone (some papers attribute it greater effectiveness than the natural micronized progesterone vaginal route, but the disadvantage is that it cannot be dosed in blood) [17].

Progesterone gel (90 mg) allows for the administration of a single dose compared to 2–3 times that are required when using vaginal tablets, thus avoiding the inconveniences of its application and discomfort derived from the discharge of part of the medication.

Subcutaneous progesterone (25 mg) (in aqueous solution) has the disadvantages of being parenteral but the advantage that absorption is ensured by being able to administer it subcutaneously or intramuscularly. It has been proven as effective as vaginal progesterone.

Other forms of intramuscular progesterones (in oily solution) have also demonstrated equal or greater efficacy in terms of pregnancy viability and progress, and a live newborn. On the contrary, intramuscular progesterones have the disadvantage of being more painful than progesterone in aqueous solution [10].

In conclusion, we can say that the type and route of progesterone administration to be used is a matter that must be individualized depending on the patient, as there are no clear advantages in terms of pregnancy success rates [18].

Start day

Regarding starting with progesterone on the day of the donor pick-up, 1 day before or 1 day later: results are worse if we start progesterone a day before pick-up. Results do not vary when we begin progesterone administration on the day of the donation or a day after.

Starting a day later has the advantage that, if we work with frozen semen and an asynchronous protocol, if there is no fertilization or it does not proceed as expected, the recipient's treatment can be postponed, and she can be matched to another donor in the same cycle [11].

Post-transfer treatment

There is not enough data regarding the duration of post-transfer treatment in the case of egg donation programs. If we assimilate it to what happens in *in vitro* fertilization (IVF) cycles, treatment should be maintained at least until the day the pregnancy test is done, about 14 days after the transfer. No differences are found in IVF cycles when progesterone is maintained until the day of the first ultrasound or later. In donation and frozen cycles, we recommend a more conservative approach by maintaining progesterone until the eighth to tenth week of gestation [18].

The usual practice is to maintain this hormone support for longer, until 12 weeks of gestation. No benefits have been shown regarding this strategy, but it has also not been shown to be harmful to pregnancy [19].

Exclusive or shared donor and number of eggs per recipient

Assigning exclusive donors to a recipient or sharing them is a decision that must be made by each clinic. The objective is to achieve the best possible result in the recipient with the fewest possible complications and the lowest possible economic cost.

Sharing a donor in a treatment cycle that is carried out mainly at the level of private medicine and which has a high cost can be interpreted negatively at the economic level. If the recipient is asked at the beginning of the cycle, she will say that she wants all the eggs for herself. However, once she has had her child, there are often difficulties in dealing with the problems surrounding the remaining vitrified embryos.

If we speak of vitrified oocytes, we can personalize with each woman the number of oocytes that will be used in her case, depending on the reproductive project and her desire or not to have remaining vitrified embryos in her specific case [15].

We consider that if a donor has a reasonable number of eggs, then she should be shared if this does not compromise the results of the cycle. The problem is defining that reasonable number. In general, the recommendation is to microinject about four MII oocytes for each blastocyst that we want to obtain for the recipient woman. Generally, it is advisable to have more than one blastocyst to be able to choose from, in the case of the first transfer, or to have at least one more opportunity to grow the family if the pregnancy is achieved after the first cycle.

Treatment complications in the recipient

Complications for the mother

In general, it is a safe procedure [1].

- Complications associated with egg donation itself: There is an increased risk of preeclampsia (between 5% and 10%, with an OR of approximately 1.5), regardless of the age or parity of the recipient, donor, and father. It is due to an immunological factor at the time of implantation and is caused by the donation itself. There is also an increased incidence of bleeding during the first trimester in donation cycles (without an increase in the miscarriage rate), which is also independent of age [20].
- Complications associated with age: The risk of preeclampsia also increases with age, especially after the age of 45. Other complications described include gestational diabetes (about 10% in women over 45) and placental alterations (antepartum bleeding, 3%; placenta previa, 1%). For the groups in which the cycles can be compared by distinguishing between women who have conceived with their own eggs and those who have conceived through egg donation, the percentages of complications are equal, so they are complications associated with the recipient's age [1].
- Cesarean section rates are increasing, exceeding 50%.
- The multiple gestation rate is related to the number of embryos transferred. Given the high gestation rates that are achieved, the transfer of more than one blastocyst would not be justified. With a single blastocyst transfer, we will have up to 2% of twin gestations per partition of the blastocyst.

Complications are identical with fresh or vitrified oocytes [7].

Complications in the newborn

The genetic risks are lower than those that would correspond to the recipient's age as they correspond to the donor's age [20].

In general, the available data indicate that there is no elevated risk for newborns, either in terms of Apgar score, admission to intensive care units, or presence of malformations. There are controversial data in relation to preterm delivery, low birth weight, and delayed intrauterine growth, and some publications indicate that these complications are more frequent [7,21].

Results

The egg donation program is the treatment that provides the best results as there are good quality eggs available given that the donors are younger.

Live birth rate per single embryo transfer slightly exceed 50% in most registries in recent years.

Furthermore, the results are not related to the age of the recipient, nor to the indications for donation or the characteristics of the semen used. The only factors that may be important are previous uterine pathology, endometrial thickness and structure, the number of oocytes received, and difficulties in performing the embryo transfer procedure [7].

Egg bank

The egg bank is one of the new ways to manage a donation program, and it makes it possible to send eggs and embryos to any location in the world [22].

The advantages it presents include the following [14]:

- Work can be scheduled in a more comfortable way for the clinics by not having to synchronize donors and recipients.
- The oocytes can complete the quarantine period.
- Donor selection can be made on a much larger basis so the match is more suitable.
- It allows for the possibility of donors with phenotypes that are not usual in the geographic area where the oocytes are going to be used.
- It makes it possible to provide each recipient with the desired number of oocytes, thus avoiding the creation of an elevated number of embryos.

The drawbacks it presents include these [14]:

- There is an increase in cycle costs by including the processes of vitrification and devitrification.
- The survival rate of oocytes post thawing is not 100% and depends on the equipment and technical skills of the laboratory.

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CHAPTER

33

Recurrent miscarriage

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Introduction

Recurrent pregnancy loss remains the epitome of controversy in the field of obstetrics and gynecology. There has been a lack of clear-cut evidence-based conclusions for this complication of pregnancy, starting with the actual definition, to the timing and types of investigations required, ending with different management modalities. The most problematic of all recurrent pregnancy loss (RPL)-related dilemmas, however, remains the most distressing two-digit number any couple that suffers from RPL could hear: up to 50% of RPL cases remain idiopathic or unexplained despite a myriad of physically, financially, and psychologically draining investigations. With recent advancements in numerous fields, primarily genetics, this number of unexplained cases of RPL has been reduced to less than 10%.

Definition

Pregnancy loss is defined as any demise prior to a gestational age ranging from 20 to 24 weeks or birth weight of less than 500 g (depending on the definition of viability). In an attempt to end the controversy regarding the vexed terminology in early pregnancy loss, the European Society of Human Reproduction and Embryology (ESHRE) issued a consensus statement in 2015 that demarcated the difference between the terms "recurrent pregnancy loss" and "recurrent miscarriage." RPL was defined as repeated pregnancy demise, whereas *recurrent miscarriage* refers specifically to repeated intrauterine demise confirmed either by ultrasound or histology [1]. Nonvisualized pregnancy losses (biochemical pregnancies or resolved/treated pregnancies of unknown location) were previously not included in the definition of RPL. Their recent inclusion, however, stems from evidence showing no difference between the negative prognoses of nonvisualized losses compared with clinical miscarriages on ensuing live birth [2].

The Royal College of Obstetrics and Gynecology (RCOG) defines "recurrence" as being three or more consecutive losses, whereas the American Society for Reproductive Medicine (ASRM) considers a minimum of two losses not necessarily being consecutive as sufficient. Most recently, and after much debate, ESHRE's final disposition paralleled that of the ASRM [3–5]. The importance of the definition of recurrence rather lies in its pivotal role in the delineation of the timing to proceed with the RPL workup. In fact, a recent systematic review and meta-analysis confirmed that no difference in the prevalence of abnormal test results exists when comparing couples with a minimum of two versus three losses [6,7], further confirming the clinical significance of limiting the definition to a minimum of two losses.

Epidemiology

Due to the aforementioned controversies in the definition of RPL, the exact prevalence is difficult to determine, with most sources estimating it at around 1%–3% [5]. The risk factors associated with RPL include, but are not limited to, the number of prior miscarriages, maternal age, and lifestyle factors. A recent population-based study of around 44,000 miscarriages showed that the age-adjusted odds ratio for miscarriage recurrence was 1.54 (95% CI 1.48 to 1.60), 2.21 (2.03–2.41), and 3.97 (3.29–4.78) after one, two, and three consecutive miscarriages respectively [8].

In regard to maternal age, the J-shaped curve described in various studies epitomizes the age-related

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increase in pregnancy loss with the risk of loss exponentially increasing starting at the age of 35 from 16.7% (for the 35–39 age group) to 56.9% in those aged 45 years or older [8,9]. This mainly emanates from higher rates of meiotic chromosome segregation errors that occur via one of three pathways: nondisjunction, premature separation of sister chromatids, or reverse segregation [10,11].

Modifiable risk factors such as low socioeconomic status, excess alcohol and caffeine consumption, smoking, body mass index extremes, and environmental pollutants have also been shown by some studies to modestly increase the risk of miscarriage [12–15]. Personal maternal history of small for gestational age and prior history of gestational diabetes, preterm birth, and stillbirth were also found to be associated with a higher risk [8].

Etiologies

Genetic causes

The prevalence of chromosomal abnormalities in pregnancy loss tissues was recently estimated by a 2020 meta-analysis to be around 48% [16]. While several previous studies showed lower chromosomal abnormality rates associated with RPL when compared to sporadic losses [17–19], this meta-analysis of 55 studies including more advanced detection technologies showed no statistically significant difference between sporadic and recurrent losses. The myriad abnormalities include but are not limited to aneuploidy, translocations, inversions, and deletions. They can either arise *de novo* or be secondary to parental chromosomal abnormalities.

Parental chromosomal anomalies

Compared to an estimated 0.7% prevalence of chromosomal rearrangements in the general population, the prevalence in patients with RPL was found to be higher at 3%–5% [20]. The most common abnormalities are balanced translocations, with 60% being reciprocal (exchange of terminal segments between two chromosomes) and 40% being Robertsonian (breakage and joining of two acrocentric chromosomes with loss of short arms) [21].

Despite carriers of balanced translocations being phenotypically normal, meiotic chromosomal abnormalities might result in homozygous or unbalanced counterparts culminating in potential spontaneous abortion. In addition, the risk of aneuploidy seems to be higher if the translocation is maternal in origin [21,22].

Embryonic aneuploidy

Accounting for around 60% of early pregnancy losses, aneuploidy in the fetus remains the most common cause of spontaneous losses [19]. The most common type, autosomal trisomy (60% of cases), is primarily due to maternal meiotic nondisjunction. Trisomy 16 accounts for 20%–30% of trisomies, making it the most common type associated with early losses. Monosomy X, on the other hand, constitutes around 20% of aneuploidies, rendering it the most common single abnormality. The remaining 20% are due to polyploidy (mostly triploidies) [23].

Parental karyotyping and products of conception cytogenetic analysis

The monumental contribution of aneuploidy to early pregnancy losses renders genetic assessment an imperative part of any RPL workup. This evaluation provides insight into future prognosis and recurrence in addition to granting emotional closure for psychologically drained couples. While the decision to proceed with genetic testing is rather straightforward, the type of testing to be pursued is rather contentious considering the baffled feud between parental karyotyping compared to products of conception (POC) cytogenetic analysis.

The ASRM recommends parental karyotyping for detection of structural genetic abnormalities, hence permitting genetic counseling with the possibility for prospective preimplantation genetic testing (PGT), amniocentesis, and chorionic villus sampling depending on prognosis [4]. ESHRE, on the other hand, does not advocate routine testing and only recommends parental karyotypes after "individual risk assessment." A prior child with congenital abnormalities, offspring with unbalanced chromosomes, or translocations found by POC evaluation are all criteria for designating the case as high risk [5]. The RCOG, however, recommends against routine testing unless POC evaluation reveals unbalanced translocations [3].

When it comes to POC genetic analysis, the discrepancy in recommendations becomes less straightforward. The genetic assessment of POC was initially accomplished using G-banded karyotyping, which has been more recently replaced with novel methods that eliminate problems with cell growth and maternal cell contamination [24]. These limitations were resolved by the introduction of the single nucleotide polymorphism (SNP) microarray technique. In fact, a study by Popescu et al. comparing 24-chromosome microarray analysis (CMA) of POC to the standard ASRM workup in identifying a cause of pregnancy loss revealed that a definite abnormality was found in 67% of patients when microarray testing only was used compared to only 45% if the ASRM workup was used [25]. The ASRM

recommendation in 2012 against the routine use of POC cytogenetic analysis was based primarily on G-banded karyotypes and only suggests its use in the following setting: if a treatable cause in the RPL workup is found, the aforementioned test can be performed to assess whether a subsequent loss was a random event or a reflection of treatment failure. The main rationale behind their standpoint pertains to the issues of maternal cell contamination, occurrence of noncytogenetic embryonic abnormalities, and failure to complete the RPL workup if POC testing revealed an abnormality [4]. ESHRE, on the other hand, also recommended against the routine use of POC testing but strongly recommended CMA testing if it were to be used for explanatory purposes [5]. Both ESHRE and ASRM acknowledged the psychological value offered by POC testing [4,5]. The RCOG issued a grade D recommendation for POC testing after the third and subsequent miscarriages [3].

Considering the huge body of conflicting evidence on the benefit of the routine use of PGT on improving live birth rates, both the ASRM and ESHRE do not currently recommend its use in the setting of RPL [4,5].

In our practice, we currently offer all RPL patients SNP microarray on POC with a subsequent miscarriage that was passed spontaneously, medically induced, or surgically extracted. We have found that many patients have a psychological benefit to understanding the reason for their loss and the elimination of feelings of guilt or inadequacy. In addition, the identification of aneuploidy has been useful in evaluation of different treatments used in our Recurrent Pregnancy Loss Center. In some cases of recurrent aneuploidy in the POC, we will discuss and counsel patients about the potential benefit of PGT.

Sperm DNA fragmentation

Elevated sperm DNA fragmentation results from numerous factors including, but not limited to, smoking, heat exposure, obesity, and advanced age. The effect of these sperm abnormalities on RPL is still controversial with a meta-analysis of cohort studies associating higher levels of fragmentation to worse miscarriage rates [26], whereas a more recent randomized clinical trial (RCT) in patients undergoing *in vitro* fertilization denied this association [27,28]. The major societies do not recommend sperm DNA fragmentation testing with ESHRE considering it for explanatory purposes solely [4,5].

Uterine factors

Uterine structural anomalies

Uterine structural anomalies whether congenital or acquired account for around 10%–20% cases of RPL [6,29,35]. These numbers signify the importance of uterine anatomy assessment as part of any RPL workup. Any diagnostic modality can be used ranging from hysterosalpingography to sonohysterography and hysteroscopy, with most guidelines currently considering transvaginal 3D ultrasonography as the preferred imaging modality [5,30]. MRI is only reserved in case of 3D ultrasonography unavailability [5].

Congenital uterine anomalies

The prevalence of congenital uterine anomalies is 16.7% (95% CI, 14.8–18.6) in the recurrent miscarriage (RM) population compared to 6.7% in the general population (95% CI, 6.0–7.4) [31]. These abnormalities are mainly associated with late first trimester or second trimester losses [32]. These developmental anomalies arise from either failure of Müllerian duct development (agenesis or unicornuate), abnormal fusion of the ducts (bicornuate or didelphys), or failure of septum resorption (septate or arcuate) [33]. Limited uterine capacity, disordered arrangement of uterine musculature (leading to cervical incompetence), and inadequate endometrial vascularization leading to impaired placentation have all been suggested as potential mechanisms for poor fertility outcomes [33,34]. The septate uterus is the most common uterine anomaly associated with RPL, yet also the one associated with the poorest reproductive outcome, having more than 2.5 times the risk of first trimester losses compared to normal controls [34–36]. The recommendations on the management of arcuate uteri is however debatable, with some sources considering them as normal variants with no effect on reproductive outcome, while others associate it with higher rates of second trimester miscarriages, preterm labor, and malpresentation [33,37,38]. Despite the complete lack of RCTs in the literature addressing the impact of the surgical correction of congenital uterine anomalies on reproductive outcomes [39,40], numerous uncontrolled trials have highlighted markedly lower miscarriage and higher live birth rates especially in those with uterine septa suffering from RPL [41–43].

Acquired uterine abnormalities

The two main categories of acquired abnormalities are intrauterine adhesions and intrauterine lesions including submucosal fibroids and endometrial polyps. Numerous studies have associated these three entities with poor reproductive outcome and have reported better outcomes when looking at miscarriages before and after surgery [44–47]. However, the lack of high-quality evidence including RCTs has led both ESHRE and the ASRM to conclude that surgical correction of these anomalies does not reduce the future risk of miscarriages and should hence be discussed thoroughly in patients with RPL [4,5,35].

We currently evaluate all patients with RPL for congenital and acquired uterine anomalies using saline-infusion 3D ultrasonography. When we identify a septate uterus, submucosal fibroids, or endometrial polyps more than 10 mm in size, we discuss outpatient hysteroscopy with our patients to correct these abnormalities.

Chronic endometritis

Chronic endometritis (CE) is defined as a persistent inflammatory state of the endometrium that is mainly asymptomatic. Numerous infectious agents have been associated with CE, and these include, but are not limited to, Mycoplasma, Ureaplasma, *E. faecalis*, *E. coli* and Streptococcus [48,49]. Histologic confirmation of the presence of plasma cells, with differing diagnostic criteria described in the literature, has the potential to become the standard for diagnosing CE. Samples obtained from endometrial biopsies are then examined via immunohistochemical staining of the plasma cell proteoglycan CD138 syndecan 1 [50,51]. Hysteroscopic findings of edema, hyperemia, or micropolyposis have been considered a less invasive screening tool for CE, with histopathologic examination remaining the diagnostic gold standard [50,51]. Due to the lack of agreement on the diagnostic criteria of CE, its prevalence in patients with RPL has been reported from 9.3% to 67.6% [52]. Abnormal infiltration by plasma cells and excessive secretion of various antibodies are thought to result in impaired endometrial receptivity and implantation [53,54]. Antibiotic treatment with doxycycline has been reported to be associated with improved reproductive outcome by various observational studies [48,49]. The scarcity of high-quality conclusive evidence and the lack of RCTs have led all major societies not to recommend routine screening of CE in the workup of RPL [3–5].

Immunologic factors

Autoimmune disorders: antiphospholipid syndrome

Antiphospholipid syndrome (APS) is an acquired autoimmune thrombophilic disorder, present in around 15%–20% of patients with RPL [22]. It is defined by a set of clinical and laboratory criteria known as the Miyakis criteria, summarized in Table 33.1 [55,56]. These criteria were developed as a guideline in an attempt to standardize reporting in research studies for patients with APS.

The extensive investigations on "noncriteria" antiphospholipid (aPL) tests have directed the leading authorities on APS to recommend adding antiphosphatidylserine testing [57]. In addition to identifying an extra 5% of patients with APS not detected by criteria testing, subsequent treatment has been shown to even decrease late pregnancy complications [30,58]. The poor reproductive outcome associated with APS is not only due to thrombosis-mediated injury as previously thought. Disruption of trophoblast migration and invasion, complement activation, and human chorionic gonadotropin release inhibition are all proposed mechanisms of APS-related injury, which all occur prior to spiral arteriolar formation [59].

Conflicting evidence exists on all aspects of the optimal treatment of APS. The bulk of evidence is based on a total of four RCTs, all of which demonstrated higher live birth rate (LBR) with aspirin and heparin combination therapy compared with aspirin alone (around 70%–80% vs. 40% respectively) and a 54% reduction in miscarriage rates with combination therapy as reported by a Cochrane review [59–61]. The two trials that used low molecular weight heparin (LMWH) showed no difference in LBR

TABLE 33.1 The research classification criteria for antiphospholipid syndrome (Miyakis criteria) [55,56].

At least one clinical criterion and one laboratory criterion are required for diagnosis	
Clinical criteria	**1.** Vascular thrombosis: ≥1 clinical episode of arterial, venous, or small-vessel thrombosis **2.** Pregnancy morbidity: **a.** ≥1 unexplained death of a morphologically normal fetus ≥10 weeks of gestation **b.** ≥1 premature births of a morphologically normal neonate before the 34th week of gestation because of either eclampsia/severe pre-eclampsia or recognized features of placental insufficiency **c.** ≥3 unexplained consecutive miscarriages at <10 weeks with other exclusion of other causes of RPL
Laboratory criteria	**1.** Presence of Lupus anticoagulant on two or more occasions at least 12 weeks apart **2.** Anticardiolipin antibody of IgG and/or IgM (>the 99th percentile), on two or more occasions, at least 12 weeks apart **3.** Anti-β_2 glycoprotein-I antibody of IgG and/or IgM (>the 99th percentile), present on two or more occasions, at least 12 weeks apart

between the two arms [56,62,63]. Despite the conflict, the consensus is treatment with preconception low-dose aspirin and prophylactic heparin starting from the timing of a positive pregnancy test until 4–6 weeks postpartum [59,64]. The literature lacks large RCTs on head-to-head comparison of combination therapy using aspirin with heparin versus LMWH [64]. With both having comparable safety profiles at prophylactic doses, the main advantage of LMWH is the once daily dosing and that of unfractionated heparin is the only heparin shown to be effective in prospective trails, and it is complete reversibility with protamine sulfate [59].

In our practice, we test all patients for anticardiolipin antibodies, antiphosphatidylserine antibodies, and the lupus anticoagulant. We treat all patients with RPL and APS with low-dose aspirin, 81 mg daily, starting before conception and continuing until 36 weeks unless they have contraindications for its use. Patients are immediately started on prophylactic doses of twice daily, unfractionated heparin with a positive pregnancy test. In the unusual case of a patient who is undergoing assisted reproductive techniques to achieve a pregnancy, we initiate heparin twice daily before embryo transfer. In all cases, we continue heparin until delivery and then resume heparin treatment postpartum for 4–6 weeks. If a patient has bleeding or enlarging subchorionic hematomas in the first trimester, we discontinue the aspirin and continue the heparin as described above.

Uterine immune system and immunotherapy

Immunotherapy in the setting of RPL is based on the proposed concept of maternal immune tolerance of the fetus being a semi-allograft with subsequent miscarriage being a form of transplant rejection. Th1 cell proinflammatory domination and higher NK cell levels whether peripheral or decidual in origin have all been postulated mechanisms of the precedent rationale [65].

The profound lack of high-quality, adequately powered RCTs has led to a unanimous stand of different societies on recommending against all types of immune testing (including HLA, Th1 and Th2, anti-HY, and uterine/peripheral NK cell tests) as well as the multitude of different immunotherapy options (including corticosteroids, intravenous lipid emulsions, IVIG, and G-CSF) [5,66–68]. Despite the lack of evidence, increased financial burden, and adverse effects pertaining to immunotherapy, it continues to be used all over the world as a last resort in the perplexing path of RPL management. We do not advise our patients with RPL to have testing or any treatment for any of these theoretical imbalances.

Endocrine disorders

Thyroid disorders

Thyroid dysfunction, namely overt hypothyroidism, is known to have a detrimental effect on reproductive outcomes ranging from menstrual abnormalities and higher miscarriage rates, all the way to adverse fetal neurodevelopment [69,70]. In contrast to the aforementioned well-established associations, the effect of subclinical hypothyroidism, defined as thyroid stimulating hormone (TSH) > 2.5mIU/L, remains rather controversial. A recent meta-analysis showed no association between RPL and subclinical hypothyroidism and confirmed that with the current very limited evidence, levothyroxine treatment does not appear to improve pregnancy outcomes [71]. Another debatable issue in thyroid disorders and RPL is antithyroid peroxidase antibodies (TPO) or antithyroglobulin antibodies. The same meta-analysis evaluating 17 studies found a statistically significant association between RPL and thyroid autoimmunity (odds ratio 1.94; 95% CI, 1.43–2.64). Benefit from levothyroxine in this patient population, however, was not proven by three out of four studies including the recent TABLET trial [71,72].

The discrepancy in recommendations by the different societies regarding thyroid dysfunction screening in RPL patients is a mere reflection of the aforementioned list of conundrums. While the RCOG and ASRM only recommend screening limited to TSH testing, ESHRE's most recent guidelines strongly support screening with both TSH and TPO antibodies [3–5].

In the absence of clear clinical data, we currently screen all patients in our practice with RPL for thyroid disease using an inexpensive test for TSH. In those patients with TSH levels above 4.5 IU/mL, there is clear evidence for the use of levothyroxine to lower TSH to below 2.5 IU/mL. In those with TSH levels between 2.5 and 4.5 IU/mL, we will evaluate antithyroid antibodies and offer low-dose levothyroxine to those with positive antibody tests. All patients on levothyroxine therapy should have TSH levels evaluated in early pregnancy and have levothyroxine adjusted to keep TSH below 2.5 IU/ml in the first trimester. At least 6 weeks should pass before reevaluating TSH levels and making any adjustments in therapy. TSH should be reevaluated in the second and third trimester with levothyroxine adjusted to stay below 3.0 IU/ml. Patients should have dosages adjusted with a postpartum check 6 weeks after delivery.

Diabetes

Poorly controlled diabetes is a well-established risk factor for miscarriage with preconceptual euglycemia after treatment decreasing the risk to normal miscarriage rates [73].

Screening for diabetes with HbA1C has hence been recommended by both the RCOG and ASRM but not by ESHRE [3–5]. We currently test all RPL patients for elevated HgbA1c. Those with elevated levels are treated with weight loss, dietary modifications, and metformin. We generally continue metformin throughout pregnancy to lower the risk of gestational diabetes.

Hyperprolactinemia

Although not confirmed in humans, *in vitro* studies on animals revealed that corpus luteum maintenance is also undertaken by prolactin [74,75]. Dysfunctional folliculogenesis and oocyte maturation secondary to the effect of elevated prolactin levels on the hypothalamic-pituitary-ovarian axis has also been postulated [76].

The literature is deficient in trials on RPL in the setting of hyperprolactinemia with only a single RCT showing higher prolactin in patients with pregnancy loss (31.8–55.3 ng/mL) compared to patients with ongoing pregnancies (4.6–15.5 ng/mL, $P < 0.01$ or $P < 0.05$). This trial also assessed the potential benefit of bromocriptine treatment with a higher percentage of successful pregnancies compared to those that were not treated (85.7% vs. 52.4%, $P < 0.05$) [76]. This single study was evaluated by a 2016 Cochrane review and was rendered low quality due to the small sample size and high risk of bias [77].

As for the different guidelines on prolactin testing as part of the RPL workup, ASRM confirms the recommendation, while ESHRE only recommends testing in patients with symptoms of hyperprolactinemia (oligo/amenorrhea) [4,5]. In our practice, we screen all patients for prolactin abnormalities. If the TSH is elevated, we initiate levothyroxine until TSH levels are normal and retest prolactin. If repeat testing of prolactin is elevated, we obtain imaging of the head including the pituitary gland to rule out intrinsic and extrinsic lesions. Therapy with cabergoline is initiated to normalize prolactin levels. Once pregnancy is achieved, cabergoline is discontinued.

Polycystic ovary syndrome

A recent study of polycystic ovary syndrome (PCOS) patients, which only used the Rotterdam criteria for diagnosis, showed that the prevalence of PCOS in RPL patients was lower than previously believed (8.3%–10%) [78]. The main underlying reported mechanisms associated with RPL were hyperandrogenemia, obesity, and hyperinsulinemia with resultant disrupted implantation due to fibrinolytic response impairment [79]. Conflicting evidence on the other hand, shows that PCOS does not predict subsequent miscarriages [80]. None of the leading societies hence recommend screening for PCOS and insulin resistance as part of the RPL workup [3–5].

Vitamin D deficiency

Vitamin D was proposed to have a beneficial immunomodulatory role on reproductive outcomes by modulating the shift to Th2 cells and regulation of cytokine secretion [81]. In addition, vitamin D deficiency was reported to be associated with increased levels of autoantibodies linked to RPL [82]. A recent prospective study on preconception levels of vitamin D in over 1000 women reported that those with levels over 30 ng/ml had higher live birth rates and lower miscarriage rates [83]. Much of the data on vitamin D were not available when the RCOG and ASRM documents were written, so they have not addressed vitamin D deficiency during the RPL workup [3,4]. ESHRE on the other hand, recommends vitamin D supplementation (irrespective of RPL) but not testing [5]. We currently suggest testing for vitamin D in our patients with RPL and find the majority of patients have levels well below 30 ng/mL. Supplementation is recommended for all patients with levels below 30 ng/ml.

Luteal phase deficiency

With progesterone being the most pivotal agent in maintaining pregnancy, any disruption in corpus luteum function is considered detrimental. Luteal phase deficiency (LPD) is hence defined as disruption in the corpus luteum's ability to produce adequate amounts of progesterone for sufficient periods [84]. Classically, the diagnosis was established through histologic dating of endometrial biopsies. This has been replaced by luteal phase progesterone concentration being below 10 ng/mL. The latter method of testing has been criticized since marked fluctuations in progesterone levels are seen secondary to luteinizing hormone pulsatility. LPD testing is hence not recommended by any of the leading societies. Regardless of conflicting evidence, progesterone supplementation in patients with RPL has been found to decrease miscarriage rates when compared to placebo or no treatment by a Cochrane review (0.38; 95% CI 0.20 to 0.70) [85]. More recently, a 2020 meta-analysis also confirmed the benefit of micronized vaginal progesterone in the setting of RPL [86]. We currently advise the use of vaginal progesterone suppositories 100 mg twice daily in women with RPL who exhibit oligoovulation, irregular cycles, low mid-luteal levels of progesterone, low progesterone levels documented in prior pregnancies, or other evidence of LPD. We advise patients to continue this supplementation until they reach 10 gestational weeks.

Inherited thrombophilia

While the association between RPL and acquired thrombophilia disorders, namely APS, has been well established, that pertaining to inherited thrombophilia remains rather controversial. Inherited thrombophilia constitute a spectrum of disorders secondary to genetic mutations disrupting coagulation proteins either qualitatively or quantitatively. These include factor V Leiden (FVL), prothrombin G20210A mutation (PT), antithrombin III deficiency, and protein C (PCD) and protein S deficiency (PSD) [87]. Being the most common inherited thrombophilia, the heterozygous carrier state

of FVL is the most investigated [88]. An early meta-analysis by Rey et al. reported that FVL was associated with recurrent early fetal loss (OR 2·01, 95% CI 1·13–3·58). Similar associations with thrombophilia were also reported for PT and PSD [89]. Due to the mere fact that association does not infer causality [90] and due to the lack of consistent evidence dominated by RCTs, numerous societies have recommended against routine screening for inherited thrombophilia in patients with RPL. All guidelines recommend thrombophilia testing in patients with a prior personal history or strong family history of thromboembolic disease or additional risk factors [4,5]. Despite these recommendations, a study reported in 2014 revealed that 46%, 76%, and 94% of surveyed physicians screened patients with early single losses, at least two losses, and at least three losses, respectively [91]. As for the benefit of anticoagulation treatment in patients with RPL and inherited thrombophilia, two meta-analyses confirmed the lack of benefit in preventing further pregnancy losses and accentuated the need for further evidence [92,93]. We currently do not screen patients with RPL for inherited thrombophilia unless they have a personal history or a strong family history of thromboembolic disease.

Evaluation for recurrent miscarriage

Several groups, including our own, have proposed the use of genetic testing of POC after the second miscarriage as aid in evaluating the causes of RPL. In the first prospective study on the use of genetic testing on POC from women with RPL, we were able to assign a probable cause for the pregnancy loss and provide a likely cause of the miscarriage in 90% of all patients (Fig. 33.1) [25,30]. Moreover, the addition of POC testing

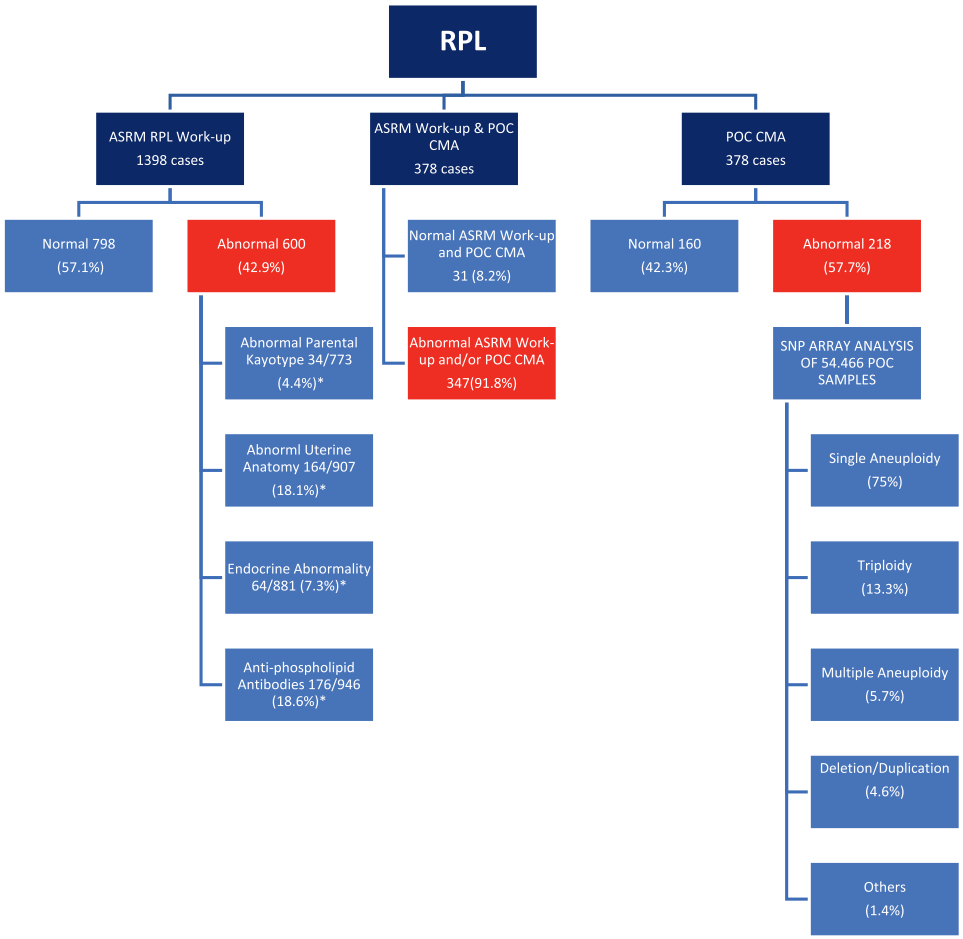

FIGURE 33.1 **Three strategies for identifying the cause of RPL [6,25,30,95].** In the left panel, if the clinician follows the ASRM guidelines, 42.9% of couples will have a potential explanation for the pregnancy loss. In the right panel, if only chromosomal microarray analysis is performed on the products of conception, 57.7% of couples will have an explanation for the loss. However, if a combined approach using the ASRM workup (without the parental karyotypes) plus a genetic analysis on the products of conception is performed, then over 90% of couples with recurrent miscarriage will have a proven or probable explanation for their loss. * Numbers do not add up to 42.9% because some patients had more than one anomaly.

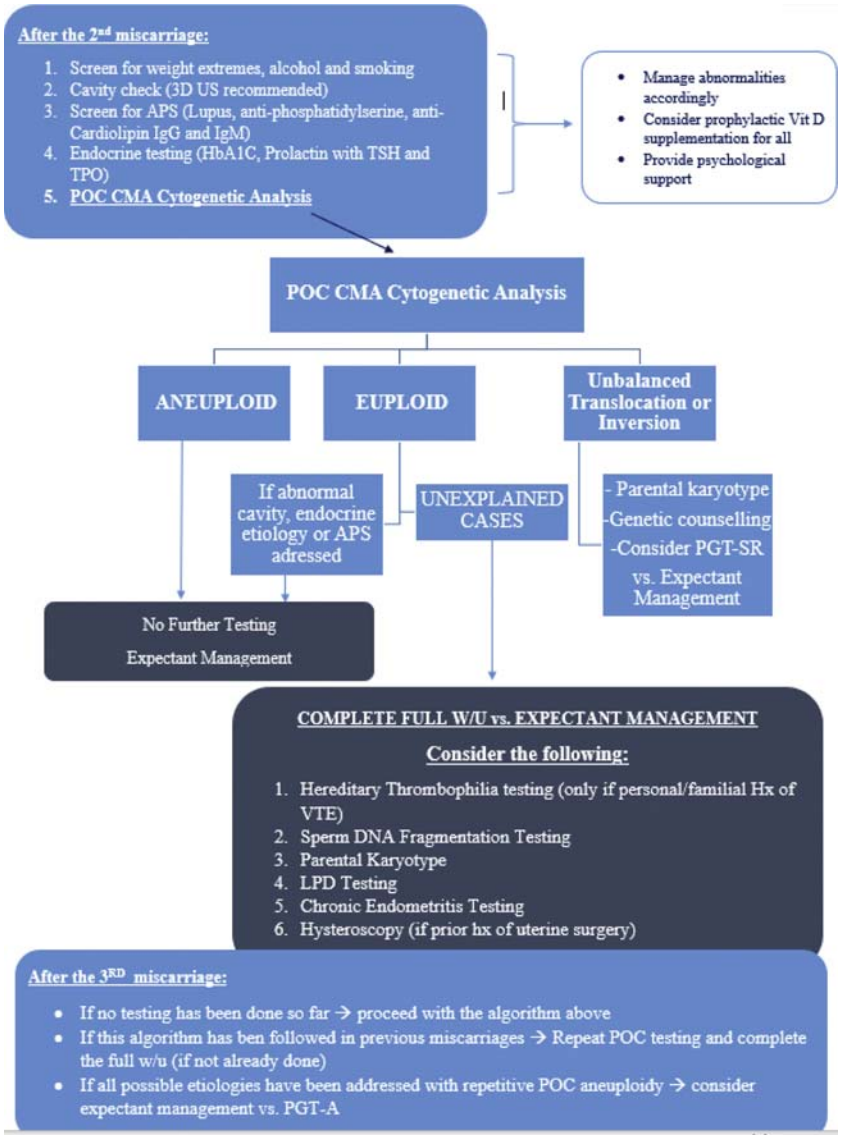

FIGURE 33.2 **Proposed algorithm for recurrent miscarriage evaluation [30].** After the second miscarriage, perform the modified ASRM evaluation with the deletion of parental karyotypes and get a chromosomal microarray on the products of conception. Based on the results of the genetic testing on the miscarriage (aneuploid, euploid, or unbalanced) follow the suggested steps. There will be a small percent of patients (<10%) who will still be truly unexplained after following the steps in this proposed algorithm. Those patients would be excellent candidates for experimental research investigations or novel treatment protocols.

has the potential to be more cost-effective compared to previous algorithms with resultant savings to the healthcare system and financially drained patients [25]. As previously mentioned, evaluation by the ASRM or CMA testing alone will provide patients an answer in only around 50% of cases. In addition, 25% of patients with abnormal POC results will also have a potential treatable cause identified by the ASRM workup [25,30]. Based on our accumulated data on the use of POC testing in patients with RPL who have also had the ASRM recommended evaluation, we are now able to provide an explanation to the vast majority of our patients. We realize that the finding of aneuploidy in POC does not provide a treatment option, but the knowledge of the cause of the loss is beneficial to both the patient and her physician. These new advancements in investigating RPL are addressed in the proposed algorithm by Papas et al., as summarized in Fig. 33.2 [30].

Conclusion

Despite the immense psychological and financial repercussions associated with any RPL workup, the prognosis remains very promising with over 50%–60% of patients ending up with a live birth [94].

The reason behind the long list of unanswered inquiries in RPL is summarized by Dimitriadis' closing statement in the most recent primer on RPL: "Assumptions rather than robust evidence have shaped our understanding of the mechanisms of recurrent pregnancy loss in some instances, and have led to a plethora of theories, unproven tests and ineffective treatments" [28].

Adequately powered and well-designed RCTs are hence peremptory in shaping our proper understanding of all aspects of RPL. Updating guidelines of different societies is also crucial since most have been outdated by many advancements since their publication dates. The main aim in the perplexed management of RPL should be to minimize any potential unnecessary harm to physically, psychologically, and financially depleted couples.

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CHAPTER

34

Repeated implantation failure

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Introduction

Implantation failure is one of the main limiting factors toward the success of assisted reproductive technologies (ART), usually linked with either maternal characteristics or dysfunction of embryo-maternal immunotolerance pathways [1]. More than 10% of infertile patients have at least two or three repeated implantation failures (RIFs) after an *in vitro* fertilization (IVF) program [2]. RIF remains a hitherto highly frustrating and distressing reproductive problem for the infertile couple [3]. Surprisingly, no clear international consensus on a definition for RIF has been reached, as yet; as a result, there are no widely approved and followed guidelines either on the diagnostic work-up nor on the therapeutic approaches. Consequently, RIF is still linked with a waste of financial resources, exposing infertile patients to undue additional health risks [4].

Definitions

The lack of a universal definition is currently well endorsed. A plethora of them have been provided during the last 25 years, focusing either on the number of—mainly fresh—failed IVF attempts or embryos used [3,5–10]. Of note, the most commonly quoted criteria include three unsuccessful fresh IVF cycles, in which one to two embryos of high-grade quality are transferred [7,8,10,11]. In contrast, in many studies and in 30% of clinicians questioned, the number of cycles assigned to the definition of RIF is reported to be two [7,12,13]. Moreover, all suggested definitions derive mainly from expert opinions, so they lack robust scientific basis. This seems sensible, as trials have included variable study populations, in terms of ethnicity, prognosis (based on age, ovarian reserve, and cases of oocyte donation), and demographic characteristics [4]. Also, the criteria on the definition have changed with the trend toward single embryo transfer and the increased use—and efficacy—of frozen replacement cycles, together with the improvements in laboratory equipment, culture media and laboratory conditions, more accurate evaluation of embryos, and improved embryo transfer techniques [14].

Thus, the incidence of RIF is difficult to weigh, as definitions and populations vary, with figures from 10% up to 33% being recorded [2,15]. As a result, there is an ongoing tendency in clinicians for using empirically both diagnostic and therapeutic interventions, irrationally complying with patients' requests. In an effort to discriminate between couples with RIF and those who do not conceive because of statistical misfortune, recent trials have focused on the use of mathematical models [3,4].

In a more practical and pragmatic approach, clinicians should confront RIF as a screening condition instead of a clear-cut diagnosis. And if they should have a definition, it could be based on "three failed IVF attempts in good-prognosis patients."

Causes

The widely recognized causes have been previously described [4,16–18]; these include lifestyle factors, quality of the gametes, uterine and adnexal pathologies, and systemic disorders, such as thrombophilia and immunological factors, vitamin D deficiency, and endocrinological disorders, together with altered expression of associated molecules and chromosomal abnormalities, both maternal and paternal, such as

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translocations, mosaicism, inversions, and deletions, along with failure of the zona pellucida to rupture after blastocyst expansion and inadequate culture conditions and technique of embryo transfer (ET). In addition, (histologically confirmed) chronic endometritis (CE) has been shown to modify decidualization of human endometrial stromal cells, through sex steroid hormone receptors' impairment [19], while its therapy could improve the IVF outcome [20]; a next-generation sequencing analysis of the microbiota in the endometrial fluid and vaginal secretions in women with RIF revealed differences when compared to women without [21]. Recently expressed theories on the real causes of RIF were based on the clinical "inability to properly synchronise the euploid blastocyst with the patient's personalised window of implantation" [22] and that both displaced and disrupted windows of implantation exist and can present independently or together in the same RIF patient [23]. In contrast, association of RIF and *MTHFR* polymorphisms has not been confirmed [24].

Diagnostic work-up

General

Various diagnostic procedures are being endorsed empirically based on clinicians' perspectives toward potential causes of RIF, such as investigation of the uterine cavity (ultrasound, hysteroscopy, endometrial biopsy), the male factor (intensified sperm analysis), or the embryo developmental potential (time lapse, preimplantation genetic testing for aneuploidy) [16].

Hysteroscopy

Hysteroscopy is one of the most common proposed procedures in RIF. It can reveal implantation failure factors, such as adhesions, CE, endometrial polyps, fibroids, and uterine malformations, while most of them can be treated [12]. Especially in cases of CE, targeted endometrial biopsy can guide both diagnosis and targeted treatment [25,26], which is mandatory before patients enter an IVF cycle, as there is data on higher live birth rates when proven CE is successfully cured [12,20,25,27–29]. There is evidence to suggest that pregnancy rates after hysteroscopy can be improved in general, albeit not miscarriages [30], and especially outpatient hysteroscopy preceding IVF in women with RIF [31]. Contrarily, when ultrasound is normal, hysteroscopy does not offer much toward this improvement [32]. For the detection of anatomic malformations, the supplementation of 2D or 3D ultrasound to hysteroscopy seems ideal [12]. The cost of the procedure, of course, still remains a challenge and a limiting factor for its widespread use [26].

Immune profile biomarkers

The study of the uterine immune profile through biomarkers toward the assessment of uterine receptivity has been carried out during the last 20 years. A recent report showed that the "immunotolerance panel" is completely changed in RIF, through the stimulation of the immune system and initiation of humoral immunity, and through the activation of inflammatory responses, such as pNK cells, Th17, and TLR signaling pathways [33]. A combination of biomarkers, including the ratios of endometrial IL-18/TWEAK mRNA and IL-15/Fn-14 mRNA and the $CD56^{+}$ cell count, was used to document the local cytotoxic/angiogenic equilibrium, and the state of activation, mobilization, and maturation of uNK cells, resulting in the association of higher live birth rates [34]. Similarly, TGf-β reduction was linked to RIF through its immunosuppressive role in pregnancy [33].

Investigation of chromosomal abnormalities

Karyotyping of both partners is a method to detect chromosomal abnormalities. It is established that the rate of fetal aneuploidy is higher in RIF [6], especially translocations (reciprocal and Robertsonian). Moreover, preimplantation genetic diagnosis aims to select the best quality embryo for ET, free of a specific chromosomal disease, followed by molecular investigative approaches, such as fluorescent *in situ* hybridization, comparative genomic hybridization, or single nucleotide polymorphisms [16,17].

Molecular assays

The "molecular signature" determines the receptivity of the endometrium. Reported paradigms include the immunohistochemistry evaluation of cyclin E and p27 [35] and the endometrial receptivity analysis [36]; of note, the high percentage of false positive results and the lack of the improvement of the primary outcomes in RIF patients do not permit the widespread usage of these techniques, as yet [26].

The investigation of *MTHFR* is important. *MTHFR* is a gene critical for the metabolism of folic acid and for the human reproduction. Its variants, such as *MTHFR 677C>T* or *MTHFR 1298A>C* are more common in patients with RIF, whereas its low activity exerts a negative effect in reproductive function and ART results [37]. Among other biomarkers, the overexpression of the proteins AMHRII and BCL6 in the endometrial tissues has been recorded in patients with otherwise unexplained RIF, so it should be tested before a new IVF cycle [26,38]. In a pilot study, the microbiota in the endometrial fluid and vaginal secretions in women with RIF through next-generation sequencing appeared significantly different compared to women undergoing their first IVF cycle [21].

Metabolomics

The study of the alterations in the metabolites level has been shown to be important for implantation and linked with infertility [39−41]. Classic examples are those of the glucose metabolism [42,43] and of the nitric oxid, L lysine and valine [44−47]. In a recent Cochrane review on the subject, the authors concluded that according to current trials in women undergoing ART, there is insufficient evidence to show that metabolomic assessment of embryos before implantation has any meaningful effect on rates of live birth, ongoing pregnancy, or miscarriage rates [48].

Reaching a robust conclusion

In a recent systematic review on the validity of conventional and modern biomarkers of endometrial receptivity, the authors included markers evaluated by ultrasound (endometrial thickness, volume, Doppler signals, and wave-like activity), endometrial biopsy (histology, molecular tests), endometrial fluid aspirates, and hysteroscopy [49]. They concluded that none of them had sufficient discriminatory value to act as a diagnostic test for endometrial receptivity based on their ability to predict clinical pregnancy. In RIF cases, robust data are lacking, driving the clinical suggestion mainly through empirical data. So far, the most accepted diagnostic approaches include hysteroscopy (and checking for CE), karyotype of both partners, screening of antiphospholipid syndrome, hormonal investigation of thyroid, diabetes, and prolactin of the female partner, semen analysis, and sperm DNA fragmentation testing.

Interventions

Endometrial injury

Endometrial injury (EI) in the cycle prior to IVF, performed by pipelle biopsy (usually) or via hysteroscopy, has been suggested to prepare the endometrium for implantation, by increasing the local cytokines involved in both wound healing and implantation processes [16,50]. Meta-analyses of randomized controlled trials (RCTs), with regard to the benefit of this method in women with RIF, have either been unable to extract results due to the substantial between-study clinical heterogeneity or have led to inconsistent conclusions [51−54]. Vitagliano et al. have demonstrated significantly higher live birth rates (LBRs) (RR 1.38; 95% CI, 1.05−1.80) and clinical pregnancy rates (CPRs) (RR 1.30; 95% CI, 1.03−1.65) in women with at least one previous failed ET undergoing EI compared with those receiving placebo or no intervention, with double luteal EI being the most beneficial method; the effect remained significant in the subgroup of women with at least two previous ET failures [54]. In contrast, the meta-analysis of Sar-Shalom Nahshon et al. contradicted these findings [53]. No differences were found in terms of multiple pregnancy, ectopic pregnancy, or miscarriage rates [53,54]. A 2015 Cochrane review assessed evidence on the benefit of EI of "moderate quality," calling for properly designed trials [55]. Moreover, the two most recent RCTs have, once again, presented conflicting results [56,57]. The first one, which showed no benefit in all participants or the prespecified RIF subgroup, included patients who underwent EI between day 3 of the menstrual cycle preceding a fresh or frozen ET and day 3 of the ET cycle [56]. In the second one, women were locally injured on the third day of cycle before only frozen-thawed ET, exhibiting significant increase in their LBRs (51% vs. 36%; $P = .032$), CPRs (64% vs. 48%; $P = .023$) and implantation rates (46.74% vs. 30.11%; $P = .001$) compared with the control group, yet also significantly higher multiple pregnancy rates (37.5% vs. 18.75%; $P = .031$) [57]. Therefore, more data are currently needed to solidify the exact clinical benefit of EI in women with RIF.

Antibiotics

The concept of antibiotic therapy in RIF is based on the high prevalence of CE in these patients [28,58,59]. Resolution of CE through therapy before proceeding with IVF has shown a significant advantage over no cure and persistent CE, in terms of LBRs and ongoing pregnancy rates [20]. Interestingly, cure of CE may further lead to comparable outcomes to women without CE [20]. Combined intrauterine infusion of dexamethasone and antibiotics has also resulted in favorable implantation rates, CPRs, and LBRs in the first IVF-ET cycle, primarily in RIF patients with both hysteroscopic and histological confirmed CE, yet without persistent CE after treatment and regardless of the results of endometrial cultures [60].

Human chorionic gonadotropin

Intrauterine injection of human chorionic gonadotropin (hCG), a molecule with a prominent role in embryo implantation and early stages of pregnancy, has also exhibited significant efficacy in improving CPRs and LBRs of women with RIF [61]. This effect has been linked to a rise of peripheral T-regulatory (T_{reg}) cells and has appeared stronger in patients with an age of <35 years and blastocyst transfer [62]. It has been further correlated with the local injury caused by the operation [63].

Immunotherapy

Intravenous immunoglobulin

Although its exact mechanism of action in RIF patients has not been elucidated, intravenous immunoglobulin (IVIG) might be a therapeutic option due to its immunomodulatory effects, including the induction of T_{reg} cell-related pathways [64]. Its use in women with unexplained infertility and RIF has been associated with significantly increased CPRs (RR 1.475; 95% CI, 1.191−1.825) and LBRs (RR 1.616; 95% CI, 1.243−2.101), as well as reduced miscarriage rates (RR 0.352; 95% CI, 0.168−0.738) [65]. Conversely, when LBRs per ET were considered, the increase appeared nonsignificant [65]. Finally, its combination with oral prednisone has been suggested as a promising approach, with evidence verifying this benefit currently lacking [66].

Granulocyte colony-stimulating factor

Granulocyte colony-stimulating factor (G-CSF) may contribute to a positive pregnancy outcome by enhancing ovarian function, correcting pathologic changes of the endometrium, and assisting in embryo implantation [67,68]. Such properties have attracted much attention toward the treatment of RIF, but relevant RCTs led to inconsistent results [69,70]. Synthesis of current data has linked both the subcutaneous and the intrauterine administration of G-CSF to significantly increased implantation rates and CPRs in women with unexplained RIF, with the second route exhibiting a slightly more positive effect [70]. A previous meta-analysis has reached similar conclusions [71]. It has also been suggested that the combination of both routes might be superior to the only subcutaneous route [72]. Finally, no robust data exists on its use as an adjunct in culture media in RIF patients undergoing IVF [73,74].

Tacrolimus and sirolimus

Treatment with tacrolimus, a calcineurin inhibitor, before or even during pregnancy has indicated favorable outcomes, in terms of CPRs, ongoing pregnancy, and miscarriage rates in the group of RIF women with elevated peripheral blood T_{h1}/T_{h2} cell ratios [75−77]. No significant obstetric or perinatal complications from its use have been detected, yet with the need for further data being mandatory [77,78]. The potential implications of sirolimus, a mammalian target of rapamycin inhibitor, on pregnancy outcomes of RIF patients is a brand-new concept. Only a recent double-blind, phase II RCT has investigated this innovative possibility, revealing significantly higher CPRs (55.81% vs. 24.24%, $P < .0005$) and LBRs (48.83% vs. 21.21%, $P < .0001$) in RIF women with elevated T_{h1}/T_{reg} receiving sirolimus compared to the control group [79].

Other immunomodulatory agents

Hydroxychloroquine has also been tested in women with RIF due to its antiinflammatory, immune-regulatory, and antithrombotic properties [80,81]. Despite its potential immunomodulatory action, through decreasing an aberrant T_{h17}/T_{reg} ratio or shifting to T_{h2} response in women with elevated TNFα/IL-10 ratio, no significant improvement of pregnancy outcomes has derived from its use [80,81]. Limited data currently exist on lymphocyte immunotherapy in RIF patients, failing to show any benefit on their LBRs [82,83].

Several studies have revealed a positive impact of the intrauterine administration of autologous peripheral blood mononuclear cells (PBMCs)—acting as an inflammatory modifier—on CPRs and implantation rates, and more evidently in women with two [84] or at least three prior ET failures [1,85,86]. The same pattern, with the effect being significant only in the subgroup with a minimum of three or even four previous ET failures, applies to studies on PBMCs after hCG activation [87,88]; in one of these, significantly higher miscarriage rates in the PBMC group were noted [88].

Intralipid constitutes a fat emulsion of soybean oil, glycerin, and egg phospholipids that is reported to possess immunosuppressive properties on uterine NK cells [89]. Low-quality evidence supports that IV intralipid has a potential positive impact on both CPRs and LBRs of women with previous implantation failure [90].

Leukemia inhibitory factor has been hypothesized to regulate the endometrial differentiation, but the only available RCT has failed to demonstrate any benefit in women with unexplained RIF [82,91,92].

Tumor necrosis factor-alpha (TNF-a) inhibitors counteract the increased T_{h1}-related cytokine secretion, held responsible for early reproduction failure [93]. The extent of TNF-a/IL-10 cytokine elevation in women being treated with adalimumab has also been correlated with IVF success rates [94,95]. While such agents as adalimumab or etanercept have been tested in patients with refractory recurrent spontaneous abortions [96,97], no evidence currently exists on their use in RIF patients.

Oral prednisone in RIF patients has been found to shift the T_{h17}/T_{reg} balance toward the side of T_{reg} and thus the direction of immune tolerance [98]. Although its underlying mechanisms are not completely understood, it has been hypothesized that response to prednisone may decrease endometrial biomarkers of immune overactivity [99]. Oral prednisolone has also been associated with a decrease in uterine NK cells, although not accompanied by a clinical benefit [100]. So far, its use cannot be recommended, until RCTs manage to associate its oral use with favorable outcomes in RIF patients [101].

Conclusion on immunotherapy

Currently available systematic reviews summarize that no or some promising benefit of immunotherapy in women with RIF might exist, all agreeing that the paucity of high-quality evidence should currently deter its routine clinical use in this subset of patients and that more carefully designed RCTs with standardized patient selection and treatment protocols are required before definite conclusions can be reached [82,102,103].

Atosiban

Atosiban is a receptor antagonist for vasopressin V_{1a} and oxytocin, and its assistance in the implantation process relies in the reduction of uterine contractile activity after ET [104]. Although a clinical benefit of atosiban in the general population of women undergoing IVF is of question, more solid evidence exists that it significantly improves CPRs, LBRs, and implantation rates of RIF patients compared to placebo or no treatment, yet without affecting multiple pregnancy, ectopic pregnancy, or miscarriage rates [105–107]. Verification of these findings requires larger studies in the future.

Low molecular weight heparin

Regardless of the presence of thrombophilia in RIF patients, low molecular weight heparin (LMWH) appears to increase the production of insulin growth factor-I and to inhibit the expression of insulin growth factor-binding protein, thus contributing to endometrial development and receptivity during the implantation window [108]. Its adjunct use has been found to significantly improve LBRs (RR 1.79; 95% CI, 1.20–2.90; $P = .02$) and reduce miscarriage rates (RR 0.22; 95% CI, 0.06–0.78; $P = .02$) in women with at least three prior IVF failures [109]. Nevertheless, these findings require careful interpretation due to small sample sizes of the available studies [109].

Freeze-all policy

Data on the implementation of freeze-all policy, meaning the elective cryopreservation of an entire cohort of embryos before their transfer in a consecutive frozen-thawed cycle, in women with RIF are currently limited, but encouraging [110,111]. In cohort studies, the policy was found superior to fresh transfer strategy, with regard to CPRs, ongoing pregnancy, and implantation rates in patients with RIF [110] and in women with at least one prior fresh blastocyst implantation failure [111]. The rationale of the beneficial effect of this policy on RIF is principally based on the bypassing of the negative effects of ovarian stimulation on endometrial receptivity, mainly the elevation of progesterone levels at day of triggering oocyte maturation [112,113], providing a more "natural" endometrial environment.

Growth hormone

Growth hormone (GH) improves both oocyte developmental potential/embryo quality and endometrial receptivity [114,115]. In RIF patients, GH administration has been associated with a thicker endometrium at day of ET and higher LBRs and CPRs compared to no cotreatment, with rates still not reaching those of non-RIF patients [116]. At this point, however, data on GH remains inadequate.

Gonadotropin-releasing hormone

Gonadotropin-releasing hormone administration prior to estrogen-progesterone preparation of the endometrium in women with RIF has not succeeded in significant improvements in pregnancy and implantation rates [117,118]. However, the addition of the aromatase inhibitor letrozole seems to reinforce its clinical benefit [118].

Assisted hatching

Assisted hatching, the artificial manipulation of zona pellucida, has been related to increased CPRs, yet also to raised multiple pregnancy rates in RIF patients receiving fresh embryos [119]. However, the small samples sizes of available studies cannot lead to safe conclusions [119]. A potentially beneficial effect only on CPRs and implantation rates has been recently suggested for laser assisted hatching [120].

Combined strategies

Combinations of clinical approaches were based on the special effects of the components. Levothyroxine supplementation has been associated with improved anti-Müllerian hormone levels in infertile patients with Hashimoto's thyroiditis [121]. Vitamin D can optimize maternal tolerance for implantation in women with RIF through both its local and systemic immunomodulatory effects and its simultaneous regulation of the T_{h1}/T_{h2} imbalanced ratio [122,123]; a meta-analysis of cohort studies showed a potential beneficial effect on the general IVF population, but not in RIF [124]. These data established the rationale for testing the OPTIMUM

treatment strategy, namely the combined treatment of CE with antibiotics, the aberrant T_{h1}/T_{h2} ratio with vitamin D and/or tacrolimus, overt or subclinical hypothyroidism with levothyroxine, and thrombophilia with low-dose aspirin [125]. The application of the OPTIMUM strategy in RIF women resulted in significantly higher biochemical and ongoing pregnancy, and lower miscarriage rates in comparison to the control group [125].

Nonetheless, combination approaches are not always beneficial. Through their cohort, Siristatidis et al. found no positive effect of the co-administration of LMWH and prednisolone on CPRs in RIF patients [126].

Other treatment options

Clinical trials on transcutaneous electrical acupuncture point stimulation and Chinese medicine methods (e.g., the "Yupei Qisun" sequential therapeutic intervention) have provided positive results [127,128]. Furthermore, anecdotal evidence supports a potential benefit of copper intrauterine device placement for two menstrual cycles at the time of hysteroscopy [129]. A retrospective study has evaluated the effect of using hyaluronan-rich medium for transferring blastocysts on CPRs and implantation rates of women with a previous implantation failure, showing no benefit [130]. Evidence also evolves on whether intrauterine infusion of autologous platelet-rich plasma (PRP) actually yields beneficial effects as an adjuvant therapeutic option in RIF patients [131–134]. While results are conflicting, a recent RCT has demonstrated significant efficacy of the method [133]. PRP belongs to the wider spectrum of "cell therapy," which also involves lymphocyte immunotherapy, PBMCs, and utilization of different stem cell types [135]. The proposed advantages of such agents include simplicity and cost-effectiveness as well as immune response regulation by reduction of cytokine production, decrease in activation of NK, T_{h1} and T_{h17} cells and induction of T_{h2} and T_{reg} cells [135]. Also, ooplasmic transfer, where various amounts of donor ooplasm are injected "as a whole" into developmentally compromised oocytes obtained from patients with RIF, led to successful pregnancies and births [136]. Although a 20-year follow up of the offspring produced by this method did not reveal any health issues [137], concerns that the mixture of two different maternal sources of ooplasm could generate high mtDNA heteroplasmy in the offspring resulting in fetal abnormalities and the lack of properly conducted trials have questioned the efficacy and safety of the method so far [138]. Finally, laparoscopy for diagnosing and treating endometriosis, especially in patients with significant dysmenorrhea or with abnormal BCL6 or miRNA testing, could be beneficial in treating RIF [26]. However, more data on their clinical utility in RIF patients is necessary.

Conclusion

RIF still remains a problem, with the absence of definition to be one of the main causes [139]. Quoting Zion Rafael, "It is a 'catch 22,' as the false diagnosis of RIF serves as a point of entry to test most of the studies that opt to prove the validity of various add-on regimens for RIF patients" [140]. Also quoting Hans Evers, "A made-up disease, refined into another made-up disease, treated by unproven treatment, you would think we now have scraped the bottom of the barrel. Wrong!" [141].

On the other hand, we should not neglect the human inability to find and establish guidelines in all above referenced aspects surrounding RIF. The purpose of this chapter was the presentation of all available to-date evidence, concerning the causes, diagnostic procedures, and treatment options.

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CHAPTER

35

Gestational carrier

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Introduction

The term gestational carrier is also commonly reported with the term "surrogate." This is defined as a woman who becomes pregnant and intends to give birth finally to a child with the predicated intention of giving away this child to another person or couple. This person or couple is often reported as "intended" or "commissioning" parents [1].

Gestational carrier has been a reality since ancient years. It is characteristic that laws of Ancient Babylon actually permitted surrogacy, potentially as a manner to avoid divorces in couples where potential maternity was impossible because of health reasons [2] One of the first described cases of surrogacy is claimed to be one described in the Book of Genesis enrolling Sarah, Abraham, and one of their servants Hagar. Since then, it has been reported that surrogacy reproductive services have been provided even based on a financial offer during all ages.

Surrogacy is a modern and increasingly important fertility treatment. It presents an option for parenthood in cases where specific medical conditions make it impossible for a woman to give birth of her own children with her own uterus. Such medical conditions may be, but are not limited to, absence or previous resection of uterus, severe uterine congenital abnormalities, and maternal medical contraindications of pregnancy. The modern aspect of gestational carrier has enrolled the use of *in vitro* fertilization (IVF) and generally assisted reproductive technology (ART) techniques in the context of the procedure. Specifically, it is therefore possible that the embryo may be produced from the genetic cells of both intended parents, even if mother may actually not carry the baby herself, while another modern option of this strategy is ovary donation with paternal sperm to achieve embryos with the contribution of paternal side. This strategy has also made possible both for homosexual couples and single men to achieve parenthood with her own contribution as embryos could be created with their own genetic cells as well.

Surrogacy may be categorized into two great types according to genetic participation of gestational carrier which are traditional surrogacy and gestational surrogacy. Traditional surrogacy is the case where the father's sperm is artificially inseminated into the surrogate mother, which leads to a baby in which the intended father and surrogate mother both participate, considering this case as a genetic or partial surrogacy. Gestational surrogacy, in contrary, is the case of full surrogacy, in which the embryo is produced by the genetic cells of both parents (sperm and eggs), so both intended parents participate equally, while the surrogate mother has no genetic connection with the child.

Another great categorization of surrogacy concerns the motivation of surrogacy mother and includes two main categories: commercial and altruistic. Commercial surrogacy concerns the case in which gestational carrier receives reward not only for the medical care that she is going to receive but also for the fact of taking responsibility of delivering a baby and giving this to intended parents. In case the motivation of the gestational carrier is only to assist intended parents to acquire a baby and she is only rewarded the cost of medical surveillance for her and her fetus, the pregnancy may be considered altruistic [3].

Indications for surrogacy

The main indications of surrogacy are the absence of uterus as well as medical conditions totally contraindicating pregnancy of intended mother.

Regarding absence of uterus, this may be due to syndromes such as Mayer-Rokitansky-Kuster-Hauser syndrome [4] or history of hysterectomy either for obstetrical or for gynecological indications. Besides, congenital uterine abnormalities, such as small

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unicornuate uterus, T-shaped uterus, or multiple fibroids are also indications.

Regarding maternal medical conditions contraindicating pregnancy, these may refer to severe renal failure, severe heart disease, such as Eisenmenger syndrome, or any other medical condition setting an obstacle for undergoing a pregnancy.

Finally, a third category of causes, which is increasingly diagnosed especially in modern times, refers to repeated miscarriage or recurrent pregnancy loss (RPL). RPL is defined as the history of multiple pregnancy losses (at least two), while it is common that such patients may often remained undiagnosed regarding their exact reason of RPL. In these patients, a gestational carrier may actually be considered the very last option to achieve parenthood [5].

Selection process and criteria of gestational carrier

The gestational carrier will be a person who is pregnant for almost 40 weeks with the intention to deliver a baby for a couple of intended parents. This demands that the carrier should rather be an ideal candidate from a medical basis, fully screened, and also controlled for any aspect related with her corporal and mental capacity to achieve a pregnancy.

A majority of existing recommendations report the following:

- Age of surrogate mother should be between 23 and 35 years old.
- Surrogate mother should be a married woman, having already delivered at least one baby for her family. Age of her baby should be over 3 years old, and the previous delivery should not have been earlier that 2 years.
- Full mental awareness of the responsibility should be accepted by the gestational carrier.

Basic screening for a gestational carrier includes the screening also used for egg donors. Therefore, full corporal and psychological control should be performed, while it is mandatory to check for her economic and criminal history. Blood exams should control for virus diseases, such as human immunodeficiency virus, hepatitis B virus surface antigen, and hepatitis C virus. Cardiologic examination with electrocardiogram should be performed. Full gynecological examination with Pap smear, mammography, and thorough pelvic and abdominal ultrasound is obligatory to control for her gynecological well-being. Finally, detailed psychological assessment from a specialist before signing the legal contract with intended parents is also of paramount importance [3].

Counseling and legal requirements

Counseling before proceeding to surrogacy is potentially one of the most basic parameters both on a medical and legal basis and should be performed in detail for both parents and gestational carrier [3,6].

First of all, the intended parents should be totally aware of all potential alternative options leading to parenthood. Furthermore, they should be aware of medical risks that may arise such as risk of multiple pregnancy, congenital abnormalities, miscarriage, and other complications that could potentially necessitate preterm delivery. The fact that gestational carrier may be an ideal candidate for pregnant does not guarantee that the pregnancy will be uneventful. Furthermore, they should be aware of their obligations to the gestational carrier regarding financial issues and the fact that many practical difficulties could arise and should be solved in the context of litigation.

The gestational carrier on her side should accept responsibility that any pregnancy could have potential risk and complications for which the intended parents may not have any medical or legal responsibility. Such risks concern both medical risks such as pregnancy complications but also psychological risks regarding the future separation from the child that she will deliver for the intended parents.

The basic goal of counseling is that both parties understand all aspects of surrogacy and accept their responsibilities but, mainly, build a relationship that is based on trust and mutual comprehension for their common goal.

After the counseling procedure, the signature of a legal contract is obligatory. As soon as the terms of contract meet all parties' expectations, the contract is signed in the presence of both parties' lawyers, and the medical process may be initiated.

Synchronization of cycle

The surrogate embryo transfer could be fresh or frozen transfer and subject to availability of the gestational carrier. As excellent vitrification techniques are now available, surrogacy cycles have become less difficult for ART clinics with a good embryology laboratory and freezing facility.

For a fresh surrogate transfer, the surrogate and the intended mother cycle may be synchronized with oral contraceptive pills or progesterone pills, or the surrogate may be put on agonist injection for flexibility of transfer dates.

The surrogate is started on estrogen tablets from the third day of her cycle for around 10 days. On reaching

of minimum 8 mm, she is then put on progesterone supplementation for 3 or 5 days before a planned cleavage stage or blastocyst transfer, respectively.

Obstetric care of surrogate

Once a pregnancy is confirmed in the gestational carrier depending on the facility of the ART clinic, she either stays in the surrogate house or at her home. The concept of surrogate house has recently caught a lot of attention for various reasons. A surrogate house is a place where the surrogate stays for her entire antenatal period till the date of delivery, and all her medical and personal requirements are taken care of. The obstetrics care of surrogate is extensive due to the preciousness of the pregnancy. She stays under the supervision of 24-h nursing staff along with dietician, physiotherapist, counselors, and gynecologist for her medical care. It is due to this care and available facilities that intended couples have taken up more liking toward the concept of a surrogate house. Although staying at a surrogate house is a preferred practice these days, it could be emotionally worse for the gestational carrier and her entire family as she has to live away from her own family; however, during her stay at a surrogate house, the surrogate can go home for few weeks during pregnancy, and her family members can also visit her at the surrogate house. Staying at a surrogate house should be optional for the surrogate mother; she should be given a choice.

There are no specific guidelines specifically regarding follow-up of a gestational carrier pregnancy. We therefore rather follow rules set for follow-up of any other common pregnancy. Carriers may undergo obstetrics assessment every 20 days till the date of delivery, obstetrics scans at 6–8 weeks, anomaly scan at 11–13 weeks, anomaly scan and 3D-4D at 20–22 weeks, and growth scan at 28 weeks and 34–36 weeks. Any additional scan is subject to the obstetric need.

The intended couple is sent regular updates regarding the surrogate's pregnancy in the form of her weight gain, vitals, fetal growth, and antenatal investigation reports and scans. Postdelivery, the surrogate is kept under observation for a minimum of 15 days before discharge.

Risks associated with surrogacy

The major risk of a surrogacy pregnancy derives from the use of ART techniques rather than surrogacy itself. We should always take into account that the medical profile of the gestational carrier is potentially ideal, having previously undergone detailed physical and psychological examination. Therefore, it is predominantly risk from ART methods characterizing risk in surrogacy pregnancy, the most important one being the risk of multiple pregnancy.

The main strategy that is strongly suggested by relative medical societies (ASRM and ESHRE) in an effort to avoid multiple pregnancies is the policy of single embryo transfer (SET). SET is considered to decrease the possibility of twin pregnancy to that possibility of the common population, about 1%–3% of cases. However, it seems that only one out of five medical infertility centers have established SET as their standard policy in infertile couples. It is, though, encouraging that according to recent publications the number of medical centers following this policy is increasing, but it leaves a lot to be desired before achieving the intended standards according to medical societies' recommendations [7].

Apart from multiple pregnancy, the main other risks increased in an ART pregnancy and thereafter in a surrogacy pregnancy are hypertensive disorders of pregnancy, namely hypertension, preeclampsia, and eclampsia, risk of preterm labor and thereafter prematurity, gestational diabetes, intrauterine growth restriction, as well as other minor ones such as urinary infections and stress incontinence.

Finally, we should also highlight the dimension of psychological impact that surrogacy pregnancy may have. The obligation of the gestational carrier to relinquish the child may actually lead to emotional disorders, which is rather reasonable to a point; however, it is by definition the actual and final goal of a surrogacy pregnancy. It seems, however, that even if emotional problems may be presented, they are quickly and easily overpassed in the consequent weeks after giving the child to the intended parents [8].

Ethical, religious, and financial concerns about the surrogacy procedure

The surrogacy procedure represents a modern medical reality, and it is thereafter reasonable that major ethical, religious, and also financial issues may be raised as always characterize the apparition of modern medical methods.

On an ethical basis, there have been many different ethical concerns expressed, but the predominant one actually concerns the fact that surrogacy procedure is performed 90% of the time in a context where wealthy people are actually considered to buy and potentially take advantage of their economic status over people that are necessitated to undergo a pregnancy not intended for them as a manner to earn their living. Terms such as "exploitation, commodification, and coercion" may be found in the literature [9,10], when

attempting to characterize the interaction between intended parents and gestational carrier. However, a reasonable argument against this theory is that every human being has the legal right to decide what is for their advantage and may freely participate and sign a legal contract, taking full responsibility of the tasks they accept.

The second major ethical aspect raised is associated with the parenthood status, especially of mothers, for women involved. The future relationship between the gestational carrier and the intended mother may be an issue of discussion, while models enrolling both women in the role of mothers have actually been discussed, without yet having great implication. Finally, another ethical issue concerns the child's rights to be aware of his or her genetic parents and gestational carrier as well. On the one hand, each human being could actually have the right to be aware of the person that carried them during pregnancy. On the other hand, since by law and definition this carrier has absolutely no right to the child, it is controversial whether this child's right is actually conflicted by the contract initially signed between two parties.

Religious concerns around surrogacy exist and are rather strongly discouraging for intended parents. The Catholic Church firmly states that every technique, including ART techniques, that causes a dissociation between the husband and the wife is not acceptable. However, this also concerns not only surrogacy pregnancies, but also cases of egg donation. Therefore, this is not an issue against surrogacy indeed, but a general issue around compatibility of ART techniques with Catholic Church statements and teachings [11]. The Orthodox Church, which is the predominant church in Greece, where actually surrogacy is permitted by law, claims similar things, but the issue of additional embryos produced by IVF procedure is also an issue firmly posed in the conversation around surrogacy. Islam has a similar approach, but implications on people finally choosing surrogacy are considered much more severe compared with other religious. Finally, it is only the Jewish who have accepted surrogacy only in the context of full surrogacy in which embryos are produced by the genetic cells of intended parents and the gestational carrier is only used because of her uterus and not additionally as a donor of genetic cells [12,13].

It is estimated that about 20,000−25,000 women search for cross-border surrogacy services on an annual basis. Countries with liberal policies may be considered as those receiving the main proportion of intended parents. Among these are the United States, Israel, Mexico, and Barbados. European couples have also preferred the United States for years for similar reasons. However countries such as Greece with a liberal policy and developed ART services may also represent a modern destination. On the contrary, there is also a significant number of people from Western developed countries that address to Asian countries through reproductive tourism programs [14].

Finally, the scale of economics around surrogacy may not be adequately estimated. The United Nations in July 2012 estimated an amount of >$400 million per year [15]. In the last 20 years, gestational carrier cycles have increased by four times, while it is characteristic that two out of three clinics in the United States offer this service according to their national registry [16].

Psychological impact with surrogacy

Surrogacy often represents the last chance of childbirth for a particular category of couples, which are besides characterized by specific medical issues resulting in unsolved infertility. This on its own poses the indication of a multidisciplinary approach for the couple, so the gestational carrier is not only within the context of a purely medical basis, but also within the context of proper psychological support, both for parents and gestational carrier.

ASRM guidelines for surrogacy clearly indicate that the physician accountable to the couple should strongly advise in favor of psychological education and counseling by a properly trained and experienced physician [17].

The basic aspect that should be always taken into account in surrogacy is the underlying relationship between intended parents and gestational carrier. Contrary to other ART methods, such as egg donorship, where parents do not actually get in touch and will be unaware of the origin of eggs, in surrogacy methods, the gestational carrier is a person that the parents are aware of, and apart from that, they have to cooperate with her on a physical and mental basis to achieve the common goal.

A nice study concerning the relationship of parents with gestational carrier was performed by Javda et al. [18]. This was a study based on semistructured interviews with intended fathers, parents, and children on four different time points over a 10-year period. Indeed, parents were first interviewed when the child was 1 year old. Collected data intended to answer the question of relationship of intended parents with surrogate, even after pregnancy delivery. Of the 42 subject families, 23 used surrogates unrelated genetically to the child. Medical conditions that lead to surrogacy were years of failed IVF cycles (43%) or lack of uterus of intended mother (38%). Nineteen were so-called traditional surrogates. Twenty-nine (69%) of the couples had not known the surrogate before arrangement, and 13 (31%) worked with a family member or friend.

According to this study's result, first of all, the majority of participants reported harmonious relationships with the surrogate mother. However, it was mentioned that frequency of contact was decreased over time, particularly in case of previously unknown gestation carrier. It was interesting that approximately 90% of children at the age of 10 that had been informed of their history had a good understanding of the condition as well as a good relationship with the gestational carrier.

As a conclusion, surrogacy families may actually have a good relationship with the surrogate mother over time. Furthermore, children do also appear to have good relationship with the gestational carrier and be well tolerant of the situation [18,19]. However, this needs intensive psychological work so the situation becomes easily accepted from every person participating in this context.

Conclusion

Surrogacy and thereafter gestational carriers represent a modern medical reality in the context of modern IVF. Main clinical indications are related with absence of uterus, medical contraindication of pregnancy, as well as RPL. Special consideration should be made to properly select gestational carrier and establish at the primary time the right and essential contract on a legal, medical, and moral basis. Results of pregnancies of gestational carriers are relatively satisfying, while the economic aspects of this policy are an issue of extreme importance. To summarize, surrogacy is a modern medical reality that gives a solution for couples with no other alternatives. Continuous education, training, and research is therefore essential to broaden our aspects in the issue.

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CHAPTER

36

Female and male fertility preservation in oncology

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Introduction

Cancer is one of the leading causes of death around the world, accounting approximatively for 10 million deaths in 2020 [1]. Cancer burden is expected to surpass 25 million new cases and 15 million deaths by 2040, due to the aging and growing of the population worldwide [2]. Regardless of sex, lung cancer is the most commonly diagnosed and lethal malignancy (18.4% of deaths), followed by female breast cancer, colorectal cancer, and prostate cancer in terms of incidence, while colorectal cancer, stomach cancer, liver cancer, and female breast cancer ranked after lung disease for number of deaths [3]. Of course the incidence of cancer is related to the human developing index (HDI) values given a wide range when comparing high versus low HDI countries, with respectively 253 and 123 cases every 100,000 habitants [4]. As an example, female breast cancer was the second disease worldwide in 2012, but most cases were diagnosed in high HDI countries, and even though not all of them would experience motherhood, most of them may have the desire to have the opportunity to think about maternity [5]. It is well recognized that fertility-related issues typically pertain to high HDI countries. In fact, cancer dimension in low HDI nations is unfortunately underestimated, and the technologies and facilities for early diagnosis and oncological treatment are not sufficient. On the counter side, in HDI countries, the major area of interest for cancer survivor regards recurrence, secondary malignancies, and the long-term consequences that can impact the quality of life. Recent advances in oncology have led to an increase in life expectancy for many types of cancers and consequently to a potential increase in the number of people requiring the maintenance of their reproductive capacity [6,7]. In this optic, the chance of having children and fertility-sparing programs globally qualify as an emerging need. As a matter of fact, in modern society, most women delay conception, resulting in an increased risk of developing a malignancy diagnosis before the fulfilment of childbearing [8]. Nonetheless, the preservation of gonadal function is a priority for the long-term health of male and female cancer survivors, even though male fertility preservation is easier when compared to fertility-sparing methods for females. In fact, in women there is a greater difficulty in obtaining and preserving the gametes and afterward establishing a viable pregnancy. Lastly, the problem of cancer and fertility raises also ethical and psychological issues that need to be addressed well in advance to ensure a successful approach. Since a strong international consensus statement should be still produced, the need for a dedicated multidisciplinary approach is mandatory to provide a clinical range of treatment options to women with cancer and a fertility issue.

Multidisciplinary fertility sparing team

Fertility preservation requires a multidisciplinary approach to calibrate the effect of different treatments, to predict the impact on patient fertility and evaluate the feasibility of fertility preservation [9].

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The multidisciplinary team aims to provide the best oncological, fertility, and obstetrics opportunities. Nowadays, some cancer patients diagnosed in reproductive age could follow a fertility preservation program, but in an unacceptable percentage of the cases the access to this type of service is limited or not available. Specialized figures need to be included in the multidisciplinary team able to promptly work together [10]. A collaborative approach, open communication, defined respective roles, and sharing of knowledge are crucial factors to achieve an effective fertility preservation program [11].

Gynecologist oncologists, gynecologists specialized in assisted reproductive technique (ART), and reproductive endocrinologist are permanent members of the multidisciplinary team. In consideration of the disease site, different physicians should be enrolled to propose the optimal treatment and evaluate the possibility of fertility preservation treatment (e.g., hematologist, breast cancer surgeon) [12,13]. Oncologists play a pivotal role in the tailored management. In fact, the indication for a medical treatment with cytotoxic drugs is widespread, and they are in charge to evaluate the possibility of becoming infertile after a potential fertility-harmful treatment during reproductive and pediatric age [11]. The ovaries are the most sensitive tissue in the human body to radiation damage, and the possible impairment on fertility is related to radiation dose and age of the patient [14]. All these aspects and the cumulative effect of different treatments should be discussed also with radiation therapy specialists.

A fundamental part of the multidisciplinary fertility sparing team is a gyneco-pathologist to assess the quality of ovarian and testicular tissue obtained for banking. The fertility preservation program should include also a biologist and laboratory to guarantee a positive experience in tissue banking, deep knowledge of technical effectiveness, and limits of the procedures [15]. The multidisciplinary team has the duty to manage and guarantee an open communicative approach, satisfying knowledge needs, and provide emotional and psychological support. To this purpose, specialized psychologists, mental health care providers, and social workers are significant members of a team that shares a holistic patient care service [16]. Furthermore, discussion should point out the legal and ethical issue of a fertility preservation program.

Counseling and psychological support

The experience of a gynecologic cancer and its related treatment with surgery, chemotherapy, or radiation deeply affects the psychological aspect of patients and represents a source of distress among women [17]. Infertility influences levels of quality of life and increases incidence of depression and anxiety through a distorted female identity and altered feelings about sexuality [18]. The literature demonstrates a benefit for women from a psychotherapeutic setting and the availability of a figure of support.

A face-to-face appointment should be proposed to clarify the complex interaction between fertility and cancer. Fertility preservation consultation is often the only occasion for the patient to discuss the available opportunities and to obtain informative sources as websites and brochures. The physician must remember that is impossible to know how important fertility preservation is unless they ask; in fact a patient could consider secondary their desire for a child or simply not be aware about the possibility of loss of fertility with cancer treatment. Understand the factors that each patient feels challenging is crucial to avoid decisional conflicts and reduce related psychological burden [19]. Before counseling the healthcare providers have to consider potential barriers to clear understanding of the topic: social status, any language barriers, financial concerns, and cultural and ethical backgrounds. Of note, the financial concern and unanswered questions are the main factors influencing final decision on a fertility-sparing program [20].

Eligibility for fertility sparing

A multidisciplinary team provides the adequate knowledge to analyze a specific clinical situation satisfying eligibility criteria and tailoring personalized management [10].

First of all, experts define the stage of disease and identify the optimal therapeutic options. It is important to predict the temporary and definitive impact on fertility, the risk of premature ovarian failure, and anticipated menopause in women.

Age of the patient, active presence of a male partner, and general health status need to be check to include patients able to sustain fertility preservation procedures. Safety of future pregnancies must be discussed taking in account the type of cancer and treatment [21,22]. Feasibility of a conservative treatment and disposable controlled clinical trials on fertility-sparing approach should be checked. It is fundamental to propose fertility-sparing treatment only preserving oncological safety and not delaying cancer treatment [23,24].

Cancer treatments that affect female fertility

Fertility preservation treatments have become an integral part of the counseling for women of reproductive

age and pediatric age who are preparing to undergo anticancer therapies. The effects that cancer treatments can have on the loss or impairment of fertility in patients are known, so patients must be informed of the possible consequences on reproductive life and the opportunities for preserving it, if possible. Fertility may be compromised following chemotherapy, radiation therapy, or surgical oncological treatment [25]. The effect of the most common anticancer agents in clinical practice and the potential risk of permanent amenorrhea are summarized in Table 36.1 [11].

In women, fertility can be compromised by any treatment that involves the reduction of antral follicle count, or that represents an obstacle to reproduction with direct or indirect organ damage to the ovaries, uterus, or salpinges, or by inducing an alteration of the endocrine system that regulates ovulation cycles and hormonal environment. Premature ovarian insufficiency (POI) is defined as oligo/amenorrhea for ≥4 months and follicle-stimulating hormone (FSH) levels of >25 IU/L on at least two dosages, 4 weeks apart, before the age of 40 years. Unfortunately, the reappearance of regular cyclic menstrual activity does not mean in every case the restoration of fertility. In fact, the analysis of the ovarian reserve with hormonal dosages and ultrasound evaluation is an essential component for the definition of the risk of infertility, and it should be performed both before and after anticancer treatment. Furthermore, exposure to anticancer treatments may not lead to the complete exhaustion of reproductive potential but may induce a decline in fertility such as to shorten its duration and, overall, may lead to an earlier onset of menopause. A nomogram was developed that helps in the prediction of ovarian activity after chemotherapy treatment in patients undergoing chemotherapy for breast cancer, based on the patient's age and anti-Müllerian hormone (AMH) value at the start of chemotherapy. These elements can be predictive of ovarian activity, expressed as the reappearance of the menstrual cycle 1 year after the end of the anticancer treatment: by following an appropriate validation process, the nomogram could be used to discern patients who should be considered high priority for fertility preservation counseling and procedures [26]. As for men, the chemotherapy treatment that is more aggressive for the germinal epithelium for women is represented by alkylating agents (ifosfamide, nitrosourea, melphalan, busulphan, procarbazine, carmustine, lomustine, chlorambucil), with probably a dose-dependent effect [27–29], and the platinoids cisplatin and carboplatin [30].

TABLE 36.1 Common effects of anticancer agents.

Degree of risk	Risk of permanent amenorrhea	Regimen treatment
High	80%	HSC-TX with cyclophosphamide, cyclophosphamide, busulfan, melphalan, or TBI
		EBRT >6 Gy to a field including both ovaries
		6 cycles of CMF, CEF, CAF, TAC (≥40 years old)
		6–8 cycles of BEACOPP escalated (≥30 years old)
		Procarbazine
		Chlorambucil
Intermediate	40%–60%	6–8 cycles of BEACOPP escalated (<30 years old)
		6 cycles of CMF, CEF, CAF, TAC (30–39 years old)
		4 cycles of AC (≥40 years old)
		4 cycles of AC or EC →taxanes
		6 cycles of CHOP (≥35 years old)
		FOLFOX (≥40 years old)
	30%	Monoclonal antibody: bevacizumab
Low	<20%	ABVD (≥32 years old)
		FOLFOX (<40 years old)
		2 cycles of BEACOPP escalated
		Multiagent chemotherapy for osteosarcoma (doxorubicin, cisplatin, methotrexate, ifosfamide) < 35 years old
		Multiagent chemotherapy for Ewing sarcoma (doxoribicin, vincristine, dactinomycin, cyclophosphamide, ifosfamide, etoposide) < 35 years old
		Antimetabolites and vincaalkaloids
		6 cycles of CHOP (<35 years old)
		CVP

Continued

Treatment for breast cancer

The risk of POI in women who undergo chemotherapy during the fertile period is conditioned by

TABLE 36.1 Common effects of anticancer agents.—cont'd

Degree of risk	Risk of permanent amenorrhea	Regimen treatment
		AML therapy (anthracycline/cytarabine)
		ALL therapy (multiagent)
		6 cycles of CMF, CEF, CAF, TAC (≤30 years old)
		4 cycles of AC (≤40 years old)
		Bevacizumab
Very low or absent		ABVD (<32 years old)
		Methotrexate
		Fluorouracil
		Vincristine
		Tamoxifen
Unknown		Monoclonal antibodies: trastuzumab, cetuximab
		Tyrosine kinase inhibitors: erlotinib, imatinib
		Irinotecan
		Platinum and taxane-based chemotherapy
		Immunotherapy

ABVD, doxorubicin/bleomycin/vinblastine/dacarbazine; *AC*, doxorubicin/cyclophosphamide; *ALL*, acute lymphaticleukaemia; *AML*, acute myeloidleukaemia; *BEACOPP*, doxorubicin/bleomycin/vincristine/etoposide/cyclophosphamide/procarbazine; *CAF*, cyclophosphamide/doxorubicin/fluorouracil; *CEF*, cyclophosphamide/epirubicin/fluorouracil; *CHOP*, cyclophosphamide/doxorubicin/vincristine/prednisone; *CMF*, cyclophosphamide/methotrexate/fluorouracil; *CVP*, cyclophosphamide/vincristine/prednisone; *EBRT*, external beam radiation therapy; *EC*, epirubicin/cyclophosphamide; *HSC-TX*, hematopoietic stem cell transplantation; *MTX*, methotrexate; *TAC*, docetaxel/doxorubicin/cyclophosphamide; *TBI*, total body irradiation.
Modified by Lee SJ, Schover LR, Partridge AH, et al. American Society of Clinical Oncology recommendations on fertility preservation in cancer patients. J Clin Oncol. 2006;24(18):2917–2931. doi:10.1200/JCO.2006.06.5888.

many factors, which are individual, genetic, and based on ovarian reserve prior to the start of treatment and the age of the woman. The risk of amenorrhea after exposure to six cycles of CMF (cyclophosphamide/methotrexate/fluorouracil) is 33% for patients under the age of 40, but 81% for older ages. The following chemotherapy regimens are associated with a lower incidence of posttreatment amenorrhea: doxorubicin/cyclophosphamide (AC), doxorubicin/adriamycin and cyclophosphamide followed by paclitaxel (ACT), 5-FU/doxorubicin/cyclophosphamide (FAC) and FACT. With these schemes, the risk of amenorrhea is however related to age: it is 10%–20% under 30 years of age and 13%–68% for older ages [31,32]. Adjuvant endocrine therapy does not appear to have a direct gonadotoxic effect, but the prolongation of this treatment is associated with a negative effect on fertility due to the natural decline of the ovarian reserve that occurs during the treatment over the years. Therefore, discontinuation of endocrine therapy after 2–3 years for the search for pregnancy may be considered, and possibly resumed later [33,34]. Treatment with trastuzumab appears to be safe from a gonadotoxic point of view, but solid data on the effect of targeted treatment on fertility are lacking [35].

Treatment for Hodgkin lymphoma

In many countries, early stages of Hodgkin lymphoma are treated with ABVD, intermediate stages with 2× BEACOPP escalated plus 2× ABVD and advanced stages with 4–6x BEACOPP escalated. In other countries, ABVD remains the standard treatment also for advanced stages. BEACOPP escalated is associated with a high risk of POI: after eight cycles of escalated BEACOPP the frequency of amenorrhea is 51.4% for women of less than 30 years of age and 95% for older ages. The risk is low for the ABVD scheme [36–38]. Radiation therapy represents a further gonadal damage, which can irreversibly compromise reproductive function if the pelvis is included in the treatment field [39]. A fertility preservation counseling is indicated for women of age <40 years undergoing a chemotherapy of high risk for gonadal dysfunction (e.g., six-cycle BEA-COPP), but also of intermediate and low risk. The synchronous treatment with gonadotropin-releasing hormone (GnRH) agonists is recommended for its beneficial effects on the reduction of gonadal damage, compared to few side effects. In specialized centers, the removal of ovarian cortical tissue is an experimental treatment that plays a role in preserving fertility in these patients, since the risk of lymph node metastases from Hodgkin lymphoma is negligible (after cancer staging and an anesthesiology evaluation) [40–42]. In summary, the combination of several treatments aimed at preserving fertility is feasible in young patients with Hodgkin lymphoma for whom good prognostic factors have been identified and if the time available allows the application of such treatments before starting chemotherapy.

Ovarian and uterine exposure to radiotherapy

Exposure of the ovaries to a dose of 5–20 Gy is sufficient to induce permanent gonadal dysfunction, regardless of age. However, with advancing age, a greater loss of ovarian reserve is observed due to exposure to irradiation at lower density: at 40 years of age, most women report irreversible ovarian damage with doses of 5–6 Gy [43–45]. Dose fractionation seems to have a

less detrimental effect with a greater probability of recovery of ovarian function [46]. It is advisable to carry out a preliminary assessment of the ovarian reserve to estimate the risk of gonadal damage before radiotherapy, since the ovarian reserve is conditioned by variable interindividual factors, and age is not the only element to take in account: others are BMI, smoking, exposure to environmental pollutants, genetic factors, previous surgeries, endometriosis.

Ovarian reserve evaluation tests are dosage of AMH, FSH, estradiol, inhibin B, luteinizing hormone (LH), progesterone, and antral follicle count [23,47–49]. This evaluation should also be repeated after the completion of the therapy to define the probability of conception and possibly support the woman in the reproductive process: the reappearance of menstrual cycles in fact does not necessarily mean the restoration of fertility. Table 36.2 describes the clinical effect of exposure to specific doses of radiotherapy on the ovarian pool of follicles [50,51].

In addition to the ovaries, the uterus can also be damaged following radiotherapy: exposure represents a risk factor for fertility, for early and late miscarriage, for preterm birth, or for intrauterine growth restriction (IUGR) [44,51]. It is calculated that after total body irradiation (TBI) with a median of 10 Gy, pregnant women may deliver a fetus with low birth weight (less than 2500 g) in 30% of cases, compared with the 10% of control cases [52]. Radiotherapy exposure during childhood results in a more detrimental effect on uterine function compared with exposure in adulthood [53], as seen in Table 36.3. For instance, after a radiotherapy of 25 Gy or more during childhood a pregnancy is not advisable, and the upper limit in adulthood is 45 Gy. In the event of pregnancy, the gestation should be monitored by an experienced obstetric team.

TABLE 36.2 Clinical effects of radiotherapy on ovarian pool of follicles.

Radio-toxicity on ovaries	Radiotherapy exposure (dose in Gy)
No relevant effects (any age)	≤0.6
No relevant effects if < 40 years old	≤1.5
Depletion of 50% of follicle pool	2.0
60% of risk of ovarian insufficiency (age 15–40 years)	2.5–5.0
Effective sterilizing dose at birth	20.3
Effective sterilizing dose at 10 years	18.4
Effective sterilizing dose at 20 years	16.5
Effective sterilizing dose at 30 years	14.3
Effective sterilizing dose at 40 years	6.0

Effective sterilizing dose: the radiotherapy dose that reduces the ovarian follicle pool to less than 1000 follicles in 97.5% of women.
Modified by Irtan S, Orbach D, Helfre S, Sarnacki S. Ovarian transposition in prepubescent and adolescent girls with cancer. Lancet Oncol. 2013;14(13):e601–e608. doi: 10.1016/S1470-2045(13)70288-2 and Wallace WHB, Thomson AB, Kelsey TW. The radiosensitivity of the human oocyte. Hum Reprod. 2003;18(1):117–121. doi:10.1093/humrep/deg016.

TABLE 36.3 Radiotherapy effect on the uterus.

Radio-toxicity on uterus	Radiotherapy exposure (dose in Gy)
Significant risk of miscarriage, premature delivery, low birth weight (<2500 g), IUGR	12 (exposure in adulthood)
Pregnancy not advisable	>25 (exposure in childhood)
Pregnancy not advisable	>45 (exposure in adulthood)

Modified by Teh WT, Stern C, Chander S, Hickey M. The impact of uterine radiation on subsequent fertility and pregnancy outcomes. Biomed Res Int. 2014;2014. doi:10.1155/2014/482968.

Cancer treatments that affect male fertility

Over the years the number of cancer survivors has increased due to progress in diagnosis and treatment and thanks to continuous innovations. In Italy every day are diagnosed about 30 new cases of tumor in patients under 40 years old. Most frequent tumors in this age range in males are represented by testis, melanoma, hematological malignancies (leukemia and lymphoma), thyroid, and colorectal cancer. Chemotherapy, radiotherapy, and biologic therapies significantly improved survival in patients affected from tumor but can generate side effects. Infertility could be one of them, and this is a problem of growing importance considering improved prognosis and life expectancy in young oncological patients [54]. Infertility is defined as not being able to conceive after 1 year (or longer) of unprotected intercourse, but not only cancer therapy can damage fertility; in fact other nonmalignant conditions in males can also negatively impact fertility. Patients affected by autoimmune disorders that have to be admitted to therapy with alchilant agents, immunosuppressants, nonsteroidal antiinflammatory drugs, and salicylate or urologic pathology complicated with ejaculations problems should have trouble in conceiving [55]. Tumor may influence reproductive function determining direct and indirect effects on spermatogenesis. Primary effects induced by neoplasm that can impact infertility are a systemic inflammatory status with altered cytokines production and a consequent increase in temperature that determine alterations in sperm

quality [56], and stress and anxiety that can also determine variations in hormonal secretion of LH and testosterone, involved in hormonal deficiency. Secondary effects are due to gonadotoxic cancer treatments: chemotherapy, radiation, and surgery that can lead to temporary or permanent sterility. This is in function of the type of malignancies, dose intensity, duration and type of treatment, age of patient, anatomic site, and previous treatments. Measurable effects secondary to antineoplastic treatments are represented by reductions of sperm count and alterations in motility, morphology, and DNA integrity. Many studies have investigated the preexistent reduced quality of seminal sperm in patients affected by leukemia, lymphoma, and testicular cancer, which may be due to an alliterated secretion of proinflammatory interleukins in circle and to an autoimmune response [57]. Testicular cells, especially rapidly dividing germ cells, are highly sensitive to irradiation- and chemotherapy-induced damage; consequently spermatogenesis can be disrupted. The potential for eventual recovery will depend on survival of spermatogonial stem cells within the testis [58]. Three main types of cells develop in the testis: germ cells, Sertoli cells that support the developing germ cells, and Leydig cells that produce testosterone. Male germ cells together with Sertoli cells are the main actors of spermatogenesis, which occurs in the seminiferous tubules, and the process of forming spermatozoa approximately takes 70 days. LH and FSH regulate testicular function. LH stimulates the Leydig cells in the testes to make testosterone that stimulates spermatogenesis in the seminiferous tubules. FSH incites Sertoli cells to stimulate spermatogenesis. Testicular damage can affect germ cells or Sertoli and Leydig cells, determining a depletion of the proliferating germ cell pool. Adults testes actively produce spermatozoa and are therefore very susceptible to such damage, so testes are vulnerable before, during, and after puberty [59]. Obviously, combination treatment with radiotherapy and chemotherapy will induce more gonadotoxicity than either modality alone.

Chemotherapy side effects

Gonadal toxicity caused by chemotherapy is due to the fact that it targets rapidly proliferating cells. Mutations occurring early in stem cell spermatogonia cause permanent damage in spermatogenesis respect to mutations in later stage spermatogonia, which leads to transient disruption. Chemotherapy-induced damage is a function of the agent administrated and on the cumulative dose received. Because of their rapid turnover, germ cells are much more sensitive to damage by chemotherapy than the Leydig cells (more resistant because of their lower turnover rate) and can lead to an effect that in most cases is transitory but in some cases is irreversible and can determine severe azoospermia. Chemotherapeutic agents in fact can penetrate Sertoli cells and damage gonocytes, but Leydig cells may also incur damage following chemotherapy, resulting in alliterate secretions in LH and FSH and subsequent hypogonadism [60]. Spermatogenesis is very sensitive to damage by alkylating agents such as cyclophosphamide, ifosfamide, cisplatin, chlorambucil, mechlorethamine, procarbazine, and busulfan. This damage tends to occur in a dose-dependent manner and can be additive when multiple agents are used in a treatment regimen. Up to 24% of fertile patients treated with chemotherapy will show persistent azoospermia or severe oligozoospermia [61]. Target therapy and immunotherapy drugs can affect cancer cells differently from standard chemo drugs, but very little is known about consequences on fertility or problems during pregnancy. Table 36.4, detailing the risks of infertility after chemotherapy exposure during childhood or adulthood, can help clinicians to better refer patients for fertility preservation options.

Radiation therapy side effects

Radiation therapy is one of the most common treatments for many cancers in men of reproductive age. Actively dividing cells show higher radio sensitivity, which is the reason why the testis is one of the most radiosensitive tissues, so a very low dose of radiation can cause significant impairment of its function. Damage can be caused during direct irradiation of the testis or from scattered radiation in the treatment of adjacent tissues (prostate cancer, rectal cancer, bladder cancer, anal canal carcinoma), and the effect of radiation exposure depends on dose, fractionation schedule, and the field of irradiation. Gonad shielding can be used during radiation therapy but is only possible with selected radiation fields and anatomy. Testicular irradiation can impair spermatogenesis by a direct loss of germ cells because of a damage to spermatogenesis supporting Sertoli cells or determining a disfunction in testosterone producing Leydig cells [62]. When the testis is exposed to radiation, sperm count begins to decline, leading to transient or permanent infertility depending on the dose received, and function of the testes may be significantly impaired by very low doses of radiation. Radiation therapies begin to affect spermatogenesis gradually from 0.1 to 1.2 Gy and can cause a permanent gonadal damage at 4 Gy [63]. The Leydig cells of the testis are remarkably more radioresistant than germinal epithelium and are only injured by high therapeutic radiation doses. They are more sensitive before puberty onset than adult age, and their function is usually preserved up to 20 Gy in prepubertal boys and 30 Gy in

TABLE 36.4 Risk of male infertility after chemotherapy based on age at exposure.

Agents (cumulative dose for effect)	Class of anticancer drugs	Risk on fertility after adulthood exposure	Risk on fertility after childhood exposure
Chlorambucil (1.4 g/m^2)	Alkylating agents	High (prolonged azoospermia)	High
Cyclophosphamide 19 g/m^2)			High (7.5 g/m^2)
Procarbazine (4 g/m^2)			High
Melphalan (140 mg/m^2)			High
Cisplatin (500 mg/m^2)			High
Busulfan (600 mg/kg)	Alkylating agents	Moderate (likelihood of azoospermia but always given with other sterilizing agents)	High
Ifosfamide (42 g/m^2)			High (4 g/m^2)
Carmustin (300 mg/m^2)	Alkylating agents		Low
Dactinomycin	DNA intercalating		Low
Carboplatin (2 g/m^2)	Alkylating agents	Low	Moderate
Thiotepa (400 mg/m^2)			
Doxorubicin (770 mg/m^2)	DNA intercalating		Moderate
Cytarabine (1 g/m^2)	Antimetabolite		Moderate
Vinblastine (50 g/m^2)	Spindle poison		Low
Vincristine (8 g/m^2)			
Dacarbazine	Alkylating agents	Temporary reduction in sperm count	Moderate
Daunorubicin	DNA intercalating		Moderate
Mitoxantrone	DNA intercalating		Moderate
Bleomycin	DNA strand breaks inducer		Low
Etoposide	Topoisomerase II inhibitor		Low
Fludarabine	Antimetabolite		Unknown
Fluoracil			Low
Mercaptopurine			Low
Methotrexate			Low
Thioguanine			Unknown

Modified by Delessard, et al. Exposure to chemotherapy during childhood or adulthood and consequences on spermatogenesis and male fertility. Int J Mol Sci 2020;21: 1454–76.

sexually mature men. Doses of more than 1.2 Gy are known to increase the recovery time of spermatogenesis [64], and reductions in sperm count following damage to the testes by radiation doses of up to 3 Gy have been noted after 60–70 days. According to a survey, the recovery of spermatogenesis may start at least 9 years after treatment [61]. Irradiation increases sperm DNA fragmentation that may continue for up to 2 years after treatment. Therefore, it affects fertilization rates even after spermatogenesis recovery [65].The most severe postradiation sperm cell damage occurs between 4 and 6 months after radiotherapy completion [14], and return of fertility is a result of proliferation and regeneration of stem cells that have survived. Cranial radiation therapy can also interfere with fertility. Specifically, radiation to the hypothalamic-pituitary axis causes impaired spermatogenesis and hormone production. Gonadotropin deficiency can be the result of a reduced GnRH secretion from the hypothalamus and consequent decreased release of LH and FSH, or from a direct damage to the pituitary (Table 36.5).

Surgery effects

As expected, gonadal surgery can interfere with the production of sperm and reproductive hormones, negatively impacting fertility. Unilateral radical orchiectomy with inguinal approach is the standard treatment for testis tumor, but after this half of patients present reduction in sperm concentration during the first few months, and 10% of patients with preoperative normal sperm counts will become azoospermic [66]. Partial orchiectomy has become a favored option in selected patients as a method to preserve hormonal and sperm cell production. In addition, retroperitoneal lymph node dissection, an important component of the multimodal treatment of this cancer, can generate a potential damage

TABLE 36.5 Clinical conditions describing male fertility complications after irradiation.

Condition	Degree of exposure	Complication
Testis direct irradiation Seminoma (stage I) Acute lymphoblastic leukemia (testicular relapse) Soft tissue sarcoma (deep and high grade)	High (>3 Gy)	Permanent infertility
Testis scattered irradiation Prostate cancer Rectal cancer Anal canal cancer Bladder cancer Testicular cancer Hodgkin lymphoma	Moderate (1.5–3 Gy)	Permanent infertility
Pituitary gland cancer	High (>24 Gy)	Hypothalamic/pituitary dysfunction
Acute leukemia (prophylactic cranial irradiation)	Moderate (<24 Gy)	Hypothalamic/pituitary dysfunction

Modified by De Felice F, Marchetti C, et al. Radiation effects on male fertility. Andrology 2019;7:2–7.

in fertility because it can lead to onset of retrograde ejaculation and ejaculatory failure. Fortunately the incidence of these complications has been mitigated with the advent of modifications in surgery with nerve-sparing techniques [67]. Males can also suffer from erectile dysfunction and the inability to achieve or maintain an erection following pelvic surgery for colorectal cancer. This occurred in 16% of patients after surgery for rectal cancer. Also, surgery on the glands of the hypothalamic-pituitary axis can impact fertility. In fact, surgery on hypothalamus and pituitary can interfere with the gonadotropin-releasing hormone area and with the gonadotropin-producing area.

Techniques for fertility preservation of female patients

Oocyte and embryo cryopreservation

Oocyte cryopreservation and embryo cryopreservation are considered the standard techniques for fertility preservation in female cancer patients [10,22,68]. Oocyte cryopreservation requires needle aspiration of the follicular contents performed at the completion of a follicular stimulation cycle with subsequent cryopreservation of the mature oocytes. When the woman will have a desire for pregnancy, the oocyte thawing and fertilization (ICSI) is carried out with subsequent embryo transfer. In embryo cryopreservation, on the other hand, the fertilization of the oocytes takes place the same day of the pick-up; then the embryos are frozen, and the frozen-thawed embryo transfer is performed when the woman will have the desire for pregnancy. This second technique requires the presence of a partner. There are countries (e.g., Italy) where embryo cryopreservation is not allowed for this purpose, and the only method available is oocyte cryopreservation. Both procedures require some time to induce controlled ovarian stimulation (COS) before anticancer therapy is initiated. Treatment with gonadotropins takes about 2 weeks to complete, and an assessment of its feasibility must be performed before programming, considering the possible postponement of the start of chemotherapy and the theoretical negative impact that stimulation could have in cases of hormone-sensitive tumors [69,70]. For some illnesses, a delay of 2 weeks prior to anticancer therapy is not possible (e.g., acute leukemia). Ovarian stimulation techniques have adapted to the need for a timely start of gonadotropin treatment, and the application of random stimulation meets this requirement [71–76]. It is well established that random start protocols, inducing luteolysis, can in fact be started at any phase of the cycle, without the need to wait for the follicular phase of a spontaneous cycle. However, the completion of the stimulation cycle up to pick-up and cryopreservation requires approximately 2 weeks of treatment. Every effort must be made to reduce the risk of complications related to cryopreservation procedures, particularly the risk of inducing ovarian hyperstimulation syndrome (OHSS). These patients in fact are often at a young age with good ovarian reserve indices, so the risk is not to be neglected at all. The induction of final trigger for oocyte maturation with GnRH analogs instead of hCG is a method that reduces the incidence of OHSS. During COS the level of estrogen increases: this aspect can theoretically represent a risk in women with hormone-sensitive cancer (estrogen receptor and progesteron

receptor positive breast cancer), even if it is a short-term exposure. For this reason, protocols have been developed with the association of letrozole [77] or tamoxifen with COS [78]. To reduce the increasing estrogen concentrations during ovarian stimulation, the addition letrozole 5 mg/day (2.5 mg BID) is recommended from the second to third day of the menstrual cycle, starting with stimulation, and for the duration of the therapy with gonadotropins, up to the day before triggering; thereafter, the intake of letrozole is resumed after the oocyte pick-up and until estradiol values lower than 50 pg/mL are reached. The studies have not shown increased malformation rates in children after low dose stimulation with letrozole. In a series of relatively small studies, no differences on the quality of frozen oocytes and embryos are detected, and pregnancy rates have been similar to those expected in noncancer women undergoing *in vitro* fertilization (IVF) [21,79,80]. To date, data available from scientific studies show that even in hormone-sensitive tumors the COS procedure associated with the intake of letrozole is a safe and feasible technique for patients with hormone-sensitive breast cancer. Similarly, exposure to two consecutive cycles of COS and oocyte pick-up was tested, aimed at increasing oocyte recovery, with reassuring data on safety; on average, the double stimulation took 33 days to complete. The oocyte or embryo cryopreservation technique should therefore be proposed to all women for whom it is feasible to postpone the initiation of anticancer treatment for 2–3 weeks.

Two methods of cryopreservation for oocytes and embryos are available: slow freezing and vitrification [81]. To date, the vitrification technique is associated with better results, which in many centers is comparable to fresh cycles in terms of pregnancy rate [82,83]. However, whatever the technique adopted in the center, the success rates are operator dependent, and differences of outcomes are identified mainly based on the patient's age at egg pick-up and the number of mature cryopreserved oocytes [84]. Physicians should inform women about the success rates of embryo and oocyte cryopreservation procedures: a live birth rate (LBR) ranging from 20% to 45% is reported in cancer patients with embryo cryopreservation and frozen and thawed embryo transfer [85]; with cryopreservation of oocytes, LBR is about 50% in women under the age of 35% and 23% in women over 36 years of age [86]. It has been calculated that the number of oocytes needed to get pregnant (live birth) is 12 for women aged 30–36, and 30 for women aged 36–39 [87]. Women over the age of 40 and/or with low ovarian reserve should be advised that fertility preservation techniques have reduced efficacy. In some cases, cancer patients may have a weaker response to COS [88] than noncancer patients undergoing IVF with similar ovarian reserve: this was reported in a retrospective observational study showing fewer oocytes recovered [89]. In fact, several factors must be considered with the choice of stimulation protocols made in cancer patients, such as random start protocols, low-dose stimulation protocols, exposure to letrozole or tamoxifen, or possible factors that can reduce the ovarian reserve (as observed for carriers of g*BRCA* mutation) [90,91]. A more recent technique, which is still to be considered experimental, is represented by the collection of immature oocytes. This method does not include a follicular stimulation phase or a minimum stimulation of 3–5 days. The oocyte pick-up technique is the traditional one, but it is not preceded by triggering; the immature oocytes taken can be matured *in vitro* and therefore cryopreserved, or they can be directly cryopreserved at the stage of germinal vesicle or metaphase I and will undergo the process of maturation after thawing before IVF. The advantage of this method is the lack of exposure to spikes in high estrogen levels and the short time needed to complete the procedure. The data available to date demonstrate a lower success rate of this technique compared with standard cryopreservation of mature oocytes [92–95].

Cryopreservation of ovarian tissue

The ovarian tissue cryopreservation technique finds particular application in prepubertal patients. In adult women, it is experimental and alternative to the more established techniques of oocyte and embryonic cryopreservation [96]. This technique is particularly interesting for those patients for whom a postponement of the initiation of anticancer therapy is not feasible, since it does not require hormonal stimulation and can be performed at any time during the menstrual cycle. The technical preparation times are those that precede the organization of a common laparoscopy with ovarian biopsy/unilateral salpingo-oophorectomy. A comprehensive preliminary anesthetic evaluation is required. It is a technique that is finding more and more widespread diffusion, but it is still considered experimental and may be performed in reference centers. It allows the preservation of fertility and ovarian hormonal function and does not require the presence of a partner [10,22]. More than 300 women worldwide have undergone the procedure, and ovarian function restoration was achieved in 95% of cases within 4–9 months [97]. It has been shown that cryopreservation of ovarian tissue can give positive results even if the sample is performed after one line of chemotherapy [98,99], with cases of restoration of hormonal and reproductive function. A limitation of this technique is represented by the age of the patient at the time of the surgical removal [100], since after the age of 35–38 the loss of antral follicles in the

ovarian cortex is significant; in addition, another risk is the presence of occult metastases to ovarian tissue in high-risk tumors, such as metastatic peritoneal tumors, leukemia, or ovarian tumors [101,102]. For this reason, however, it is mandatory to associate a biopsy ovarian sampling to perform the histological examination and to carefully consider the application of this technique in cases at risk. In particular, in highly aggressive hematological diseases, this technique should not be considered. Even before ovarian tissue reimplantation, a careful histological analysis is indicated with the methods available to exclude the presence of tumor cells [103,104]. The neoplasm that is safest for this technique is breast cancer, where the incidence of metastases found in cryopreserved ovarian tissue in the studies carried out was zero [105,106]. To obtain the removal of ovarian tissue, the removal of fragments of the ovarian cortex (by means of large bilateral biopsies) or of an entire ovary can be performed. The tissue sampling and cryopreservation techniques necessarily imply the loss of a quota of primordial follicles, estimated to be at least 25%. Although the tissue retrieval technique can also be performed locally, the pathological analysis and cryopreservation phase must necessarily be centralized [107]. Replanting can take place in an ortho-topic or hetero-topic position. The transplantation of ovarian tissue generally takes place on the residual ovary or in a fold of the pelvic peritoneum; this method theoretically allows spontaneous conception and makes the tissue more accessible for oocyte pick-up in case of IVF. The LBR after ovarian tissue cryopreservation ranges from 18.2% to 40% in literature [108–110]; the differences in the results of the different studies are due to the limited number of case series and the age difference in the patients undergoing the procedure.

GnRH agonists

A therapeutic strategy aimed at protecting the ovary from the toxic effect of chemotherapy treatments is represented by the temporary suppression of ovarian function by administering GnRH analogs. This therapy may reduce the risk of POI and its associated fertility- and endocrine-related consequences. Therefore, it may also be of value in patients without a desire for pregnancy and not interested in fertility preservation. The mechanism by which this treatment is able to have a gonadoprotective effect is not entirely clear [111], but the rationale for its use is based on the hypothesis that the resulting pituitary downregulation and "inactivation" of the ovarian activity would lead to a reduced sensitivity to cytotoxic effects. However, activation of the primordial to secondary follicles is gonadotropin-independent, so a protective influence cannot be plausibly explained this way [112]. In addition to the decrease of FSH secretion, the protective effect is achieved with the reduction of ovarian perfusion, as a consequence of the state of hypoestrogenism induced by the therapy [113]. A temporary ovarian suppression during chemotherapy achieved by administering a GnRH agonist (starting at least 1 week before the initiation of systemic cytotoxic therapy and continued for the duration of therapy) is the only strategy that has entered clinical use. Several randomized phase II and III clinical trials investigated whether GnRH analog therapy was actually beneficial for the protection of ovarian function in women undergoing chemotherapy: most of the women enrolled were affected by breast cancer, but there were also cases with ovarian cancer or hematological diseases [114–120]. Most meta-analyses since 2011 and 10 out of 14 randomized clinical trials on patents treated for breast cancer showed a significantly lower rate of POI occurrence after chemotherapy accompanying GnRHa administration. The risk can be reduced by about half, admittedly in a heterogeneous data situation. The protective effect was observed in both patients with hormone receptor-positive and -negative disease and was irrespective of patient age at the time of treatment or type and duration of chemotherapy [121]. In premenopausal women with hematological malignancies, the efficacy of this strategy was investigated in four randomized trials, but none showed a protective effect with the use of a GnRH agonist during chemotherapy [111]. A 2018 meta-analysis including three trials showed no significant difference in POI rates or posttreatment pregnancies between patients that received GnRH agonists administration or not during chemotherapy in the court of 109 patients treated for lymphoma [122]. In premenopausal women with other solid tumors, only one randomized trial including 30 patients with ovarian cancer is available [123]: a significant reduction in POI rates was observed with the use of a GnRH agonist concomitant to chemotherapy, and a significant influence on the likelihood of a later pregnancy has not been proven. Side effects are reported during the use of GnRH agonists during chemotherapy, all related to the transitory hypoestrogenism, e.g., higher incidence of menopausal symptoms (hot flushes and sweating), but those are of low severity grade in the majority of cases and are reversible; bone loss is not a relevant side effect as far as the GnRH agonist treatment lasts for 6 months or less. In women affected by hormone receptor-positive breast cancer, the use of GnRH agonists is not associated with detrimental survival outcomes, and subsequent ovarian function suppression should be considered part of the adjuvant endocrine treatment in these patients [124,125]. Based on the available evidence, the most recent guidelines published by ASCO, NCCN, ESMO, and BCY3 agree on this issue: a

temporary ovarian suppression with a GnRH agonist during chemotherapy should be considered a standard option for ovarian function protection in premenopausal breast cancer patients undergoing adjuvant or neoadjuvant systemic chemotherapy. In premenopausal women with other malignancies who are candidates to receive chemotherapy the available data are limited, but the use of a GnRH agonist may be discussed considering its other beneficial medical effects on the menstrual cycle and prevention of menometrorrhagia. For all patients interested in fertility preservation, temporary ovarian suppression with a GnRH agonist during systemic chemotherapy should not be considered an alternative to cryopreservation techniques: a GnRH agonist can be administered, but only following cryopreservation procedures or when these surgical options are not accessible.

Fertility-sparing surgery in gynecological cancers

Conservative treatment for cervical cancer

The definition of the indications for a conservative treatment in case of cervical cancer requires careful clinical, instrumental, and histological evaluation and an extensive counseling with the patient. This is suitable for conservative surgery patients under 40 years of age, wishing to preserve fertility, with a cervical cancer diagnosed at an early stage (FIGO stage IA1, IA2, IB1), with a tumor less than 2 cm of maximum extension [126]. Many publications have shown that the size of the lesion >2 cm is associated with an increased risk of recurrence following radical trachelectomy [126]. The removal of the primary lesion is adequate if a free margin of at least 5 mm from the endocervical margin is identified on the pathological preparation to reduce the risk of local recurrence. The eventual involvement of the endocervical margin requires a subsequent treatment, which could lead to the abandonment of the conservative approach if it were impossible to extend the local exeresis. The presence of lymphovascular space involvement correlates with the risk of extension to parameters and represents a risk factor to be considered in the choice of the therapeutic approach, but it does not preclude the possibility of a conservative treatment. A review of retrospective studies involving a total of 1117 patients undergoing radical hysterectomy shows that the risk of parametrial infiltration in patients with low-risk disease (lesions <2 cm, negative lymph nodes, <50% of stromal invasion) is less than 1% so does not justify the morbidity of radical surgery [127]. Furthermore, the results of a multicenter study with 30-year follow-up showed that part of the parametrectomy does not affect survival in patients with tumors <2 cm undergoing radical hysterectomy [128]. Simple conization or trachelectomy are alternatives to radical trachelectomy and may be a treatment option, with less iatrogenicity [129]. The goal of the treatment remains to remove the entire involved tissue keeping free margins, reducing the morbidity related to radical surgery. The literature confirms the safety and efficacy of this approach as long as an accurate pathological analysis of the size of the tumor lesion is done and the surgical excision obtains free margins. To avoid relapses or deaths in this category of patients, a meticulous preoperative evaluation of the imaging and histological examination of the biopsy sample is of fundamental importance [130].

The evaluation of the possible presence of lymph node metastases is substantial and is part of the surgical staging for invasive cancers. Therefore, lymph node staging, even in the case of conservative surgery, must be performed. Both sentinel lymph node technique and systematic bilateral pelvic lymphadenectomy can be used. Histology is another fundamental element for evaluating the feasibility of conservative surgery; the histologies associated with the worst prognosis are the following: adenosquamous, clear cell adenocarcinoma, gastric-type adenocarcinoma, and neuroendocrine tumor [126]. According to recent studies, the adenocarcinoma histotype has not been associated with a higher recurrence rate than the squamous histotype in the case of conservative surgery. The risk of ovarian metastases is an aspect to consider in the choice of surgery: the adenocarcinoma histotype is associated with the risk of 8.2% of ovarian metastases, compared to 0.4% of the squamous histotype [131].

Considering radiotherapy-induced toxicity on the gonads, craniolateral transposition of the ovaries, marked with metal clips for radiotherapy planning, must be considered. It is known that an exposure to pelvic radiotherapy (EBRT) of a dose of 14.3 Gy is sufficient to induce a complete loss of ovarian reserve in 97.5% of women over 30 years of age, and that exposure of the uterus at a dose greater than 45 Gy results in the loss of reproductive function of this organ and is not compatible with a subsequent pregnancy [128]. Neoadjuvant chemotherapy and, above all, radio chemotherapy are harmful treatments for the preservation of fertility. However, selected cases can be treated with neoadjuvant chemotherapy to achieve a reduction in the size of the primary lesion (<2 cm), to reduce the risk of nodal and parametric metastases. Exposure to platinum however represents a gonadotoxic element to consider, and ovarian reserve should be assessed both before and after completion of primary treatment [132]. Neoadjuvant chemotherapy followed by conization and lymph node stages of action can represent a conservative therapeutic approach aimed at including high-risk cases suitable for demolition surgery, but at present the long-term

outcomes are not known, and it is not a standard technique: these elements should be discussed with the patient [133].

For cases of FIGO IB1 cervical cancer >2 cm or higher stages, hysterectomy with bilateral salpingo-oophorectomy is indicated, and most centers do not consider fertility preservation a reasonably safe treatment option from a prognostic point of view [128].

In summary, the possible conservative therapeutic indications in the case of early stage cervical cancer are the following:

- *In situ* cervical carcinoma: Cone biopsy or large loop excision of the transformation zone are techniques that do not impair fertility.
- Micro-invasive FIGO IA1 cervical carcinoma with one risk factor or FIGO IA2 cervical carcinoma without risk factors: Cone biopsy is a procedure eligible for fertility preservation if a complete resection of the primary tumor is achieved.
- FIGO IA1 with more than two risk factors or FIGO IA2 with more than one risk factor: Lymph node staging is required, radical trachelectomy is feasible, and prophylactic cerclage should be considered.
- FIGO IB1 with <2 cm without risk factors: Radical trachelectomy is possible in selected cases.

A fundamental requirement for the feasibility of a conservative surgery is the referral to an experienced cancer center. The choice of the surgical approach often does not follow the criteria of evidence-based medicine but is related to the experience of the center itself. The conservative surgery techniques available are cold blade conization, simple trachelectomy, vaginal or abdominal radical trachelectomy, and laparoscopic and robot-assisted trachelectomy [134].

Women treated conservatively may also have difficulty conceiving spontaneously (frequency reported in the literature of 14%–40%); infertility can be caused by cervical stenosis or other cervical factors, making it necessary to promptly initiate the patient toward ART [135]. The first trimester abortion rate in these patients is not substantially higher than in the general population. One of the possible obstetric complications in pregnant patients who have been treated with conservative surgery is the risk of preterm birth and premature rupture of the amniochorial membranes [136]. The cause is attributable to mechanical and infectious factors, or consequent to a cervical incontinence that can become evident in the second to third trimester, and to a predisposition to ascending infections due to alteration of the cervical mucus. Cervical cerclage is a procedure that is being considered a prophylactic treatment of cervical incontinence in these patients. Patients are advised to have a waiting period of at least 6–12 months for pregnancy planning following conservative surgical procedures. To try to reduce the risk of obstetric complications, several interventions have been proposed, including routine screening for genital tract infections, prophylactic use of antibiotics, bed rest, reduced physical activity, administration of glucocorticoids of routine to accelerate fetal pulmonary maturation in case of preterm birth, and prophylactic cervical cerclage [135]. However, there is no evidence that these measures are effective in preventing complications and preterm births in these patients. Vaginal birth should be avoided due to the high risk of birth injuries of the residual cervix, with possible lateral extension in the direction of the uterine vessels. For this reason, a caesarean section is suggested between 37 and 39 gestational weeks [135].

The conservative treatment of cervical cancer is therefore the product of a personalized choice shared with the patient and requires in addition to the surgical and medical oncological evaluation the support of an obstetric consultant, infertility specialists, psychologists, and a rigorous follow-up. Follow-up schedules can be individualized considering prognostic factors, treatment modalities, and estimated risk of recurrence [137]. There are no standardized guidelines for the follow-up of patients undergoing conservative surgery. In general, intervals of 3–4 months are recommended for the first 2 years, then from 6 to 12 months up to 5 years and annually thereafter. A close long-term follow-up is required for these patients since most relapses occur on the residual cervix and can be recovered with curative intent. Follow-up is clinical, cytological, colposcopic, and possibly bioptic, assisted by the search for high-risk human papilloma virus (HPV). HPV vaccination is recommended for all women undergoing trachelectomy/conization to reduce the risk of future reinfection and the risk of tumor recurrence on the residual cervix.

Radical surgery could be proposed at the end of the woman's reproductive life, particularly in high-risk HPV-positive patients. To date, no data are available to compare the long-term outcome of patients who have undergone hysterectomy once their reproductive desire has been satisfied, compared to patients who have continued follow-up.

Conservative treatment for ovarian neoplasms

It is estimated that 12% of malignant ovarian neoplasms arise in patients of childbearing age, and most of these tumors are diagnosed at an early stage with a 5-year survival greater than 90% [138]. There are clearly no prospective, randomized, controlled clinical trials available to date comparing demolition surgery and conservative surgery. However, there are many data in the literature that support the belief that conservative surgery may be an adequate option in the treatment of some gynecological cancers in young women [139]. They can be evaluated for conservative treatment if a

complete surgical staging has been performed and if the following conditions are present:

- early stage borderline tumor with no invasive implants;
- epithelial cancer stage IA–IC, serous histotype G1–G2;
- epithelial cancer stage IA–IC, endometrioid histotype;
- epithelial cancer stage IA–IC, mucinous histology, expansile growth pattern;
- stage IA clear cell tumor;
- unilateral epithelial cancer stage IA–IC1, serous histotype G3;
- ovarian germline cancer (fertility-sparing surgery is considered the gold standard for these patients);
- early stage ovarian stromal cancer;
- absence of contraindications to pregnancy;
- surgical treatment, follow-up, and pregnancy monitoring performed in centers with adequate oncological experience;
- compliance with follow-up.

Every surgical treatment on the ovaries involves a loss of the ovarian reserve of an extent that is not easy to predict. It is therefore advisable to evaluate ovarian reserve indicators before and after conservative surgery and to discuss pregnancy planning with the patient, possibly applying ART techniques if indicated. Fertility-sparing surgery clearly involves preservation of the uterus, and biopsy of any suspected uterine lesions is recommended during primary surgery. If pregnancy is possible and desired, the patient should be informed and encouraged to conceive upon completion of primary surgery.

Ovarian borderline tumors

The standard treatment includes surgical removal of the uterus and bilateral salpingo-oophorectomy, associated with peritoneal staging and the identification of any invasive/noninvasive implants. If the woman has not completed her reproductive life, the hypothesis of conservative surgery can be discussed only in the presence of complete surgical staging, in the absence of invasive implants [140]. In the case of unilateral cancer, surgery involves unilateral adnexectomy, a treatment that is burdened by a lower rate of recurrence than the single enucleation of ovarian lesions. In case of bilateral ovarian involvement, supplementation of adnexectomy with enucleation can be considered, or multiple tumor enucleations can be performed. In case of removal of only the tumor localizations with preservation of both ovaries, some studies report a higher recurrence rate, up to 65% [140]. A recent follow-up study of a case series of ultraconservative surgery of borderline bilateral serous histotype tumors has shown that bilateral enucleation of the masses, compared with the combined adnexectomy with the enucleations on the contralateral ovary, does not significantly increase the recurrence rate, but on the contrary, it significantly increases the fertility rate [141]. In the current state of knowledge, the most significant risk factors for prognosis (DFS) are execution of multiple enucleations, micropapillary histological pattern, and CA125 value $>$ 300 mg/dL at diagnosis [142]. We also remind that borderline tumors can recur as invasive or low-grade malignant forms. Overall, conservative surgery is burdened by a higher relapse rate, even if overall survival is not conditioned [139]. In fact, the overall mortality rate does not appear to be related to the type of surgery, but rather to the stage of the disease at the time of diagnosis, which is 0.7% in stages I and 2% in advanced stages. 85% of relapses develop in the ovary, and in these cases it is possible to discuss with the patient the hypothesis of further conservative treatment, if there is space for enucleation [142]. Oocyte cryopreservation should not be performed before primary surgery in case of borderline ovarian cancer due to the risk of rupture of the capsule of tumor lesions with intraperitoneal spillage. This treatment can only be proposed in case of complete remission after adequate conservative surgical therapy [143]. However, if pregnancy is possible and desired, the patient should be informed and encouraged to conceive upon completion of primary surgery. If gestation were not in the woman's upcoming projects, oocyte cryopreservation can be discussed. There is no evidence that oocyte stimulation treatments induce an increased risk of tumor recurrence. On the other hand, cryopreservation of ovarian tissue is not safe from an oncological point of view [143]. The usefulness of performing radical surgery to complete the patient's reproductive desire is still doubtful: some authors suggest demolition surgery only in case of relapse in patients who do not further renew the desire for conservative treatment or in mucinous histotypes for their greater propensity to relapse in an invasive form. Since the follow-up must in any case be prolonged for at least 10 years from the complete response, and is conditioned by the previous surgical treatment performed, the opportunity to preserve the genital apparatus must be discussed with the patient [144].

Invasive epithelial ovarian cancer

Only 20% of ovarian invasive epithelial neoplasms are diagnosed at an early stage [145]. This disease occurs in less than 10% of cases in reproductive age. The surgical approach involves a complete oncological staging performed with laparotomic approach and in selected cases laparoscopically (only in specialized centers) [146,147]. The standard surgery of the initial forms (IA–IC) involves the following surgical steps: peritoneal washing, hysterectomy, bilateral adnexectomy,

infracolic omentectomy, multiple peritoneal biopsies, pelvic and para-aortic lymphadenectomy up to the left renal vein. The goal of up front surgery in the initial stages is to obtain the complete removal of the neoplastic mass, without rupture of the tumor capsule, and the detection of any localization of occult disease in the upper abdomen and retroperitoneum [145,148]. Tumor spillage leads to an upgrade in the clinical stage affecting prognosis. Appendectomy is indicated in mucinous histotypes and in case of macroscopic involvement [147]. Conservative surgery can be discussed in selected cases, and only when cancer surgery has been adequate to provide complete disease staging, in referral centers for the management of this disease. Fertility preservation surgery can be considered in these cases: early stage cancers (IA−IC) with serous G1 and G2 or endometrioid or mucinous (expansive growth pattern) histotype, and stage IA clear cell histotype [149]. The patient should be advised that high-grade serous histology is associated with a higher relapse rate and also a worse prognosis in case of recurrence. Therefore, fertility-sparing surgery may be proposed in the case of serous G3 histotype only in stage IA−IC1 in selected cases and must always be evaluated with caution: this histotype is in fact the only prognostic factor that correlates with survival, and the conservative approach does not seem to affect it since recurrence is very often extraovarian [149].

According to recent ESMO ESGO recommendations [145], adjuvant chemotherapy may not be identified in the low-risk stages of recurrence undergoing complete surgical staging, such as serous low-grade stage IA, endometrioid G1 and G2 stage IA, and mucinous with expansive growth stage IA. Furthermore, chemotherapy should not be recommended in patients with an isolated diagnosis of serous tubal intraephitelial carcinoma (STIC). Patients eligible for adjuvant therapy, on the other hand, may receive a carboplatin-based monochemotherapy for six cycles or a carboplatin and taxol polychemotherapy whose duration will be modulated in relation to the risk factors [150]. In conservative surgery, as already explained, complete surgical staging must be guaranteed and unilateral adnexectomy in the primary tumor site is indicated; the ovary not involved in neoplasia must not be biopsied (if macroscopically normal) in order not to compromise the ovarian reserve or induce iatrogenic damage. The disease recurrence rate after conservative surgery reported in the literature is as follows: the recurrence rates for the serous histotype are 7% in stages IA G1 and 11% in IA G2, IC G1, and IC G2. The relapse rate appears to be about three times higher in grade 3 [151]. In the latter, recurrence occurs in 95% of cases in the extraovarian site. In the low-grade forms, the recurrence is often found to develop on the residual ovary. Overall, the relapse rates are very similar to those reported in patients undergoing radical surgery [152]. The onset of relapse is an indication for radical surgery. Upon completion of the reproductive process, in all patients treated conservatively, it is advisable to complete the therapeutic surgical treatment, considering that relapses can occur even after 10 years from the primary diagnosis and in high-risk cases. Women with nonmucinous and nonborderline epithelial ovarian neoplasm must also be subjected to genetic investigations for the search for *BRCA*1/2 mutation [91]. Completion surgery is especially recommended in women positive to *BRCA*1/2 germline mutation, who will have to undergo the removal of at least the residual annex; removal of the uterus is not specifically indicated and is to be reserved in cases with associated uterine pathology [153]. The application of oocyte or ovarian tissue-preservation techniques has no indication in invasive tumors [149].

Conservative treatment for endometrial hyperplasia and cancer

According to the 2020 ESGO/ESTRO/ESP guidelines for the management of patients with endometrial cancer, a conservative approach could be considered for women of reproductive age with desire of pregnancy in the following cases: atypical hyperplasia, endometrioid intraepithelial neoplasia, stage IA grade 1 endometrioid carcinoma without myometrial invasion and without genetic risk factors, such as Lynch syndrome or carriers of *BRCA* germline mutation [154]. In these cases, the prognosis after progestogen treatment with histological follow-up is excellent. It is necessary to refer these patients to synthetic centers, where also an expert in reproductive medicine is available to possibly support pregnancy planning. Patients who wish to do so should be advised that the fertility-sparing procedure in early endometrial cancer has not been substantially demonstrated in randomized trials, which are currently impractical due to the small number of existing cases [155]. This is not the gold standard of treatment at present. Available data on conservative surgery outcomes are derived from retrospective studies describing variable rates of success and relapse [156]. The 2017 review conducted by Wei et al. [157] on 28 studies and 1038 women with early endometrial cancer or atypical complex hyperplasia treated with progestogen only (MA, MPA, or LNG-IUD) showed a 9% recurrence rate and a pregnancy rate of 18% with a LBR of 14%. The pregnancy rate appears lower with the use of the levonorgestrel intrauterine device. The 2012 systematic review by Gallos et al. [158] (34 articles, 408 patients) shows a regression rate for atypical hyperplasia and endometrial cancer of 76.2%, with a recurrence rate of 40.6% and a viable pregnancy rate of 28%. Another review by Zhang et al. [157] of 2017 (54 papers, 1152 patients) reports a complete response rate to the combined treatment of

hysteroscopic resection and progesterone of 98%, with LBR of 52.57%, and recurrence rate of 4.79%. Patients should agree to undergo a close follow-up and be informed that during the course of treatment or follow-up the decision may turn toward nonfertility sparing treatment, even if doubts arise about the presence of concomitant ovarian neoplasm or extrauterine involvement. There are very few data on patients with grade 2 stage IA endometrioid carcinoma without myometrial invasion who have received fertility-sparing treatment with the combined oral medroxyprogesterone acetate + levonorgestrel intrauterine device [159]. Although the results are encouraging, this treatment should only be considered by experienced oncologists using well-defined protocols with detailed patient information and careful follow-up. The first diagnostic step, which may also have a therapeutic role in the first instance, is represented by the hysteroscopic biopsy, based on its higher concordance with the final histology compared to D&C [160]. Although hysteroscopy appears to be associated with a higher rate of positive peritoneal cytology, it does not appear to have a negative impact on survival [161]. Performing the hysteroscopy, an accurate assessment of cervical infiltration and separate endocervical sampling are indicated. To date, immunohistochemical molecular testing is part of the diagnostic and prognostic evaluation of endometrial cancer and must be considered for risk stratification and for the therapeutic decision-making [160]. The instrumental evaluation of the local infiltration of the lesion, but also of the suspected ovarian and lymph node involvement, can be done through an expert vaginal ultrasound examination, instead of pelvic MRI. Its high diagnostic performance allows to detect myometrial invasion and cervical stromal invasion with respect to the final pathological examination [154]. Ultrasound should be done by an experienced sonographer. The possibility of a synchronous ovarian neoplasm through the use of Ca 125, US/MRI, and where indicated also with the execution of a staging laparoscopy with pelvic washing and possible biopsy of suspicious lesions should be excluded [154].

To date, available data suggest that the most successful therapeutic approach for patients who are candidates for conservative treatment is hysteroscopic resection of the primary uterine lesion, followed by progestin treatment for 6–12 months, with significant rates of neoplastic regression. Progestin treatment is represented by the following oral therapy regimens, equally recommended: continuous oral intake of medroxyprogesterone acetate (400–600 mg/day) or megestrol acetate (160–320 mg/day) [159]. Treatment with levonorgestrel intrauterine device in combination with oral progestins with or without gonadotropin-releasing hormone analogs can also be considered. The progestin treatment must be continued for at least 6 months, at the end of which the response to treatment and tumor regression must be verified histologically. There do not seem to be any advantages in continuing the treatment for 12 months. To assess response, hysteroscopic guided biopsy and imaging at 3–4 and 6 months must be performed [162]. If no response is achieved after 6–12 months, standard surgical treatment is recommended. The treatment can be continued, in case of regression of the lesion, as a maintenance treatment for women who wish to delay pregnancy [163]. From the cessation of progestin treatment, the pregnancy should take place in a limited time interval. For this reason, women who fail to achieve a spontaneous pregnancy within 6 months of discontinuing therapy should be prioritized for medically assisted procreation support at centers of experience. IVF has been proposed to reduce the time to pregnancy before a completion of surgery [164]. Hormonal stimulation for oocyte cryopreservation also appears to be possible, if stimulated estradiol levels are reduced with an aromatase inhibitor or an antiestrogen and ovulation induced by a GnRH agonist.

In case of nonregression to the treatment or noninvasive or minimally invasive intrauterine recurrence, the continuation of conservative treatments in highly selected cases, under strict surveillance, can be considered. However, hysterectomy and bilateral salpingo-oophorectomy is recommended in these cases. This treatment should also be proposed in the case of regression with conservative treatment after childbearing, due to a high recurrence rate. In case of hysterectomy, preservation of the ovaries can be considered depending on age and genetic risk factors [154].

Techniques for fertility preservation in males

Infertility is a significative side effect of cancer treatment and other nonmalignant conditions in males, but many patients do not receive adequate information or referrals to reproductive specialists prior to starting cancer treatment. For patients with a high risk of becoming infertile due to cancer treatment, evidence-based guidelines for fertility preservation have been provided: all patients of reproductive age that are candidates for gonadotoxic therapy have to be informed about the risk of related infertility and on the existing possibilities; in fact, more than a third of male patients surviving from cancer in adolescent age will become azoospermic after therapy. These patients have to be addressed to a special counseling for fertility preservation that requires specific competence and a multidisciplinary approach (oncologist, surgeon, radiation oncologist, reproduction specialist, and psychologist). This appropriate counseling has to be proposed as soon as possible and a

preferential and rapid access has to be organized. Nevertheless, it has been shown that the expected number of patients who should bank sperm before cancer treatment is consistently lower than the expected number of cancer cases in young men [165]. This should be because of reluctance to delay initiation of cancer treatment, difficulties in communicating, lack of knowledge, and concerns regarding the costs of freezing sperm [166]. The network between oncologists and fertility specialists has to be encouraged with the aim to incorporate fertility preservation as a routine aspect of health care. Sperm cryopreservation (sperm banking) should always be the first-line option for men of reproductive age, while in prepubertal boys, dialogue with patient and parents is shown to be critical for informed decision-making. Parents and patient must be informed that surgical removal of testicular tissue is an invasive procedure still at an experimental level, so the inclusion criteria should be restricted to patients at significant risk of treatment-induced testicular damage and subsequent infertility.

Semen cryopreservation

Cryopreservation of ejaculated semen represents an efficacy and simple strategy to preserve fertility in young postpubertal male patients before starting gonadotoxic treatments and should always be considered. Collect of seminal sperm for cryoconservation is simple and does not comport a delay in the beginning of cancer treatment. This opportunity should be also offered to the peripubertal subject (from 11 years old) who has already start masturbation, so it is theoretically possible to freeze spermatozoa obtained after sperm collection. These techniques have to be proposed even when semen quality is not so good, like often happens in oncological patients. It is essential that this procedure is done as soon as possible, before starting gonadotoxic therapy, because the quality and DNA integrity should be compromised even after just one cycle of therapy. Semen can be cryopreserved for adolescent boys in more than 80% of cases [167–169], and just in 4%–13% of cases, impossibility to collect sperm has been reported. Otherwise semen samples obtained in adolescence are frequently of poor quality [170], so ideally it should be necessary to collect multiple (two or three) samples for having sufficient biologic material with an abstinence period of at least 48 h, but this is not always possible [171]. Cryoconservation can reduce semen quality, and it is important to inform the patient about the possibility that after de-freezing, no sperm will be available. In patients already azoospermic before starting therapy or with severe oligozoospermia, necrozoospermia, or ejaculation disorders, the only acceptable strategy is a testicular sperm extraction (TESE) and a subsequent cryopreservation. Posttreatment, TESE has also been successfully used to obtain sperm in up to 50% of cases of persistent azoospermia in patients in which the option of cryopreserving was not considered or with a previous cryopreservation failure [167]. In cases of ejaculation failure, an attempt to search for spermatozoa in a urine sample could be proposed. Other methods described for retrieval of spermatozoa in adolescents include penile vibratory stimulation and electro-ejaculation. After cryopreservation, stored spermatozoa can be used for IVF, especially intracytoplasmic sperm injection (ICSI), the inject of a single sperm directly into the cytoplasm of a harvested egg, so the problems of low sperm count and poor sperm motility can be bypassed [172]. In rare cases, IUI technique (intrauterine insemination) could be considered, but only if semen quality and quantity is permissive, but success rate is lower [173]. Several studies demonstrated the efficacy of these strategies in preserving fertility, showing a pregnancy rate about 49% [174]. A possible limit for expiration of the cryoconserved material has not been established, but pregnancy after 28 and 40 years has been reported [175]. Anyway data from several studies demonstrate that just a minority of the patients (5%–16%) will effectively use the cryopreserved semen [174].

Gonadic suppression

Differently than in women, hormonal treatment to protect gonads does not seem to be a successful strategy in men. Some authors evaluated suppression of the hypothalamic-pituitary-gonadal axis by administration of GnRH analogs before and during chemotherapy, with the aim of suppressing spermatogenesis and protecting rapidly dividing germ cell populations [176]. No clinical relevance has been demonstrated, so this does not seem to be a valid option for fertility preservation in males [177].

Cryopreservation of testicular tissue

In prepubertal boys the only chance to preserve fertility is cryopreservation of testicular tissue; in fact it is not possible to cryopreserve sperm from seminal fluid because no mature sperm yet exist, and at this age, therapy can damage and completely suppress stem cells. This is the age range most affected from tumor and in which incidence is constantly growing: in Italy, in fact, about 15 boys under the age of 15 receive a diagnosis of tumoral pathology every day. To prevent infertility or conditions associated with prepubertal germ cell loss, cryopreservation of testicular tissues containing spermatogonial stem cells is a promising experimental strategy that is being tested in many European countries. In prepubertal boys

who do not produce spermatozoa or in peripubertal boys who have already started treatment and did not have the opportunity to bank sperm beforehand, testicular tissue freezing or suspension of immature testicular cells including spermatogonial stem cells appears to be an acceptable alternative to preserve reproductive and hormonal testicular function. Testicular biopsy is performed surgically combined with other procedures requiring anesthesia and is preferably unilateral. At present various methods of reimplantation of testicular tissue to restore fertility have been proposed, and others are still in development in mouse and primate models to evaluate the safety and efficiency of the technique [178]. A controversial aspect of transplantation is the possible presence of malignant cells in the removed tissue, but there are just a few data about this possibility. For these patients, spermatogenesis *in vitro* could be an excellent option to restore fertility. Anyway these techniques are still at a research stage, and additional evidence is needed regarding the optimization of protocols for cryopreservation and strategies to minimize the risk of disease recurrence from reintroducing residual malignant cells in the cryopreserved testicular tissue [167].

Techniques for fertility preservation in pediatric patients

Thanks to the excellent results obtained in terms of survival for cancer in childhood and adolescence, it is essential to try to ensure an adequate quality of life for these patients, and preservation of fertility is a key point. The evaluation of the potential for gonadotoxicity and the appropriateness of fertility preservation techniques before undertaking cytotoxic therapies in any pediatric cancer patient is essential to limit long-term damage to the gonads. As previously described, in patients after puberty prior to treatments, sperm preservation in males as well as oocyte cryopreservation in girls are considered the gold standard and should be always offered before starting treatments. Cryopreservation of gonadal tissue, both ovarian and testicular, is the only viable alternative in prepubertal patients but is still considered experimental [179]. Ovarian tissue cryopreservation has emerged as a safe and effective option for these children but must be offered only by centers with the laboratory and surgically advanced expertise, and optimal use of cryopreserved tissue for fertility and hormone replacement is under active investigation [180]. Testicular tissue cryopreservation offers a great potential, and the results are promising in animal models. Nonetheless, it is still experimental, and there are not published studies reporting development of sperm following transplantation of prepubertal human testis tissue or spermatogonial stem cells [181].

Pregnancy after fertility preservation in cancer survivors

Data from the literature show that female and male cancer survivors have significantly reduced chances of posttreatment pregnancies compared with the general population. Posttreatment pregnancy rates are highly dependent on the type of cancer, with the lowest rates reported for men with a history of acute leukemia or non-Hodgkin lymphoma and for women with a history of breast or cervical cancer [182]. After the completion of an oncological treatment, the counseling about the feasibility and safety of pregnancy may consider factors related to the patient (and couple) and the disease itself. The main problems concern the potential negative influence of previous exposure to anticancer treatments on the occurrence of congenital anomalies or obstetric complications, and the possibility that a pregnancy could have a detrimental prognostic effect, in particular in the case of hormone-sensitive tumors. There is an increased risk of developing obstetric and birth complications for female cancer survivors in terms of increased risk of prematurity (RR 1.56; 95% CI 1.37–1.77), low birth weight (RR 1.47; 95% CI 1.24–1.73), elective (RR 1.38; 95% CI 1.13–1.70) and emergency caesarean section (RR 1.22; 95% CI 1.15–1.30), assisted vaginal delivery (RR 1.10; 95% CI 1.02–1.18), and postpartum hemorrhage (RR 1.18; 95% CI 1.02–1.36) [183]. Timing of conception after the end of the treatment is related the risk of these complications, which appears to be higher when the interval is short: women who conceive $\leq$1 year after starting chemotherapy for any cancer have higher risks of preterm birth, and a close monitoring of these pregnancies is recommended [184]. In patients receiving different anticancer treatments for breast cancer a specific wash-out period should be considered before conception: 3 months for tamoxifen [185] and 7 months for trastuzumab [186]. Although the literature is controversial and relies on register-based studies, a slightly increased risk of congenital abnormalities has been reported in offspring of male cancer survivors (3.7% vs. 3.2%; RR 1.17; 95% CI 1.05–1.31) when either cryopreserved sperm or fresh posttreatment sperm was used [187]. Data from mostly retrospective studies support the safety of conceiving following adequate treatment and follow-up of patients with breast cancer [188] including ER-positive disease. At the moment, there are no reliable data about the safety of a temporary treatment interruption to have a pregnancy in those women treated for breast cancer who are candidates for 5–10 years of adjuvant endocrine therapy. When this option is discussed, patient wishes and age, availability of cryopreserved gametes, and individual risk of recurrence are issues of main

importance that must be evaluated. Following delivery, adjuvant endocrine therapy should be resumed to complete the recommended 5–10 years of treatment: there is an international multicenter trial ongoing (POSITIVE trial, estimated study completion in December 2028) aimed to investigate if temporary interruption of endocrine therapy, with the goal to permit pregnancy, is associated with a higher risk of recurrence in positive-receptor breast cancer. A relevant topic in counseling with patients/couples is the feasibility and safety of ART following anticancer treatment in cases where the patient did not have access to fertility preservation strategies at the time of diagnosis and/or where there are difficulties with spontaneous conception: significantly lower LBRs with ART with the use of autologous oocytes were described for cancer survivors compared with healthy women (24.7% vs. 47.7%) [189]. The efficacy of ART was lower in particular among breast cancer patients (14.3%), while the best results were registered in melanoma survivors (53.5%). In women with hormone-sensitive cancers, such as patients treated for hormone receptor-positive breast cancer, the potential detrimental effect of ART on survival outcomes must be discussed. The available safety data are reassuring for the use of ART at the time of diagnosis, but at the moment, data are limited to counseling breast cancer survivors about the safety of using ART during oncological follow-up, particularly when ovarian stimulation is needed: retrospective data suggest that women who had been diagnosed with breast cancer and completed treatment have no increased risk of relapse if they gave birth after conceiving with IVF, but the evidence is limited to draw solid conclusions in this setting and more research is needed [190].

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CHAPTER

37

Psychological impact of infertility and ART procedures

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Introduction

The psychosocial consequences for people experiencing infertility and fertility-related treatment can be significant and long lasting and have an impact across a number of life domains, including close relationships. It is therefore important that healthcare professionals are mindful of this when interacting with people seeking treatment and ensure psychosocial care is routinely implemented as part of the patient's treatment plan. Psychosocial care is defined as care that enables couples, their families, and their health care providers to optimize infertility care and manage the psychological and social implications of infertility and its treatment [1]. In this chapter we provide an overview of work on the psychosocial impact of fertility problems and the ways in which people adjust, the impact of fertility-related treatment, and posttreatment implications, including the consequences of unsuccessful treatment.

In this chapter we present the literature as a timeline from before, during, and after fertility treatment, to illustrate the varying impacts that these different stages can bring. We focus on research with heterosexual couples where primary or secondary infertility is the reason for seeking treatment, while acknowledging the importance of recognizing minority groups and people in different circumstances (for example, those experiencing infertility as a result of cancer treatment).

The psychosocial impact of infertility

As well as the recognized physical challenges associated with fertility treatment, infertility represents an unanticipated life crisis for many people and requires psychological, emotional, and spiritual care [2,3]. There is evidence that the psychological effects of involuntary infertility are similar to those of cancer, heart disease [4], and HIV/AIDS [5]. The adjustment to infertility is described as one of the most stressful experiences a person can undergo, along with divorce and the death of a loved one [6,7], and infertility has also been reported to contribute to various negative psychological sequelae including isolation, frustration, depression, anxiety, hopelessness, guilt, and feelings of worthlessness, failure, and inadequacy [8,9]. Infertility can cause significant disruption to a couple's or individual's plans [10], pose threats to an individual's self-identity, and treatment can cause significant disruption and expense [11].

Research generally indicates that the psychosocial impact of infertility is greater for women than men, with this pattern seen across a range of psychosocial constructs, including higher levels of depression, distress, anxiety, and stress, and lower levels of self-esteem [12–16]. Women report higher rates of moderate to severe anxiety but anxiety in both women and men accounts for significant variance in sexual infertility [17]. Variation in the psychosocial impact of infertility may be related to a number of factors, including a longer duration of infertility and more previous *in vitro* fertilization (IVF) attempts being related to worse quality of life scores [14], and high levels of education and reported social support coping in women related to higher quality of life and psychological health in women [18]. Similarly, risk factors for depression and anxiety have been found to include being aged over 30, lower education levels and occupational activity, diagnosed with male infertility, and longer duration of infertility [15]. Despite evidence of a greater impact for women, there is a growing indication that men do also experience a significant negative impact of infertility. A review of qualitative and social science literature produced core

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themes of infertility such as a crisis of masculinity, the stigma of infertility, men's strong emotive responses, and a desire for fatherhood [19]. Issues relating to masculinity and stigma are seen in other work, along with the impact on mental health, self-esteem, and support [20].

The concept of infertility-related stigma [21] has been described in women experiencing infertility in a number of countries, including Bangladesh [22], Israel [23], and the United States [24], but it may be particularly prevalent in those living in highly pronatalist or patriarchal societies where childbearing is seen as a woman's fundamental role and also in cultures where families are more involved in each other's lives [25]. Women report reacting to perceived stigma in different ways, for example, through resisting and surviving individually [22] or by coping and managing, and using selective disclosure [23].

Coping and adjustment and the couple unit

Infertility will affect both partners, regardless of the cause, so it is important to consider the impact and the experience of infertility at the couple level. In recent years there has been an increased interest in dyadic adjustment, which refers to the way an individual perceives their relationship with an intimate partner, and dyadic coping, which conceptualizes the way in which couples cope with stressors [26], such as infertility. Dyadic analysis of couples' responses (e.g., Actor Partner Interdependence Model) [27] has been developed to examine the influence of each partner's patterns of responses and congruence between partners' coping styles on outcomes, rather than comparing the overall scores of women and their partners.

When studies of dyadic coping and adjustment in infertility are examined, both similarities and differences between female and male partners' use of coping strategies, patterns of adjustment, and outcomes are evident. For example, women's use of meaning-based coping (e.g., personal growth) lead to reductions in their own personal, social, and marital distress levels but increased social distress for their partners, and men's use of meaning-based coping led to increases in their own social distress scores and reductions in their partner's personal distress levels [13]. Women's use of active-confronting coping (e.g., expressing feelings) was related to increased partner marital distress, and increased use of active-avoidance coping in both women and men (e.g., avoiding pregnant women) was related to more personal, marital, and societal distress for both partners [13]. Where couples were incongruent in their use of distancing (women scoring low, men scoring high), significantly higher levels of distress and lower levels of marital adjustment were seen [28]. Both women's and men's own marital satisfaction scores were related to their own depression scores, but partner marital satisfaction scores were only significant for women [29]. Crucially, no overall difference was seen between women's and men's depression and marital satisfaction scores in this study, which demonstrates the importance of examining dyadic relationships. In a study of Portuguese couples, own perceived dyadic coping was positively related to marital adjustment, but partner perceived coping was only significant for women [30], suggesting a key support role for men in women's adjustment.

Despite the difficulties experienced by couples, marital adjustment is generally high [12], and as Schmidt [31] points out, the experience of infertility can strengthen as well as put strain on a couple's relationship, due to the communication involved in managing fertility-related problems [32].

Psychosocial impact during treatment

Advances in assisted reproductive technologies (ART), such as IVF, intracytoplasmic sperm injection (ICSI), and third-party reproduction can offer hope to many couples. However, such treatments are not necessarily accessible to all those in need, with legal restrictions across jurisdictions limiting treatment options. Affordability may also be a key barrier for some people, which can be exacerbated by geographic disparities existing in terms of government-funded treatment availability. Fertility treatment is an incredibly physically, psychologically, and financially demanding process [33]. A 2016 national UK survey via the fertility support group, Fertility Network UK, reported that 54% of the 865 respondents had to pay for some or all of their treatment, with 10% spending more than £30,000, in some cases as much as £100,000 on treatment (the average was £11,378), and almost one-third of those having government-funded treatment still reported having to pay for some additional treatments or tests [11]. Combining the demands of employment and treatment may also be problematic. Estimates suggest that during an IVF/ICSI cycle, six in 10 patients report treatment-related absences from work, and on average, patients miss 23 h of work [34], with higher estimates of some patients needing more than a week off work during a treatment cycle [11]. Such an impact on women's working lives may have financial implications for securing sufficient funds for future treatment plans.

Accessing ART not only has a financial impact, but it also impacts relationships, lifestyle, and physical and emotional well-being [35]. The National Institute for Health and Care Excellence [36] recommends that

couples having difficulty conceiving should be informed that stress in the male and/or female partner can affect the couple's relationship and is likely to reduce libido and frequency of intercourse, which can further contribute to their fertility problems. The 2016 Fertility Network UK survey found that 90% of the 865 respondents reported feeling depressed, with 42% of respondents having experienced suicidal feelings as a result of fertility problems and/or treatment [11]. Those most in danger of experiencing high levels of distress and suicidal feelings were those who had unsuccessful treatment, who spent longer trying to conceive, who experienced some relationship strains, and who had less support from friends and family and their employer [11].

The National Institute for Health and Care Excellence [36] has produced clinical guidelines on fertility care that outline the importance of offering counseling before, during, and after investigation and treatment, as fertility problems themselves, and the investigation and treatment for fertility problems, can cause emotional stress [36, p. 6]. Obviously, specific treatment programmes have distinct implications and considerations that cannot be covered in detail in this chapter. However, there are significant points in a patient's fertility journey that may increase emotional stress and anxiety, necessitating psychological support interventions.

The Human Fertilisation and Embryology Authority, the UK regulator for fertility services, has produced a patient support pathway: *Good Emotional Support Practices for Fertility Patients* [37], which aims to ensure excellent support is offered to all patients consistently. This pathway identifies examples of good practice approaches that can be explored, tailored, and refined by the clinical team (Fig. 37.1).

FIGURE 37.1 Emotional support pathway human fertilisation and embryology authority (2018). *Reprinted with permission https://portal.hfea.gov.uk/media/1406/patient-pathway-final-01.png.*

This image clearly demonstrates the importance of placing the patient at the center of their care, encouraging shared decision-making and ensuring good communication throughout a patient's fertility journey. A full explanation of all procedures and interventions is required, using language and terminology that can be clearly understood. If the woman's partner is in attendance, it is important that the partner is involved in all communications and feels part of the overall experience.

Some ART programs, such as IVF, require women to undergo numerous invasive testing procedures such as vaginal ultrasounds, hormonal blood tests, as well as daily hormonal drug injections to maximize the number of oocytes retrieved. The drug regime can evoke a variety of side-effects including depressed mood [38,39], and some evidence suggests that agonist treatment contributes to anxiety and mental distress [40,41].

While these medical interventions can be arduous and physically challenging for women, the unpredictable response to such drug regimes, and the uncertainty of success, can add to the psychological burden of infertility treatment [9]. Most patients have been reported to experience some degree of emotional distress during treatment [42–44), and around 23% discontinue their program prematurely because of the perceived burden of treatment [45]. The oocyte retrieval, embryo transfer, and the 2-week waiting period before the pregnancy test are stressful periods for patients [43,46,47]. If a positive result is not indicated at each of these key stages, patients face further upset and distress after expensive, intensive, and exhausting treatment [48–50]. If a pregnancy does not occur after a treatment cycle, evidence suggests that for some, treatment failure is followed by strong negative emotional reactions, mainly depression, that may last for 6 months [51]. Continuity and consistency of care is paramount to ensuring the patient's, and her partner's, experience during and after fertility treatment is a positive one. The provision of patient-centered care throughout the treatment cycle by a core group of practitioners with whom the patients become familiar is associated with better patient well-being [52,53].

Providing preparatory information before the start of treatment has been recommended to increase adherence [54], reduce anticipatory anxiety and stress [55], and increase patient knowledge about treatment-related issues [56]. The European Society of Human Reproduction and Embryology [57] also suggests the use of a screening tool (SCREENIVF) to determine the possible emotional impact of the treatment. SCREENIVF [58] is a screening instrument developed for fertility patients, consisting of five items on state anxiety, five items on trait anxiety, seven items on depression, five items on social support, and 12 items on cognition regarding fertility problems. Patients are asked to read each statement provided and

encircle the number (1–4 indicating nearly never, sometimes, often, nearly always) next to the statement that most closely matches with how they felt during the last week. Patients were defined as at risk when their scores on one of the five risk factors showed clinically relevant problems. This questionnaire can be used before the start of each treatment cycle to assess patients' risk factors for emotional problems after the cycle and indicate referral of patients at risk of experiencing clinically significant psychosocial problems to specialized psychosocial care (infertility counseling or psychotherapy).

Ending fertility treatment

Fertility treatment may end for several reasons: because the person being treated becomes pregnant, the treatment is not successful and there is no likelihood of pregnancy occurring, or the person or couple decides to seek alternative ways of family building, such as adoption. Within these possibilities there will be variation in the impact experienced by those undergoing treatment; some will accept the end of treatment, and for others the transition will be more problematic, and there may be differences in experience within the couple unit. In this section we present research on the implications of ending treatment and the longer-term outcomes beyond undergoing fertility treatment.

Unsuccessful treatment often signals the end of hope [59] and the ending of treatment without pregnancy can lead to significant negative psychosocial impacts on those undergoing ARTs across a range of outcomes [42]. This can include mental health and well-being [60] and quality of life [61], with women generally experiencing greater impact than men (61–63; see Johansson et al. [64] for an exception), with unresolved grief evident for both women and men [65]. Those with better adjustment to unsuccessful treatment generally have more options and social support, better emotional and physical health, and less reliance on emotion-focused coping, with better adjustment also seen in couples who adopt [66]. The use of meaning-based coping is also shown to be related to less personal distress in women and marital distress in men [62]. However, overall, sexual satisfaction has been found to decrease over time, with a significant long-lasting impact on sexual relationships for couples where treatment is unsuccessful [60].

The long-term process of adjustment to infertility may be seen as an existential crisis that needs to be addressed, necessitating the creation of a different life and a rethinking of the self [67], with those affected needing to accept their situation, both rationally and emotionally, before being able to move on [60]. Infertility may be perceived by some as a personal failure with loss of identity. It is important for practitioners to demonstrate sensitivity and awareness of the importance of this loss, and to signpost women and their partners to counseling and support services to help them grieve their losses [68]. Despite the difficulties faced by those who experience unsuccessful treatment, evidence suggests that couples often experience a strengthening of their relationship or are able to maintain a stable relationship throughout the treatment period [69,70], with IVF largely viewed as a positive experience even when unsuccessful [61,70].

For those whose fertility treatment is successful, research indicates that the long-term psychological impact is lessened significantly for both women [42,64] and men [64]. However, psychosocial stress still exists, with pregnancy-specific anxiety prevalent in women [71–73] along with reduced quality of life compared to women who conceived naturally [71]. The time between a positive pregnancy test result and the first antenatal appointment can be a time of heightened anxiety, as women may experience more anxiety than women who conceive spontaneously [72]. Anxiety may be related to concerns about their baby surviving, possible damage occurring during childbirth, and separation from their baby after birth [73]. Qualitative research with women who conceived after treatment highlights the complex nature of this pregnancy-specific anxiety, with women expressing difficulty in shedding their infertile identity (of which feeling unprepared and anxious was a significant part) while feeling they had no right to complain [74]. Reasons for this pregnancy-specific anxiety include the length of the infertility period and number of ART attempts (repeated IVF attempts and losses meant women approached pregnancy as a stressful time), facing the need for gamete donation, risk of medical conditions and complications, and the nature of social support [75]. Anxiety is often greater for multiple births, which may be related to the perceived risks compared to single pregnancies, the additional work of caring for multiple babies [76], and higher maternal expectations with multiple pregnancies after IVF/ICSI than natural conception [77].

Recommendations for clinicians

The evidence presented in this chapter outlines significant stressors that individuals and couples may encounter in their fertility journey, which require an empathetic response from healthcare providers involved in their care. It is essential for practitioners

also to recognize and acknowledge diversity in individuals seeking fertility care; this may include people with disabilities, same-sex couples, single people, those seeking fertility preservation, women seeking treatment for medical or social reasons, heterosexual couples, transgender people, and those looking to pursue surrogacy arrangements [68].

While acknowledging that each person's fertility journey is completely individual, we offer the following recommendations for providing emotional support to address the common psychosocial needs identified within this chapter:

- Create a patient-centered environment, ensuring those receiving infertility care are treated with dignity and respect and involved in all decisions about their treatment options and well-being.
- A visible "patient support policy" that outlines how the fertility clinic ensures patients and donors receive appropriate psychosocial support throughout their fertility journey, which is embedded in all interactions, may encourage the uptake of emotional support and counselling.
- Be an active listener, recognizing the patients' and partners' perspectives, feelings, and needs, so these can be incorporated into their care.
- At the initial appointment, take a psychological health history, including previous trauma and/or loss, as well as a physical health history, so a timely counselling referral or intervention can be initiated if required.
- Provide comprehensive preparatory information about assisted reproductive technology (ART) procedures and lifestyle behaviors that may negatively affect patients' general and reproductive health to decrease infertility-specific anxiety and stress and help patients better prepare for their ART program.
- Discuss the emotional aspects of every stage of treatment together with any strategies for minimizing negative impact.
- Look out for and recognize signs of stress and distress that patients may display, for example irritability, impatience, anxiety, nervousness, lack of concentration, tearfulness, restlessness. nail biting, lack of eye contact, or the appearance of disinterest or disengagement [68], as further intervention or referral may be necessary.
- Ensure patients are consistently involved in decision-making, that their voices are heard, and sufficient opportunities are made available for them to discuss and clarify their treatment- or donation-related concerns.
- Practitioners need to be approachable, nonjudgemental, compassionate, and sensitive to individuals' beliefs and needs.
- Establishing an effective practitioner-patient relationship allows the woman and her partner to feel able to share their feelings and concerns in a supportive, safe, and confidential environment.
- Counselling can have a significant impact on an individual's emotional well-being by facilitating discussion of a variety of issues and concerns in a supportive and confidential environment. This provides an opportunity for people to explore their thoughts, feelings, and their relationships to reach a better understanding of the meaning and implications of any choice(s) they may make [78].
- It is important for practitioners to remain abreast of the range of fertility support groups, online forums, and therapeutic counselling carried out by trained/accredited counsellors to signpost people to relevant services.
- Patients may have clear preferences about the care they receive and the type of psychological care they wish to explore. Practitioners need to pay attention to the specific needs of each patient, incorporate them into their care delivery, and help signpost patients to the most appropriate support available.
- Staff should be sensitive to any ethnic, religious, societal, cultural, or other factors that may influence the kind of support that is appropriate for an individual.
- Practitioners must be aware of the specific needs that patients may experience at different treatment stages of their fertility journey (before, during, and after treatment cycles), so counselling and other relevant interventions can be offered, and psychosocial care and interventions can be tailored accordingly.
- Patients' emotional stress fluctuates during an *in vitro* fertilization/intracytoplasmic sperm injection cycle, with peaks at the oocyte retrieval, the embryo transfer, and the waiting period before the pregnancy test. These are key times to contact the patient and their partner to identify any concerns or emotional needs.
- Aim for continuity of care to help establish good rapport and patient-practitioner relationship to enable patients to feel able to open up and disclose their anxieties and concerns.
- Practitioners need to recognize and observe for psychological risk factors:
  - Having undergone multiple ART cycles
  - Experienced high stress during treatment

  - Long duration trying to conceive
  - Those experiencing relationship strains
  - Lack of support from friends, family, or their employer [11].
- Acknowledge risk factors for increased psychosocial needs and consider using the SCREENIVF tool before the start of fertility treatment to identify patients at risk of developing emotional problems, those more vulnerable to the demands of treatment, and patients in need of additional psychosocial care or specialized mental health services.
- Early pregnancy after ART can be very stressful, so ensure information is provided on the next steps and what emotions to expect during pregnancy following fertility treatment. A courtesy call after the initial pregnancy scan may be beneficial to allay anxieties.
- Following successful treatment, women and their partners should be encouraged to share their concerns with their midwife. Sufficient opportunities must be provided for emotional well-being checks to ensure women are monitored and supported to maintain good mental health during the pregnancy and the first year following the birth of a child [79].
- After unsuccessful fertility treatment, check what support patients and their partners have previously accessed or wish to access, and ensure they are aware of how and where to gain ongoing support to assist them in adjusting to their unmet parenthood goals.
- Discussion about future treatment should be sensitively timed and individually tailored to the patient and their partner and include the possibility of not seeking further treatment.
- Offer patients the opportunity to discuss the implications of ending unsuccessful treatment, and offer additional psychosocial care to those at risk of increased infertility-specific psychosocial distress.

Research limitations

While a significant body of research has examined the impact of infertility, treatment, and adjustment in individuals and couples, it is recognized that quality-related issues exist with this work [80]. Studies are predominantly cross-sectional, so untangling the direction of causality between variables is not possible. In addition, samples are often heterogenous and will include participants at different stages of the treatment process, with different fertility problems (e.g., female infertility, male, unexplained, joint), which can make it difficult to compare across studies. Participants are usually recruited from clinics just prior to or during treatment, so those who do not seek treatment (e.g., for financial reasons or limited access) are excluded [81]. When data were collected from a nationally representative sample of women in the United States who had not been able to conceive (including those who had not sought treatment), pregnancy intentions were found to be highly significant; when these intentions were strong and the woman had not conceived, fertility-specific distress was higher [82]. It is also possible that couples with higher marital adjustment are more likely to seek treatment [28] and therefore are not comparable with couples who do not.

While it can be difficult to determine the impact of infertility and the impact of treatment, longitudinal work by Greil et al. [49] found that women who did not undergo treatment over the 3-year period did not have increased fertility-specific distress. These findings suggest that infertility treatment is associated with distress above that experienced due to infertility, emphasizing the importance of comparing impact, coping, and outcomes between treatment and nontreatment groups. All of these points should be considered when evaluating research in this area.

Conclusion

The World Health Organization [83] has now recognized that infertility is a disease, and the reality of the devastation and grief it causes has been widely acknowledged. Fertility problems and treatment have been found to cause high levels of distress within the couple unit, while significant distress may serve to undermine treatment outcomes and adjustment to parenting [11]. The negative psychological impact of chronic infertility can be equally as serious as that seen in potentially fatal medical conditions [5]. It is estimated that the incidence of infertility is likely to continue to increase over time [84], so psychological intervention and support is an important prerequisite for people, prior to, during, and after fertility treatment. Couples may seek informational and emotional support from health professionals during the investigation and treatment stages of their infertility journey [49,85] and/or more therapeutic psychosocial and counseling support to cope with the emotional turmoil of trying to conceive [86], as well as dealing with past life experiences that may surface during this time. The importance of effective management of psychosocial issues in reproductive care is now firmly recognized as an integral component of patient care.

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CHAPTER

38

Risk, safety, and outcome monitoring in the IVF clinic

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Introduction

Objective evaluation of the *in vitro* fertilization (IVF) process is an essential part of a good assisted reproduction practice and should be a primary target of IVF clinics. For healthcare providers, monitoring clinical practice has the final goal of minimizing the rate of complications and couple dropout while maximizing live birth rate (LBR) and good obstetric outcomes. For patients, these data provide a reliable way to understand the chances of success and complications. Moreover, appropriate monitoring of risk, safety, and outcome collected in national and international registries allows analyzing and evaluating the application of IVF techniques in terms of efficacy, safety, and outcomes. These data provide objective evidence for purchasers, regulators, and politicians to guide healthcare policies.

Continuous local, national, and international monitoring of assisted reproduction and its outcomes is essential for all stakeholders to monitor and compare the effectiveness of treatments and identify safety issues [1]. Notably, increased adherence to monitoring and communication of data to registries has been observed over the years [1,2]. The European IVF Monitoring Consortium (EIM) developed and manage the European registry. Worldwide, the reference is the International Committee Monitoring Assisted Reproductive Technologies (ICMART). The last report published by EIM for the European Society of Human Reproduction and Embryology (ESHRE) refers to 2016 and analyzes data collected on an aggregate basis by 40 European countries [2]. The last report published by the ICMART refers to 2012 and includes approximately two-thirds of world-assisted reproduction activity [1]. More recent preliminary reports are available on ICMART's website [3]. To facilitate risk, safety, and outcome monitoring, the ICMART offers a Data Collection Toolbox and a Glossary, which was developed in 2017 by a global panel of more than 100 multidisciplinary experts, professional organizations, and patient representatives. This global panel provided a consensus agreement to make data collection and communication easier [3–5].

The IVF process can be monitored globally or at the level of each step: ovarian stimulation and oocyte retrieval, gametes management and insemination, embryo transfer, and cryopreservation of gametes or embryos. Monitoring these key steps is crucial to identify the source of complications or low performance of IVF clinics. Therefore, risk, safety, and outcome monitoring of the IF process include different endpoints corresponding at different levels of the process. However, global outcomes remain the most relevant, with the **delivery of a single healthy child** as the primary endpoint. Of all couples visiting infertility centers, about 35%–40% will not achieve the goal of live birth and will remain childless [6–8]. Concerning this primary global outcome, due to the segmentation of treatments and the growing number of freeze-all cycles, **cumulative delivery rate per cycle or aspiration** is becoming the primary indicator of treatment effectiveness represented by the delivery of a single healthy child [2]. Following these principles, efficacy and safety outcomes proposed by the ESHRE guidelines for IVF clinics (Table 38.1) include critical outcomes representing the entire process versus others that can be attributed to specific steps. Critical outcomes for efficacy are **LBR** and **cumulative**

Management of Infertility
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TABLE 38.1 Outcomes defined by the ESHRE guidelines for the IVF process.

Critical outcomes	Efficacy in terms of cumulative live birth rate (CLBR) per started cycle Efficacy in terms of live birth rate (LBR) per started cycle Safety in terms of moderate and/or severe OHSS
Other outcomes for efficacy	Cumulative ongoing pregnancy rate per started cycle Clinical pregnancy rate per started cycle Number of oocytes retrieved Number of MII oocytes retrieved
Other outcomes for safety	Incidence of different grades of OHSS Cycle cancellation Bleeding Infections, torsion, long-term effects on maternal/child health, Other treatment-related adverse events
Patient-related outcomes	Compliance (dropout rates) Patient burden Quality of life (QoL) Patient preferences

live birth rate (CLBR) per started cycle. In contrast, the critical outcome for safety is the rate of a **moderate or severe hyperstimulation ovarian syndrome (OHSS).** [6].

Monitoring of risk, safety, and outcome in ovarian stimulation

Ovarian stimulation is a pharmacological treatment aiming to develop multiple ovarian follicles and obtain multiple oocytes. Based on the number of retrieved oocytes, response after ovarian stimulation is classified as poor/low, normal, or excessive/high, without a universally accepted definition of these categories [6].

Nevertheless, the absolute number of retrieved oocytes is not enough as efficacy outcome. Indeed, although various cross-sectional studies suggested that "more is better" and "less is bad," randomized controlled trials did not demonstrate that the number of retrieved oocytes make a relevant difference within the individual couple in terms of LBR [6,7,9,10]. For this reason, some authors propose the **number of metaphase II (MII) oocytes retrieved** as the primary outcome of efficacy for the ovarian stimulation phase instead of the absolute number of retrieved oocytes [6,11]. Surrogates of this outcome are the **oocyte maturity rate**, which is the proportion of oocytes at the MII stage, and the **oocyte grade**, which is the proportion of oocytes with expanded cumulus, although objective criteria for assessment of these indicators are lacking [11,12]. Concerning the **proportion of MII oocytes**, the expected range is 75%–90% at 40 ± 1 h posttrigger for all cumulus-oocyte complex (COC) retrieved. Values outside this range should prompt evaluating any changes in ovarian stimulation or triggering [11]. A poor ovarian stimulation may result in a low number of retrieved oocytes and/or a high rate of immature or abnormal oocytes [11,13].

Regarding safety, the appropriate ovarian stimulation protocol and the freeze-all strategies are universally accepted to prevent **ovarian hyperstimulation syndrome (OHSS).** However, this is a global safety outcome for IVF that reflects the entire management of patients. A specific safety outcome of the ovarian stimulation phase is the **cycle cancellation** rate (Table 38.1) for insufficient or absent ovarian response to stimulation. The main risks related to inadequate ovarian stimulation are couple dropout and loss of time [6].

Monitoring of risk, safety, and outcome in oocyte retrieval

Oocyte retrieval is the step between ovarian stimulation and the laboratory phase and influences performance outcomes of the ovarian stimulation phase. The specific efficacy outcome is the **proportion of oocytes recovered (**Table 38.2**),** which was defined as the number

TABLE 38.2 Reference indicators for laboratory performance according to the Vienna consensus.

Reference indicator	Calculation	Benchmark value
Proportion of oocyte recovered (stimulated cycles)	n° oocyte retrieved × 100 n° follicles on day of trigger	80%–95% of follicles measured at ultrasound
Proportion of MII oocytes at ICSI	n° MII oocytes at ICSI × 100 COC retrieved	75%–90%

of oocytes retrieved in the function of the number of ovarian follicles seen at the ultrasound assessment. The number of collected oocytes is expected to be higher than 80%–95% of follicles measured before pick-up [11]. Follicular aspiration should be checked for the presence of oocytes with minimal time between pick-up and culture of washed oocytes. Operator, timing of retrieval, and number of collected oocytes should be documented [14].

Regarding safety outcomes, the pick-up can be associated with bleeding or infections (Table 38.1) [6]. Their monitoring is mandatory to maximize patient safety and guarantee early preventive interventions.

Monitoring of risk, safety, and outcome for semen collection and preparation

Regarding performance indicators (PIs) for an andrology laboratory, **postpreparation sperm motility** would be a valuable indicator of efficiency, reflecting the effectiveness of sperm washing procedure. It is defined as the proportion of progressively motile spermatozoa in the sperm preparation for insemination, including only fresh normozoospermic ejaculate specimens. For this reason, low values suggest problems with the preparation procedure. Potential weaknesses of this PI include unacceptably high uncertainty in the measurement of sperm motility, variability in sperm preparation methods used in different laboratories, and the possible abnormal response of sperm to preparation. The reference values are competence of 90% and benchmark ≥95% [11,15,16].

Sperm recovery rate is the percentage of progressively motile sperm that are recovered after washing compared to prewash. It provides helpful information for interoperator comparison and proficiency evaluation. Each laboratory should develop its standard for this indicator [11,16].

Monitoring of risk, safety, and outcome in laboratory

To perform all laboratory tasks ensuring patient safety and quality of care, ESHRE, in accordance with the European Commission (2019) and the Council of Europe (2013), recommended working in compliance with a quality management system (QMS), which should be revised every year [14,17,18].

PIs are necessary parameters to monitor the laboratory's contribution to patient care (ISO-15189:2012) and relevant elements of the QMS [11,14,19]. The Vienna consensus in 2017 was an attempt to categorize these indicators. Based on data gathered by national and international registries, the Vienna consensus defined for each indicator the competency (minimum performance-level values) and benchmark (aspirational values) levels. The gap between these two levels defines the "desirable range" for each indicator [11].

Each laboratory should implement a systematic approach to data collection and analysis and develop its own set of key PIs (most significant PIs to evaluate the laboratory activity; Table 38.3) [11]. According to the Vienna consensus, data collection should be done for all PIs monthly, although that is not always practical [11]. Moreover, for each key PI, the single laboratory should define the critical performance levels based on the "desirable range" for each indicator [11,14]. In addition to the overall laboratory performance, PIs should be regularly checked for the single operator to implement individual retraining [14].

In vitro fertilization

Concerning IVF, a normally fertilized oocyte should present two pronuclei (2 PN) of similar size that are closely apposed and centrally located [11,20]. The expected fertilization rate is 67% (53%–81%), based on the literature. **Total IVF fertilization rate** following IVF is calculated, including all fertilized oocytes with $> o = 2$ PN. This parameter provides an indicator of the ability of the culture system to support sperm capacitation and sperm-oocyte interaction in IVF cycles [11]. Incidence of **poor IVF fertilization** (<25% of inseminated COC with 2 PN) or **total IVF failure of fertilization** suggests a problem in sperm function or motility or oocyte activation [11,21].

Normal IVF fertilization rate is defined as the number of fertilized oocytes on day 1 (presence of 2 PN or 2 PB assessed at 17 ± 1 h postinsemination) as a function of all COC inseminated. This PI measures the efficiency of the whole *in vitro* fertilization process [11].

Failed fertilization rate for IVF cycle is defined as the proportion of IVF cycles (not including ICSI) with no evidence of fertilization on day 1 (17 ± 1 h postinsemination). It is a marker of poor gamete quality, problems in sperm processing, or an insufficient number of spermatozoa used for insemination [11].

Intracytoplasmatic sperm injection

The Vienna consensus defined four possible PIs for ICSI: normal fertilization rate, oocyte damage rate, poor fertilization rate, and failed fertilization rate [11].

Normal fertilization rate for ICSI is defined as the proportion of MII oocytes injected that are fertilized on day 1 (presence of 2 PN or 2 PB assessed at 17 ± 1 h postinjection). This indicator is informative of gamete quality and operator competence. It can be calculated in

TABLE 38.3 Key performance indicators for laboratory performance according to the Vienna consensus [11].

Key performance indicator	Calculation	Competency value	Benchmark value
ICSI damage rate	n° damaged/degenerated ×100 all oocytes injected	< o = 10%	< o = 5%
ICSI normal fertilization rate	n° oocytes with 2 PN and 2 PB ×100 n° MII oocytes injected	> o = 65%	> o = 80%
IVF normal fertilization rate	n° oocytes with 2 PN and 2 PB ×100 n° COC inseminated	> o = 60%	> o = 75%
Failed fertilization rate (IVF)	n° cycles with no evidence of fertilization ×100 n° of stimulated IVF cycles		<5%
Cleavage rate	n° cleaved embryos on day 2 × 100 n° 2PN/2 PB oocytes on day 1	> o = 95%	> o = 99%
Day 2 embryo development rate	n° 4-cell embryos on day 2 × 100 n° normally fertilized oocytes	> o = 50%	> o = 80%
Day 3 embryo development rate	n° 8-cell embryos on day 3 × 100 n° normally fertilized oocytes	> o = 45%	> o = 70%
Blastocyst development rate	n° blastocyst day 5 × 100 n° normally fertilized oocytes	> o = 40%	> o = 60%
Successful biopsy rate	n° biopsies with DNA detected ×100 n° biopsies performed	> o = 90%	> o = 95%
Blastocyst cryo-survival rate	n° blastocysts appearing intact ×100 n° blastocyst warmed	> o = 90%	> o = 99%
Implantation rate (cleavage stage)	n° sacs seen on ultrasound ×100 n° embryos transferred	> o = 25%	> o = 35%
Implantation rate (blastocyst stage)	n° sacs seen on ultrasound ×100 n° blastocysts transferred	> o = 35%	> o = 60%

PB, polar body; *PN*, pronucleus.

cycles using ejaculated spermatozoa, both fresh or frozen. It cannot be calculated in cycles using *in vitro* matured oocytes and thawed/warmed oocytes [11,20].

ICSI damage rate is the proportion of oocytes damaged during ICSI injection or degenerated between fertilization and the assessment on day 1. This is a useful indicator for the operator's competence, oocyte quality, and laboratory performance [11].

Poor ICSI fertilization rate is defined as the proportion of cycles in which less than 25% of the injected oocytes are fertilized. It can be an indicator of operator competence and gamete quality [11].

Failed ICSI fertilization rate is defined as the proportion of cycles in which none of the injected oocytes are fertilized. It can be informative of gamete quality and function, and ability of the operator [11].

In conclusion, **ICSI damage rate** and **ICSI normal fertilization rate** appear to be relevant key PIs, while poor and failed ICSI fertilization rates are not considered key PIs [11].

Embryo culture

The Vienna consensus proposed different indicators for cleavage-stage embryos: early cleavage rate, cleavage rate, embryo development rates, embryo fragmentation rate, and embryo score or grade.

Early cleavage rate is the fraction of fertilized oocytes that achieved the first round of cleavage by 26 ± 1 h postinsemination by ICSI or 28 ± 1 h postinsemination by IVF. This indicator reflects the capability of the culture system to support the early cleavage of fertilized oocytes and the vitality and quality of the embryos. There are no conclusive data about the utility of this indicator and no recommendations for target values [11,22].

Cleavage rate is an important indicator, defined as the proportion of zygotes that cleave to become embryos on day 2 (44 ± 1 h postinsemination) [11,20]. This PI reflects the culture system efficiency in supporting cellular division of fertilized oocytes and embryo viability. Its

cleavage rate should be calculated not only on the overall population referring to an IVF clinic but also for IVF versus ICSI and specific subgroups divided for female age or ejaculated versus surgically retrieved sperm [11].

Embryo development rate is the proportion of four-cell embryos on day 2 among the 2 PN zygotes (44 ± 1 h postinsemination) or the proportion of eight-cell embryos on day 3 (68 ± 1 h postinsemination) [11,20]. This PI measures the culture system ability to support cleavage and the quality and vitality of embryos. In well-defined categories of patients, this key PI reflects the overall laboratory performance [11].

The **rate of good-quality embryos** is the proportion of day 2 and day 3 embryos with high scores or grades. Many different scoring systems, based on different variables, have been suggested, but none of them is robust, and they can be used only to assess the clinic's internal quality [11,14].

Embryo fragmentation rate is defined as the proportion of day 2 and day 3 embryos with less than 10% fragmentation. This PI reflects the quality and viability of embryos, but it has been reported challenging to evaluate and highly operator dependent [11].

Embryo (or blastocyst) utilization rate is the number of embryos transferred or cryopreserved per number of 2 PN zygotes in the same cycle. It depends on the request of patients and strategies of embryo transfer and cryopreservation. Therefore, this PI dramatically varies between different regions in the world and between each clinic. For these reasons, it cannot be used as a key PI with target values [11].

In conclusion, **embryo cleavage and embryo development rates on day 2 and day 3** are critical indicators and must be used as key PIs to evaluate the IVF laboratory (Table 38.3) [11].

The Vienna consensus proposed several key PIs to evaluate blastocyst-stage embryos. The most relevant ones are blastocyst development rate, good blastocyst development rate, and day 5 embryo transfer rate.

Blastocyst development rate is the proportion of 2 PN zygotes present at the blastocyst stage at day 5 (116 ± 2 h postinsemination). It reflects the efficacy of the whole culture system and embryo viability [11,20].

Good blastocyst development rate is calculated as the proportion of 2 PN zygotes that are good-quality blastocysts on day 5 (116 ± 2 h postinsemination) [11,20].

Day 5 embryo transfer rate is the proportion of cycles with at least one utilizable blastocyst on day 5 relative to the presence of at least one 2 PN oocyte on day 1. This parameter reflects the efficacy of the whole culture system, but it depends on specific transfer policies adopted by different IVF clinics. For this reason, each clinic should develop its expected value for this indicator [11].

In conclusion, the **blastocyst development rate** is the only key PI to be used for blastocyst-stage evaluation (Table 38.3) [11].

Preimplantation genetic/diagnostic test

The key PI for preimplantation genetic/diagnostic test can be considered the **successful biopsy rate** (Table 38.3). This key PI is defined as the proportion of biopsied and tubed/fixed samples where DNA is detected. It measures the embryologist's ability to transfer the biopsied samples to test tubes, as proven by positive DNA amplification. Based on data from surveys and international databases, which reported a 91% diagnosis rate in 254,820 biopsies, the proposed reference values are competency ≥90% and benchmark ≥95% [11,23].

Cryopreservation

Blastocyst cryo-survival rate is the key PI proposed by the Vienna consensus to evaluate cryopreservation as a step of laboratory practice. It reflects operator skills and the performance of the devices used. Since no finding an embryo is a rare event, the reference rates could now reasonably be expected to be competency ≥90% and benchmark ≥99% [11].

Embryo transfer

The transfer of a single embryo is recommended to avoid multiple pregnancies [14]. From a broader perspective, the number of embryos to transfer should be decided based on embryo quality and stage of development, maternal age, ovarian response, and previous attempts of IVF treatment. However, it is generally unwise to transfer more than two embryos [14]. Because the primary objective of an IVF treatment is the delivery of a single healthy child, a twin pregnancy should be regarded as a complication. Reducing the number of transfers of two or more embryos leads to reducing prematurity associated with multiple births [2,24].

According to national legislation and patient wishes, supernumerary embryos may be cryopreserved for subsequent attempts, donated to research, or discarded [14].

Other outcomes

Regarding safety, identification of patients and traceability of reproductive cells and embryos must be guaranteed throughout all steps of laboratory practice up to

the embryo transfer. The tracing system must be consistent also when transporting reproductive cells and tissues between different laboratories [14].

Finally, an emergency plan is mandatory for the IVF laboratory, not only for the safety of personnel and patients but also for protecting all fresh and cryopreserved human material and limiting damage to equipment and medical records [14].

Monitoring of risk, safety, and outcome for the entire IVF process

Efficacy outcomes

The **primary outcome of assisted reproduction is a live-born singleton at term with normal birth weight**, which reflects both efficacy and safety of the process [25]. For this outcome, the implantation rate and the LBR are the key PIs.

Implantation rate is a key PI of the overall performance of the IVF clinic, even though it can be influenced by uterine receptivity [11]. It can be defined as the number of gestational sacs observed divided by the number of embryos (cleavage-stage or blastocysts) transferred or the proportion of fetal heartbeats detected relative to the number of embryos transferred [4,5,26,27]. The Vienna consensus agreed to use the number of gestational sacs to calculate the value of the indicator.

Reference values for transfer of day 2 or day 3 embryos are competency ≥25% and benchmark ≥35%. Reference values for blastocyst transfer are competency ≥35% and benchmark ≥60% (Table 38.3). An overall low implantation rate is a sign of a serious systemic problem in the performance of the IVF clinic [11].

LBR is defined as the likelihood of a baby being born per embryo transferred and must be considered the ultimate performance indicator to evaluate the IVF clinic performance [6,11].

However, we need to be aware that these outcomes are affected by the characteristics of the population referring to the IVF clinic. A high proportion of patients with multiple previous unsuccessful cycles or significant adverse factors (including mother age) can significantly affect the implantation rate of the center [11]. Similarly, LBR is affected by a series of maternal factors pertaining to post-implantation development, and for this reason, it does not reflect laboratory performance only [11]. The LBR varies considerably between different regions in the world [1,2,25]. On that basis, these outcomes should be adjusted for the population referring to the clinic.

Safety outcomes

According to the Royal College of Obstetrics and Gynecology and the American Society of reproductive medicine, the **most significant obstetric risk of assisted reproduction treatment is multiple gestations** with the associated risks with mother and babies, including preterm and extreme preterm birth [1,2,28,29]. Based on data of the European register of 2016, the risk of extreme prematurity and the risk of very preterm birth increase by threefold and fivefold, respectively, for twin deliveries compared to singleton deliveries [2]. For this reason, multiple embryo transfer should be reserved for patients with a poorer prognosis [1,25,30].

The rising awareness of maternal and neonatal risks related to multiple gestations may explain the decreased average number of embryos transferred in fresh nondonor IVF cycles and frozen/thawed cycles [1,2,25,31,32]. The trend toward single embryo transfer must be encouraged, considering that the multiple birth rate following assisted reproduction remains unacceptably high in most countries [1].

Moreover, IVF per se is a risk factor for obstetrics complications. **Preterm birth** is increased by an additional 23 in IVF twins over naturally conceived twins, and a twofold increased risk of preterm birth in singletons [28]. The risk of **low birth weight** is increased for IVF babies, and this outcome is not entirely accounted for by preterm birth and multiple pregnancies but seems to be partially related to IVF itself [28,33].

IVF is associated with a 30%–40% increased risk of **major congenital anomalies** compared with natural conception. Of note, the absolute risk is nevertheless low since anomalies per se are relatively uncommon [28,34,35].

OHSS is the most severe complication in patients undergoing IVF treatments. Reported frequencies are 20%–33% for mild forms and 3%–8% for moderate or severe forms [36]. OHSS is a general safety outcome, representing an overall evaluation of the appropriateness of the IVF process. Potential severe complications of OHSS are pleural effusion, renal insufficiency, and venous thromboembolism [6,37]. The driver of the syndrome is the exposure of the granulosa cells to human chorion gonadotropin (hCG) [6].

It is essential to identify women at high risk of OHSS to lower the incidence of this condition. Polycystic ovarian syndrome (PCOS), elevated anti-Müllerian hormone values (>3.4 ng/mL), the peak of estradiol over 3500 pg/mL, maturation of 25 follicles or more, and retrieval of 24 oocytes or more are associated with an increased risk of OHSS (Grade B). This subgroup of women may benefit from tailored treatments to reduce the risk of OHSS (Grade B), such as stimulation using GnRH antagonists and use of GnRH agonist to trigger oocyte maturation (Grade A). Other strategies to improve outcomes in these patients are metformin therapy in PCOS patients (grade A), aspirin administration (Grade A), use of low-dose hCG co-trigger (Grade B), dopamine agonist administration starting at the time of hCG trigger (Grade A), and luteal hormonal support

(Grade B). A freeze-all strategy is strongly recommended to eliminate the risk of late-onset OHSS [6,37].

Ectopic pregnancy is the leading cause of maternal morbidity and mortality in the first trimester of pregnancy. Incidence in patients undergoing assisted reproductive technology is 1.5%–2.1%, significantly higher than in women with natural conception [38].

Long-term outcomes for progeny and women

Progeny

As an increasing number of babies are being conceived with IVF techniques, registries should start to focus on the health of the progeny, considering not only perinatal outcomes but also long-term outcomes [2]. The physical, neurological, and developmental health of children born after IVF is undoubtedly one of the major concerns related to this aspect [28,39]. Overall, the neuromotor, cognitive, language, and behavioral outcomes in children born following IVF and ICSI appear similar to those of naturally conceived babies [28,40–43]. The only consistently increased adverse outcome reported by literature is cerebral palsy, which is not wholly accounted for by the increased preterm birth rate [28,44].

The Backer hypothesis postulates that adverse antenatal conditions can lead to long-term consequences in adulthood. Ceelen et al. observed cardiometabolic differences and increased peripheral adipose tissue mass and fasting glucose in children born after IVF. These data suggest the need for further metabolic epidemiological studies in IVF adolescents and adults [28,45,46].

Oncological risk

The delay or the inability to achieve pregnancy may increase the risk of invasive ovarian, endometrial, and breast cancer [47,48]. Conversely, fertility treatments (temporary ovarian stimulation) do not appear to increase the risk of breast cancer (Grade B), cervical (Grade B), and colorectal cancer (Grade B) [47,49]. Only studies about the risk of endometrial cancer have discordant results reporting a nonsignificant increased risk for endometrial cancer (Grade B). Thus, infertility per se and not its therapy seems to be the main risk factor [47].

There is insufficient evidence supporting in association between fertility drugs and an increased risk of melanoma and non-Hodgkin lymphoma (Grade C) [47,49]. Conversely, being pregnant at an earlier age is a significant protective factor for gynecological cancers. Moreover, fertility counseling is an opportunity to address lifestyle changes and cancer prevention strategies [47].

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CHAPTER

39

Regulation, data management, informed consent, and legal issues for ART

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Introduction

The number of assisted reproduction (AR) treatments undertaken worldwide has risen steadily since 1990. In general, AR represents a story of many stakeholders: the client, the gamete/surrogacy provider, the clinician, the religious leader, and governments, each asking very different questions of AR. For the client: how can I become a parent? How can I overcome a genetic condition? For the gamete provider: how can I obtain enough money to improve my life or help others? For the fertility agent: how can I use my experience and clinical judgment to help patients and build a career and earn sufficient profit from it? For the religious leader: how can I combine religious beliefs and technologic innovations? For governments, the main challenge has been to conciliate these often-competing goals while serving the state's economic agendas and fulfilling its basic role in determining citizenship and legal parentage. AR has been over many years the subject of public inquiries, religious and legal cases, often leading to passionate public debate. The current global legislative map is complex and diverse [1]. As the International Federation of Fertility Societies observes, "The position adopted for various issues is dependent on different social, cultural, and political norms," [2] where each government has its own jurisdiction on what to regulate and how to do it, intended to facilitate it, to limit it. or sometimes to do both at once.

Patient counseling and informed consent have both legal and ethical dimensions, which can be in tension with one another when an emphasis on completing consent forms supplants a focus on effective treatment conversations. Patient counseling is best understood as both a process and a relationship that lasts for the duration of the patient's treatment experience. Patient counseling is especially critical within assisted reproductive technology (ART), where treatment decision-making entails many unique choices that go beyond medical decisions, such as determining prior to treatment what should happen to surplus embryos, should patients die or become divorced.

The aim of this chapter is to provide an overview of ART regulation, presenting its major legal issues, and to underline the specific aspects of quality or data management and informed consent in ART treatments.

ART regulation

Technologic advances in AR have provided ways to separate the creation of children from heterosexual intercourse and enabled families to come into being who potentially have no genetic ties, even though, initially, AR was intended as a therapeutic treatment only for infertile couples. Due to political, ethical, and social reasons related to AR practice, each country has a different perspective when it comes to ART. Several papers have already pointed out how many factors contribute to these differences, including financial issues (affordability, treatment costs), customary law, cultural and belief dimensions. In addition, individual and professional options may play different roles in different societies [3–5].

ART regulation in European countries

In 1999, with the purpose to organize *in vitro* fertilization (IVF) data collection, highlight gaps, and update the information, the European Society of Human

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Reproduction and Embryology established the European IVF Monitoring Consortium. At the present time, 43 European countries are included in the consortium [6]. All the annual reports published so far mirror the huge diversity of the use of AR techniques in Europe. The last detailed survey was published in 2020 [7], showing great variation not only in terms of organization and treatment outcomes, but also regarding the availability of techniques for infertile individuals [8]. In addition, the survey not only confirms the great diversity across countries, but also highlight differences within the same country. This does not mean an actual lack of legislation, as ART practice is regulated by legal norms in all European countries (including European Union directives in member states), in spite of the absence of specific legislation in a few. The collected data shows that accessibility to ART is limited only to infertile couples in 11 of the 43 countries, with 30 countries offering ART to single women and 18 to female couples. In only five countries the access to ART is permitted to all patient groups (infertile couples, single women, male and female same sex couples). Sperm donation is allowed in 41 countries and egg donation in 38. Simultaneous donation (sperm and egg) is accepted in 32 countries and embryo donation in 29. Surrogacy is possible in 16 countries. Excluding marital/sexual situation, another main limitation criteria is female age: minimal age is set at 18 years and maximum ranging from 45 to 51, with no age limit in some countries. Regarding third-party donation, in some countries there are some constraints in the number of children/families born from the same donor and in the maximum number of egg donations. Great differences across countries are shown concerning donor anonymity: strict anonymity, anonymity just for the recipients (not for children when reaching legal adulthood age), mixed system anonymous and nonanonymous donations, and strict nonanonymity. Another topic that displays marked variations across countries is public funding and limits to the provision, with only four countries providing no financial assistance at all [7].

The level of acceptance of ART and its usage in each country depends on several aspects (financial, social, cultural, and religious), and few papers have focused on the role of implicit cultural normative values. Indeed, the link between women's higher educational status and delayed childbearing is well established. However, although the variation in the proportion of highly educated women across nations is a reality, no statistically significant relation between the percentage of middle-aged women with tertiary education and ART usage has been reported [4]. On the other hand, the study conducted by Billari et al. [9] concludes that the higher the social age norm for childbearing is, the greater is the availability of ART clinics. The association between cultural factors and the prevalence of ART has also been studied by Präg and colleagues [5]. The results of their study show a positive linear correlation between the average ART normative approval in a country and the number of treatments performed. Access to ART and its usage are also influenced by couple and gender requirements, which have also great social relevance. In this context, third-party donation and surrogacy are clear examples. These factors also govern financial assistance and restrictions [10], which are quite variable among countries. So far data on these significant social circumstances are very scarce and limited to only empirical information [11]. Regarding the preservation of reproductive potential, at the present time no legal specific legal dispositions are in place. Virtually, cryopreservation of gametes, embryos, and gonadal tissue is performed all over Europe, with some exceptions when embryos are involved. However, oocytes cryopreservation (vitrification) for nonmedical reasons is not permitted in eight countries [7]. All this data, however, must be considered with caution. They describe a very complex reality, whose legislation represents a constant topic of debate and undergoes continuous evolution.

The role of religion

In reproductive politics, religion represents one of the most influencing forces in ART regulatory history. AR has, indeed, highlighted and complicated the role of religious authorities, presenting new dilemmas. However, the views on ART and its use may vary within the same religion. In most religions (Christianity, Islam, Judaism, Hinduism, and Buddhism), the conservative view prohibits the use of ART and third-party reproduction, while the liberal religious view allows them under certain circumstances [12–16]. The case of Italy's Law 40/2004 highlights the strict connection between religion, politics, and legal dynamics that have accompanied the history of ART since the 1970s. In 2004, the Italian government passed its first and only piece of legislation on ART. The law reflected, above all, the Catholic Church point of view, where the welfare of the human embryo should take precedence over other possible concerns. The law banned embryo experimentation, preimplantation genetic testing of embryos, as well as embryo freezing in all but exceptional cases. It prohibited surrogacy, the use of donor gametes, and the access to ART to same sex couples and single women, making Italy one of the most restrictive countries in the world when referring to ART options.

Cross-border reproductive care

An emerging dilemma on the global healthcare agenda is represented by cross-border reproductive care (CBRC), also called fertility tourism or reproductive tourism. It is a complex global phenomenon through which many people are traveling internationally to obtain fertility treatment. Usually, patients travel abroad to less legally restrictive countries to overcome legal restrictions in the home country [17,18]. The most common forms of fertility treatment are IVF, intracytoplasmic sperm injection (ICSI), sperm donation, egg donation, embryo donation, commercial surrogacy, preimplantation genetic diagnosis (PGD), sex selection, and fertility preservation. Despite IVF and ICSI being legally permitted almost all over the world, the legal restrictions concerning the access to these technologies vary among countries (e.g., sexual/marital status, female age, number of embryos to transfer), finally promoting "fertility migrations." Due to the higher success related to fewer regulations, some countries are considered optimal destinations (USA, Spain, Czech Republic, Denmark, Belgium, and Israel) [19]. Another common reason for CBRC is gamete donation. Denmark is a very well-known market for sperm donation due to its liberal legislation and the appeal of its donor population to those needing donor sperm, particularly in Europe. Its legislation also allows the use of both anonymous and directed sperm donation for lesbian and single women [20]. Spain and Czech Republic are the most common destinations for egg donation, which is still illegal in many countries worldwide [19]. Spain alone makes almost half of all egg donation procedures in Europe [21]. The same thing happens with embryo donation, which in some countries is also allowed for unmarried couples, as well as single, gay, and transgender individuals (Spain, Czech Republic, Belgium, USA, and Russia) [22]. Altruistic or commercial surrogacy represents a reproductive option for women affected by congenital malformations or by diseases that interfere with normal pregnancy. Also cancer survivors who underwent gonadotoxic chemotherapy and radiotherapy may seek surrogacy [23]. Due to ethical and religious reasons, surrogacy is not allowed in many countries. In Europe, thanks to its liberal laws regarding fertility treatments, only Russia permits surrogacy [24]. PGD and sex selection are other common reasons for CBRC. In Europe these techniques are not allowed, except for selective cases (genetic and inherited diseases). The major destination for patients seeking PGD and sex selection is the United States [25]. Advances in cryopreservation have enabled successful international shipment of frozen gametes (oocytes, sperm, embryos) from one country to another, increasing the potential of a global CBRC market. Recently, the so called "social freezing" (elective egg freezing or oocyte cryopreservation for nonmedical reasons) has gain popularity in clinical practice because it represents a way for single women to delay childbearing while preserving their fertility [26]. However, fertility preservation options are not allowed in many countries, and the most common destinations for this type of fertility tourism are Belgium, Denmark, Germany, United States, and Israel [27].

Unquestionably, CBRC raises many ethical, economic, and social debates, which sometimes are the same old, unresolved questions related to ART in general. In addition, CBRC involves many different parties (patients, doctors, brokers, donors, surrogates) with their own goals and ethical or social dilemmas.

Quality and data management

Quality and data management represent two key aspects for a successful fertility clinic with a high standard of care. To achieve that, a strong quality management system (QMS), which includes quality policies, quality assurance, and quality control, is required. Keeping a high-quality standard of care is a continuous process that implies regular risk assessment and audit to ensure the highest expectation for both patients and staff members. Quality should be embedded within the organization and constantly supported by full managerial supervision. This leads to troubleshooting via a cycle of continuous improvement, which enhances both quality and patient and staff satisfaction. A fertility clinic that operates through well-established QMS is effective and implements its quality level on a daily basis. The four-step quality management tool of "plan-do-check-act" (also known as the Deming Cycle) is often used in many industries to control and improve processes and procedures [28].

To achieve an optimal data management and to ensure consistency throughout all procedures, documentation control is imperative, especially in a fertility clinic. Good document control guarantees that all staff members operate following the same standard operating procedure (SOP), using the same recording methods (e.g., forms, worksheets, reports) to minimize interoperator variations. It is advisable that only current and validated versions of documents should be available. If new documents or forms are approved, all staff should have access to latest and up-to-date form. Adherence to older versions of SOPs could have a negative impact on patients' care, their gametes/embryos, and on staff members. Any deviation, accident, or event from an agreed process should be recorded as a nonconformance to ensure traceability. Staff should be given the possibility to justify deviations, and if required, SOPs

should be updated accordingly. Recommendations for documents and data management are as follows:

- Allocate every document with a code to allow traceability (e.g., SOP-XXX).
- Each time a document is reviewed the previous version should be archived for reference only, and access should be restricted.
- Documents should be modified by someone with expertise and peer reviewed before approval.
- Documents should be available all times to staff members in a format that cannot be amended (e.g., PDF versions).
- Documents should have the same template (consistency and ease of use).
- Where applicable, references should be present.

Informed consent

Informed consent represents the decision of a patient to undergo a therapeutic treatment and improves patient participation in making decisions about their health. This is the most important and delicate moment of the treatment since it contains information on therapeutic options. In ART, communication and the understanding of the information received are crucial issues, due to their medical, ethical, and psychological implication [29]. Patients consented for AR treatments are fully informed about risks, benefits, and ethical considerations before starting treatment. The document provides a basis of uniform knowledge of the topic for all ART patients and could facilitate their access to information, thus making their understanding more meaningful. Nevertheless, many reproductive specialists and lawyers expressed doubts about patients' understanding of the balance of risks, benefits, and alternatives to ART.

Given the complexity of informed consent documents in ART, the American Society of Reproductive Medicine has created a model template to facilitate the process for ART patients/couples [30]. Patients are often unaware of what therapy protocol they will follow even if they have signed the informed consent. Most of the time they are confused, but their expectations are very high to overlook the treatment. Many couples undergo ART without understanding the real implications of treatments in their personal health or the health of future children born through ART. Do patients really read the informed consent? Why do patients often sign consent without reading and asking questions? Are informed consents too complex for them? At the present time all these questions are still unanswered. For this reason the informed consent process could be organized as a three-step (three meetings) process, thus allowing couples to have the time to be acquainted with ART issues. In ART centers, physicians and psychologists should collaborate harmoniously, favoring patients' medical and emotional needs.

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CHAPTER

40

Impact of COVID-19 on ART (Assisted Reproductive Technologies)

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Introduction

An unknown pneumonia was reported the first time in Wuhan, China, in December 2019 related to a virus of the coronavirus family [1]. The pandemic of SARS-CoV-2 responsible for the disease called COVID-19 represents the most exceptional health, social, economic, and humanitarian crisis known to humankind since the H1N1 flu of 1918. It has affected 213 countries, infecting over 100,000 people daily worldwide, with hundreds of thousands of deaths [2]. The manner of transmission for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is through direct person-to-person contact by respiratory droplets released when someone with the virus coughs, sneezes, or talks [3]. A possible way of transmission through the fecal-oral route is demonstrated by the presence of the virus in urine, feces, and tears [4]. COVID-19 affects the respiratory system, and people with COVID-19 present some symptoms in the beginning of the illness including dry cough, fatigue, fever, breathing difficulties, and muscle pain, and these symptoms may develop to pneumonia, loss of taste and smell, diarrhea, and lymphopenia [5–7].

When the virus enters the host cell by binding to angiotensin-converting enzyme 2 (ACE2) receptors, it is predicted that cells expressing ACE2 receptor in different tissues and organs have the risk of being affected [8]. ACE2 receptor is expressed in many tissues and organs including lungs, intestine, kidney, testis, and many others [8]. An important and interesting topic that emerges in the COVID-19 era is the ability of the virus to affect male and female reproductive abilities and whether pregnant women with COVID-19 are at increased risk of fatality or comorbidity. When the fusion of the virus with the target cell membrane occurs, the virus releases its genome, and using the host cell organelles to replicate its RNA, it releases new mature virion to target other cells (Fig. 40.1) [9,10].

SARS-CoV-2 and reproductive system

So far, there is no evidence that this disease can be transmitted through sexual secretions and the pregnant mother to the child, but there are doubts and suspicions in this regard [11,12]. In a report from a Wuhan university hospital in China, none of the serum or throat swabs of the newborns of six parturients with confirmed COVID-19 displayed SARS-CoV-19 according to reverse-transcription polymerase chain reaction testing. However, their neonatal umbilical blood did display virus-specific antibodies. Five infants had elevated IgG concentrations and two newborns had IgM antibodies. Unlike IgG, the larger macromolecular IgM does not usually pass through the placenta from the maternal compartment to the fetus. In another study, of mothers with SARS, abnormal weights and pathology were observed in the placentas of two patients infected with SARS-CoV in the third trimester. It has been speculated that the IgM detected in the neonates could have evolved from the abnormal or damaged placentae or, on the other hand, possibly could have been generated by the neonates in response to transplacental viral infection [13,14].

The ACE2 receptor, used by SARS-CoV-2 for infectivity, is overexpressed in the testes and male reproductive system [6,7]. Previous evidence on another virus of Coronavirus family (HCoV-229E) has been revealed in vaginal discharge [8]. Therefore, even if there is no clear evidence that SARS-CoV-2 could be transmitted through sexual secretions, and such concerns about many and more common infectious diseases, there is a perceived risk of infertile couples to affect the process of continuing

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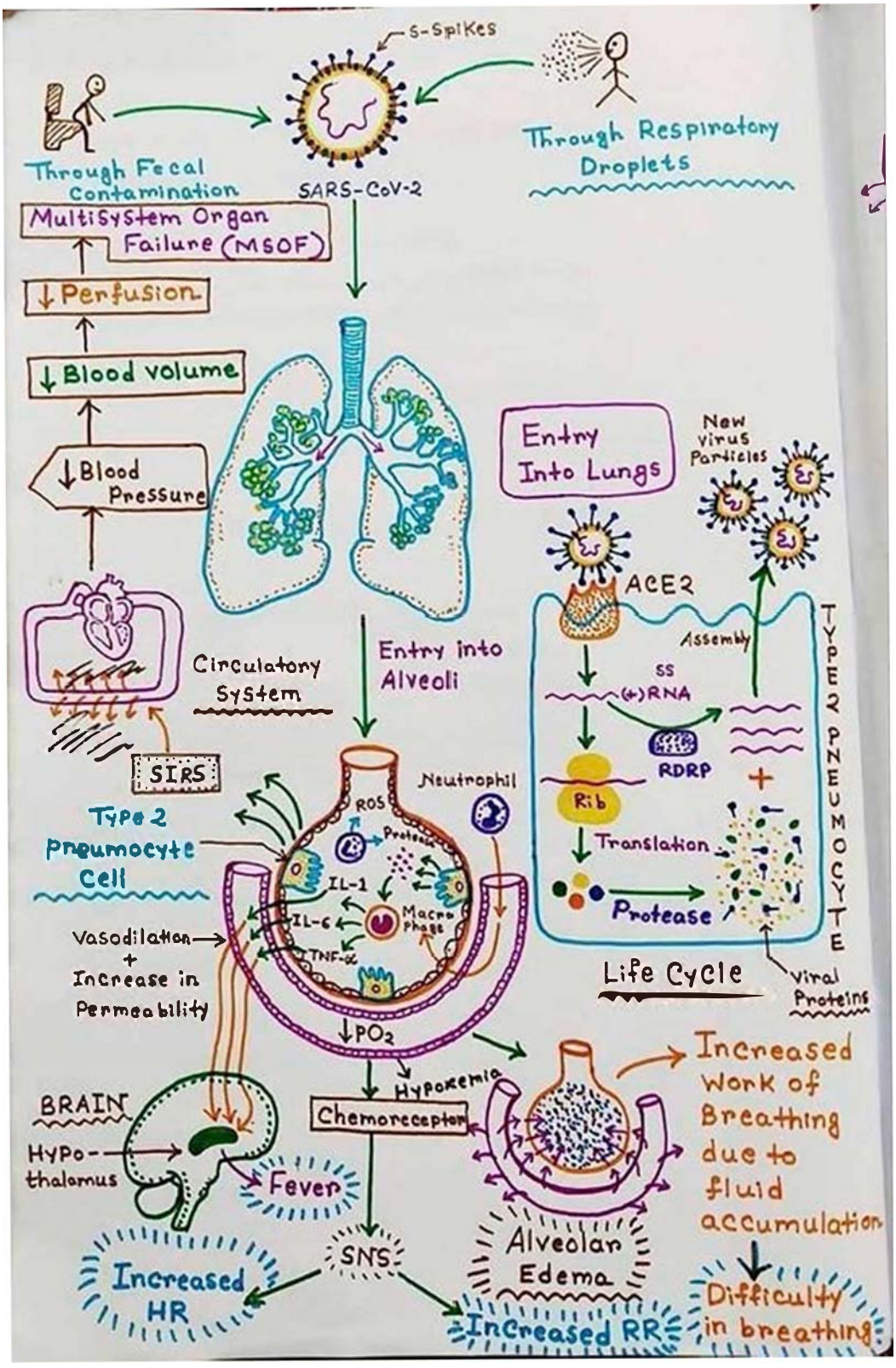

FIGURE 40.1 SARS-CoV-2 pathogenesis. *By permission K. Lehman, PhD.*

infertility treatment [9]. Worldwide, the rate of primary infertility in women aged 20–44 is estimated at 1.9%, even if the prevalence of infertility varies from country to country [10]. According to data collected by the World Health Organization in 2004, there are 187 million infertile couples in developing countries except for China. If these first treatments do not work or are considered inappropriate, these couples are recommended to use assisted reproductive technology (ART). One of these methods is *in vitro* fertilization (IVF), which accounts for more than 99% of ART. When starting treatment, couples must endure a variety of treatments, including ovarian stimulation, regular monitoring, egg retrieval, embryo transfer, and the use of progesterone supplements [11]. Therefore, couples can be expected a stressful experience of infertility because they endure the pressure of time-sensitive treatments and the fear of failure [12]. IVF couples are more likely than non-IVF couples normally to report an unstable relationship due to length and treatment expectations [13]. Before the COVID-19 pandemic, some viral infectious diseases such as Zika virus infection, which can cause pregnancy abnormalities and microcephaly in the fetus [14], influenced the attitude and decision of infertile couples to continue infertility treatment with ART [15].

Some questions in the practice of ART

Due to the fact that the transmission of SARS-CoV-2 has imposed restrictions to personal freedom and partial or complete lockdowns have been implemented to safeguard public health, with a noticeable impact on reproductive practice [16], and in consideration of recent evidence of the risk in pregnancy [17], some of these important aspects need to be evaluated:

1. At time of pandemic peak, it is debated if there is a rationale for an accurate identification of infertile women who are time-sensitive as suggested by some experts' opinions. Effective personalization of stimulation based on maternal age and ovarian reserve [18] is mandatory for prevention of ART-related risks such as ovarian hyperstimulation syndrome, complications associated with egg retrieval, and multiple pregnancies, when preventing complications and limiting burdens for national health systems [19].
2. Should laboratory procedures change in consideration of risks of transmission for biologists or gametes or embryos?
3. Is it useful to personnel of ART clinics or patients, with the aim to realize COVID-19-free centers, to perform diagnostic tests and with what kind of recurrence or timing with respect to ovulation induction or endometrial priming? Some clinics arranged an informative pre-triage questionnaire for all access to identify risks factors, and this evaluation could have avoided the access to COVID-19 patients

Indeed, at this time we do not have an answer validated by literature data for all questions.

The impact of COVID-19 on ART care in Europe and the United States

The experience of the last months of the COVID-19 pandemic shows conflicting evidence related to couples' decisions to seek infertility treatment. A recent study investigated the attitudes and consciousness of infertile couples regarding candidate therapy about continuing treatment during COVID-19 outbreak [20]. In this study among 92 patients (46 couples), we found that more than 60% of individuals did not have a reduced motivation to continue treatment. One-third of patients who had a decreased motivation to continue treatment felt the need because of their infection and the transmission of the disease to the fetus and others. Also, in the present study, there was no significant relationship between the presence of COVID-19 symptoms and the level of awareness of couples.

So, with the aim to contribute to a useful management of fertility health providers for diagnosis and treatment of infertile couples, all over the world, human reproduction societies published suggestions for managing patients who currently are or will be undergoing infertility treatments through ART. The International Federation for Fertility Societies recommended on March 12, 2020, that patients who are considering pregnancy or who are currently undergoing fertility therapies should consult with their personal physician for planning further steps [21]. In the same period the American Society for Reproductive Medicine (ASRM) published a bulletin suggesting that patients who are highly likely to suffer from COVID-19 (i.e., patients who tested positive for SARS-CoV-2 or who have been exposed to confirmed COVID-19 cases within 14 days of onset of their symptoms) should consider freezing oocytes or embryos and avoid embryo transfer until they are symptom-free; however, this recommendation was emphasized to not necessarily apply to suspected COVID-19 cases as symptoms of COVID-19 closely resemble those of other more common forms of respiratory disease [22]. On March 17, 2020, the ASRM published a new document named "Patient Management and Clinical Recommendations During the Coronavirus (COVID-19) Pandemic" in which the key recommendations were as follows: 1. Suspend initiation of new treatment cycles, including ovulation induction, intrauterine inseminations, IVF, including retrievals and frozen embryo transfers, as well as nonurgent gamete

cryopreservation. 2. Strongly consider cancellation of all embryo transfers whether fresh or frozen. 3. Continue to care for patients who are currently "in-cycle" or who require urgent stimulation and cryopreservation. 4. Suspend elective surgeries and nonurgent diagnostic procedures. 5. Minimize in-person interactions and increase utilization of telehealth [23].

The European Society of Human Reproduction and Embryology issued a statement on March 14, 2020, detailing that so far, only a few cases of COVID-19 during pregnancy have been reported, so the respective data must be interpreted with caution as no information is available regarding potential effects of COVID-19 infection during the initial stages of pregnancy; furthermore, medical treatment administered to severe COVID-19 cases may include drugs that are contraindicated during pregnancy [24]. The same publication advised that all patients considering or planning treatments, independently of confirmation or suspicion of COVID-19 infections, should avoid becoming pregnant at this time and consider deferring pregnancy by freezing oocytes or embryos for embryo transfer at a later point [24].

The Italian Society of Human Reproduction, in the country with the highest spread of the disease in the first wave, issued a statement, on March 10, 2020, detailing that it is advisable to conclude the procedures that have been started to date, inviting and suggesting that patients postpone the performance of the services until the end of lockdown [25].

So, assisted reproduction centers in various countries, according to different organizational models, have implemented and, in some cases, changed the procedures for accepting and managing the infertile pair and their treatments (Table 40.1).

The impact of vaccine for couples who want a pregnancy or for responsible parenting

In Italy since March 3, 2020, in three successive waves, COVID-19 infections and hospitalizations have reached record levels, with the result of more than 3,400,000 cases and over 105,300 deaths from COVID-19 confirmed at the Ministry of Health as of March 21, 2021.

At the moment, public health measures to mitigate and control the pandemic continue to rely heavily on the use of masks and means of personal protection, social distancing, and frequent sanitization measures that present critical issues in minimizing the spread and existence of COVID-19 disease.

It is hoped that widespread vaccination will further and definitively limit the viral spread and shorten the duration of the pandemic and its impact on its morbidity and mortality.

TABLE 40.1 IVF protocol modifications pre- and post-COVID-19.

Pre-COVID-19	Post-COVID-19
In-person consultations	Telemedicine consultations
In-person meetings with reproductive nurses and medical staff	Virtual meetings with reproductive nurses and medical staff
Unrestricted travel with no personal protective equipment	All staff were issued and encouraged to wear masks on their way to and from the center; all patients were issued masks to wear at the center if they did not already have one
Hepatitis B, C, HIV, and syphilis tests prior to stimulation	Consider addition of SARS-CoV-2 testing prior to start of stimulation (if positive, do not start)
Multiple visits during stimulation (typically 5–7 visits before egg retrieval)	Space out visits during stimulation where appropriate (3–4 visits before egg retrieval); temperature checks at each visit (patients and staff)
Crowded waiting rooms	Patients immediately roomed after checking in; vital signs and blood draw done while in the exam room; seating and location of staff were changed to maintain >1 m distancing wherever possible
Rapid turnover of ultrasound examination room	Empty waiting room, thoroughly wipe down surfaces, longer interval between procedures
Partner encouraged to accompany patient at visits, egg retrieval, and transfer	No partners or visitors (encourage use of video-telephone products)
Sperm production on site in small collection room	Off-site sperm production
Signed consent forms with common pens	Electronic consent forms with clean pens available if needed to sign forms

Currently, four vaccines are authorized and recommended in Europe and the United States to prevent COVID-19.

The COVID-19 Pfizer and Moderna vaccine

Pfizer and Moderna vaccines are both mRNA vaccines that do not contain live viruses. Both vaccines require a series of two injections at intervals of 21 days (Pfizer-BioNTech) or 28 days (Moderna). Vaccines provide mRNA in cells near the injection site. This mRNA instructs the body's cells to replicate the coronavirus Spike protein. This protein, in turn, is recognized by the body as foreign, generating protective antibodies. The mRNA itself is rapidly degraded and does not enter the nucleus of the cell. In particular, the Pfizer-BioNTech COVID-19 vaccine is a vaccine against mRNA modified with lipid nanoparticles. The lipid coating of nanoparticles binds to the cell membrane, facilitating the entry of the mRNA segment into the cell. Rarely, some individuals may be allergic to a part of the lipid nanoparticle known as polyethylene glycol, a common component in other injectable medicines. As a result, caution is advised when administering the vaccine to those individuals who have experienced severe allergic reactions to previous vaccines or injectable drugs.

The COVID-19 AstraZeneca and Johnson vaccine

The vector (a different, harmless virus) will enter a cell in our body and then use the cell's machinery to produce **a harmless** piece of the virus that causes COVID-19. This piece is known as a Spike protein, and it is only found on the surface of the virus that causes COVID-19. Then, the cell displays the Spike protein on its surface, and our immune system recognizes it does not belong there. This triggers our immune system to begin producing antibodies and activating other immune cells to fight off what it thinks is an infection. At the end of the process, our bodies have learned how to protect us against future infection with the virus that causes COVID-19. The benefit is that we get this protection from a vaccine, without ever having to risk the serious consequences of getting sick with COVID-19. Any temporary discomfort experienced after getting the vaccine is a natural part of the process and an indication that the vaccine is working.

The COVID-19 AstraZeneca vaccine and Johnson vaccine are intended to prevent COVID-19 disease and have been evaluated in clinical trials in people aged 18 and over. It is designed to prepare the immune system to identify and counter the coronavirus (SARS-CoV-2) responsible for COVID-19 disease. The vaccine consists of a replicable chimpanzee adenovirus (ChAdOx1, Chimpanzee Adenovirus Oxford 1) that is modified to convey genetic information intended to produce the Spike protein of the SARS-CoV-2 virus. The technology of the viral vector used for this vaccine has already been successfully tested and is used to prevent other diseases. The COVID-19 AstraZeneca vaccine is administered in two injections, in the muscle of the upper arm. People who have been vaccinated with the first dose of AstraZeneca COVID-19 vaccine should receive the second dose of the same vaccine to complete the vaccination cycle, ideally during the 12th week, and in any case at least 10 weeks after the first dose.

After administration, the modified and modifiable adenovirus binds to the surface of human cells and penetrates the nucleus of the cell. There it provides the genetic code to produce the Spike protein of the coronavirus. Circulating immune cells (T cells) recognize Spike protein stimulus and induce a cellular immune response and the production of virus-neutralizing antibodies. The immune system also produces cells with defensive memory against the coronavirus Spike protein, facilitating recognition and rapid immune response in case of future exposure to COVID-19. Vaccination then introduces into the cells of those who vaccinate only the genetic information needed to build copies of the Spike protein. Adenovirus is not able to replicate so cannot spread in the organism.

In pregnancy or during ART treatments, the information should contain the finding that the evidence, on the use of the vaccine in pregnancy and in women who want to get pregnant, is considered sufficient today to be able to recommend the routine use of the COVID-19 vaccine.

The vaccines cannot replicate and consequently cannot create a condition of infection for the woman or for the fetus. We can say that the vaccine is not contraindicated either before or even during pregnancy, especially if the woman's health condition combines pregnancy with other risk factors such as advanced age, diabetes, cardiovascular disease, or obesity that could make her at more serious risk of COVID-19.

If the woman seeking pregnancy or already pregnant is in a clinical or environmental condition vulnerable to COVID-19, the option of the vaccine should be addressed and discussed in a sufficiently short time with her obstetric gynecologist or general medicine doctor. The following are the clinical conditions that could expose the woman to a high risk of serious complications with COVID-19:

- maternal age (>35 years)
- patients with diabetes or obesity
- patients with serious respiratory problems including cystic fibrosis and severe asthma sufferers
- sickle cell anemia patients

- patients undergoing immunosuppressive therapies that can significantly increase the risk of infection
- patients suffering from dialysis or chronic kidney disease
- patients with significant congenital or acquired heart disease
- in addition, all patients working in healthcare environments or social care facilities, including residential homes, should face the vaccine option in a short time because the risk of exposure to COVID-19 could be high, even if their health is not at risk of serious complications with COVID-19

Patients with a history of severe anaphylactic reactions or severe allergy, or who are already aware that they are allergic to one of the components of the available vaccine should consult with the gynecologist-obstetrician or their general practitioner. As with all vaccines, this should also be administered under strict medical supervision. People who experience a severe allergic reaction after receiving the first dose of vaccine should not receive the second dose. Allergic reactions (hypersensitivity) have been observed in some subjects who have been given the vaccine. Since the vaccine has been used, there have been very few cases of anaphylaxis (severe allergic reaction).

It is evident that personal protective measures must remain firmly in force until the conclusion of the entire vaccination path of the general population due to the following:

1) it is not yet known whether a vaccinated individual can spread the virus if infected with SARS-COV-2;

2) Protection starts about 3 weeks after administration of the first dose of AstraZeneca COVID-19 vaccine and persists up to 12 weeks. However, up to 15 days after administration of the second dose the protection may be incomplete. In addition, as with all vaccines, vaccination with AstraZeneca COVID-19 vaccine may also not protect all vaccinated subjects.
   - Pregnancy-seeking patients with fertility treatments have no reason to postpone or even avoid pregnancy, just as already pregnant patients do not need to avoid the parenting project before or after vaccination.
   - Patients who conceive in the window between the first and second dose of the vaccine may be offered the second dose of the vaccine at the appropriate interval.
   - Doctors should promote vaccination to patients, their communities, and the public. Preliminary data suggest that populations most at risk of serious COVID-19 disease may sometimes be hesitant to get vaccinated, and specific efforts to increase vaccine administration in these communities should be undertaken. Additional COVID-19 vaccines using different platforms are being developed.

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Index

'*Note:* Page numbers followed by "f" indicate figures, "t" indicate tables and "b" indicate boxes.'

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Management of Infertility

A Practical Approach

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Management of Infertility: A Practical Approach offers an accurate and complete reference for the management of infertility and a robust step-by-step guide for assisted reproduction technologies (ARTs), including how to plan, design and organize the clinical setting and laboratory. It also provides an evidence-based, complete and practical description of the available methods for diagnosis and management of male and female infertility.

This book is designed to help researchers, students, and clinicians worldwide to gain a complete knowledge about both basic and advanced knowledge for the diagnosis and management of infertility and related disorders.

Key Features

- Provides step-by-step description about how to design, plan and organize an Assisted Reproductive Technology (ART) unit and laboratory
- Discusses both male and female factor infertility, providing a complete guide for the diagnosis and treatment of the different causes of infertility
- Addresses all the techniques of assisted reproduction and in vitro fertilization, discussing their use in different clinical settings

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