

# Forty years of IVF

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## INTRODUCTION

**Craig Niederberger, M.D.**

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1978 was a remarkable year (1). The Nobel prize was awarded for the discovery of cosmic microwave background radiation that furnished the first direct evidence of the big bang and that time itself in our universe had a starting point. The very first online exchange forum, the predecessor of all public communications, like blogs and social media, made its debut in Chicago. Sony invented the Walkman, the first portable stereo and the father to the iPod. But without a doubt the technological advance that rivaled humans walking on the moon some 9 years earlier was the birth of the first IVF baby.

That human gametes could be fertilized outside of the mother's uterus in an instance where reproduction would be possible no other way, leading to an embryo that could then develop as a fetus in the mother and born live changed the world. Millions upon millions of children have been born since that time who simply could not have been without the development of IVF.

This monograph chronicles the arc of development of IVF that began 40 years ago and follows the new questions asked by the beginning of this new technology and the possibilities that it created. It is designed not to be an exhaustive data-driven compendium, but a readable narrative of what has happened in the past, what is happening at present, and what may happen in the future. For those who are students of reproductive medicine and who did not experience living through the entirety of these past 40 years, it tells the story of how and why you do what you do. For those who predate 1978, it serves as a thrilling ride through all of the varied roads that IVF paved.

We begin with the laboratory, as that is where the heart of IVF was born and where the foundations of our field developed. At present our IVF laboratories appear totally different from those in 1978. Initial implantation rates that were <5% per embryo replaced were continuously increased, at present, to rates >50%. The low implantation rates of the early days was problematically addressed by increasing the number of embryos replaced into the uterus and aggressive ovarian stimulation protocols, unfortunately often uncontrolled, introducing the two main complications of IVF in these 40 years—multiple pregnancies and ovarian hyperstimulation syndrome (OHS). These challenges were intensively reported, studied, and almost completely resolved.

Access to these early embryos in the laboratory also introduced a series of medical techniques that have been tremendously useful and provide new pathways to fight against disease by applying preimplantation genetic testing. In the future perhaps we will be able to treat genetic diseases by genomic editing.

Other techniques were developed to preserve fertility in men and women with life-threatening illnesses for whom the use of life-saving but gonadotoxic treatments may compromise their future fertility. Cryopreservation of the male gamete predated IVF, but oocyte cryopreservation was far more challenging, which was finally solved a quarter century into the story of IVF. Freezing the oocyte has not only been effective in preserving fertility, but has also given practitioners the ability to split the reproductive cycle into different steps to improve outcomes and reduce complications, such as OHS. In many ways, at present, oocyte cryopreservation has changed the way we perform IVF.

The protocols of controlled ovarian hyperstimulation (COH) have also undergone substantial evolution, mainly due to the research and development of new therapeutic agents. But we also have learned that overly aggressive management of the ovaries was detrimental to a woman's reproductive system. As a result, at present, the way we manage COH is completely different from 40 years ago. The way we monitor patients and perform oocyte retrievals has also changed—the development of vaginal ultrasound in the 1980s has been a fundamental pillar in the advancement of IVF.

Arguably the most important advance since 1978 in the trajectory of IVF was the ability to insert a single sperm into an ovum and achieve a live birth. Intracytoplasmic sperm injection (ICSI) made possible biological parenthood when only a few sperm were available in the ejaculate, and ultimately the previously unthinkable source of sperm, the testis. But this technique raised its own questions: which single sperm should one choose, how, and why? We continue to search for answers to these fundamental challenges that this remarkable reproductive tool engendered.

Of course IVF did not just fundamentally change reproductive possibilities for a mother, it did so for the father as well. From evaluating male reproductive potential to therapy, IVF opened avenues of investigation, created dilemmas, and opened doors previously unimagined. With the ability to use gametes obtained directly from the testis, we now can truly probe medical therapies as we enter an age of controlled spermatogenic stimulation.

We conclude with the big picture, how IVF created legal and ethical challenges, and how mental health and psychology play central roles in this new world of reproductive technology. Finally, luminaries in our reproductive medical societies, our places of vigorous interaction in moving our field forward, describe how they came to be and how they are propelling us into a better future.

We hope that you enjoy reading this monograph as much as we enjoyed making it, and that wherever you are in the arc of reproductive medical history, it will give you insights into the remarkable field that gives birth to the unborn.



# ADVANCES IN THE IVF LABORATORY

## HOW THE EMBRYOLOGY LABORATORY HAS CHANGED!

Jacques Cohen, Ph.D., H.C.L.D.

The human assisted reproduction laboratory has undergone near-complete metamorphosis since the early days of IVF (2). The increasing success rates with this technology are owed in large part to these changes, including improvements in quality control and embryo culture systems. Equally important have been the advances in personnel training and exchange of ideas and communication, which is not surprising as IVF matured during the information age. The first IVF laboratories were essentially improvised, sometimes fitted into existing surgery suites. Before 1985, there were no “add-on” procedures, no egg donation or surgical sperm retrievals, and no cryopreservation or micromanipulation. At that time, there was little governmental oversight with few licensing requirements. There was no specialized clinical or laboratory training, therefore, skills were acquired mostly through apprenticeships. The procedures in the laboratory were performed by researchers with experience in experimental embryology and veterinary science. These pioneers were establishing rules and principles, which evolved into international guidelines and standard operating procedures.

As astounding as the relatively quick rise of IVF may have been, 40 years ago IVF was rarely viewed as an obvious treatment for subfertility. Despite numerous obstacles—most important, a lack of public funding—assisted reproduction is now well established. Its path seems to follow Moore's Law with linear increases in implantation rates, being just shy of 1% annually, corrected for maternal age (3). This linear progression has been relatively constant since the early days and may predict a time in the near future when there is no need for multiple attempts at pregnancy. It seems that IVF will become an obvious form of safe reproduction, the means to avoid deleterious mutations, inheritable or de novo, and to allow prospective parents to build their families with forethought and deliberation. This future is possible because of initiatives that started in the laboratory in 1978.

Before IVF was accepted as standard treatment for infertility, there was little to no concern about the laboratory environment, from cleanliness of work surface areas to air quality in the confines of the laboratory. Although not typical, one did come across improvised laboratories where staff was allowed to smoke and even enjoy a meal in between cases! The first dedicated clinical suite and laboratory to exclusively perform IVF was built in Cambridgeshire, United Kingdom, in the village of Bourn in 1980 by IVF pioneers Robert G. Edwards (scientific director) and Patrick Steptoe (medical director) and their senior team, which included Jean Purdy (laboratory quality control manager), John Webster (senior consultant), and Alan Dexter (financial director). The move to this private setting represented extraordinary courage, as it had taken hundreds of attempts to achieve two births before

the planning of this dedicated facility (4). Other clinics were opened as well, at the Royal Women's Hospital in Australia (Alex Lopata and Ian Johnston) and at Monash University (Carl Wood and Alan Trounson), both in Melbourne, with some government financial support, and in London, United Kingdom (Ian Craft) using private funding. At the Eastern Virginia Medical School in Norfolk, Virginia (USA), pioneers Howard and Georgiana Jones opened the first US-based facility using funds provided by the university. Other countries, such as India, Austria, France, Holland, Sweden, and Spain, followed swiftly and established their own clinics. By 1985, a new discipline was emerging, a field that was for the first time referred to as assisted reproductive technology or ART.

### Laboratory equipment and instrumentation: from bell jars to time-lapse incubators

Just as formulation of the cell theory was intricately linked to the development of the microscope, IVF and its associated technologies have relied on engineering efforts by many key individuals who have played important roles but unfortunately are rarely remembered. For instance, in 1850, John Lawrence Smith, a faculty member at what is now Tulane University (New Orleans, LA, USA), engineered the inverted microscope. Robert Chambers from New York University (USA) invented the first micromanipulator for cell microsurgery in 1912. The first incubators were used for hatching chicken eggs and date back to ancient Egypt. In the 19th century, this changed to heated bell jars. Carbon dioxide incubators date from the 1960s, and warm-jacketed incubators were developed in the 1970s.

In vitro fertilization-specific instrumentation began to be introduced in the late 1980s, and this process is ongoing with many companies now specializing in the area. The pioneering laboratories relied completely on equipment and materials that were designed for somatic cell tissue culture and not human (or mammalian) gametes and embryos. This is illustrated by the presentations and discussions of the first international group of IVF clinicians and biologists, to convene at Bourn Hall in 1981, to discuss the emerging IVF technology. The 26 attendants came from Basil (Switzerland), Cambridge (United Kingdom), Gothenburg (Germany), Kiel (Germany), Manchester (United Kingdom), Melbourne (Australia), Norfolk, Virginia (USA), Paris (France), and Vienna (Austria). In her chapter on methods of fertilization and embryo culture in vitro in the proceedings of this first conference on clinical IVF, Jean Purdy wrote that, “The equipment needed in a tissue-culture laboratory has been described extensively by Paul (1970)” (5). This was reflective of a conspicuous lack of specialized equipment and disposables for IVF. Egg collection kits and ET catheters, as well as a pump that allowed gentle aspiration of follicular fluid (FF) from ovarian follicles were among the first IVF-specific instruments/devices to be developed. For the laboratory, laminar flow workstations equipped with heated surfaces were engineered. A benchtop incubator was later invented by

David Mortimer and colleagues in Australia as an alternative to “big box” incubators to provide a more stable and controlled culture environment. Incubators have continued to evolve and improve and the present-day embryologists not only culture embryos for longer periods of time, and with more confidence, they can also watch development frame by frame thanks to incorporation of time-lapse microscopy into incubation systems. Close observation of embryos through superior microscopy has contributed to an understanding of the morphology and timing of developmental events and the ability, albeit with limitations, to select/deselect embryos for transfer and cryopreservation.

### **Culture media and culture systems: from simple salt solutions to complex optimized culture media**

Tissue culture media were first developed nearly 150 years ago by Ludwig and Ringer. These were simple salt solutions, which were based on the properties of serum/blood plasma. Knowledge was gained during those years about the biochemistry of metabolism in mammals and humans, particularly osmolarity, pH, and temperature. This knowledge provided the basis for modifications to the simple salt solutions and successive generations of culture media, which were considerably more complex. The second generation of culture media was developed in the 1970s, mimicking the female reproductive tract environment. This was followed by a third generation of media, which was designed to optimize growth in vitro, to some extent ignoring existing formulations and the “back to nature” principle behind those formulations. To formulate these new media, the performance of each ingredient was evaluated separately using a “simplex optimization” process. This was first developed for mouse embryo culture by Professor John Biggers at Harvard University. His work was supported by the US National Institutes of Health, in part aiming to develop culture media for use in the human while circumventing the moratorium still in place on human embryo research. Variations on the second-generation media, called sequential and third generation simplex optimized-derived media are still in use at present and seem equally effective in supporting development of human embryos through 6 or 7 days of culture in vitro. The departure from home-brew culture media and the acceptance of commercially manufactured media, which is strictly regulated by governmental agencies, very likely has contributed to improvements in laboratory and clinical outcomes by virtually eliminating inconsistencies, manufacturing errors, and batch-to-batch variability.

Culture systems have evolved too. In the 1930s, Carrell flasks (Gregory Pincus) were used to culture embryos or perform in vitro insemination. In the 1950s, experimental embryologists like John Hammond and Wesley Whitten switched to test tubes. In 1963, Ralph Brinster introduced the culture of eggs and embryos in small droplets of culture medium under a layer of paraffin oil (6). With some modifications, this ingenious “micro-drop” method using the Petri dish has become the most widely used and successful system for culture of mammalian embryos in vitro. Brinster’s contributions were for a long time unappreciated by human IVF spe-

cialists; early on, nearly all practitioners used either organ culture dishes or small test tubes for culture of human gametes and embryos. But eventually, by the mid-1990s, most IVF laboratories adopted the “closed” under-oil culture system. There is little doubt that this system has provided a better, more stable environment and significant advantages for embryonic growth, which were lacking in the open culture systems of the past. This in turn has led to increased efficiency and efficacy of embryo culture.

### **Staffing the IVF laboratory: from experimental to clinical embryologists**

Clinical embryology did not evolve in a vacuum but against a backdrop of a 150-year history in experimental embryology, cryobiology, and other related fields. When Bourn Hall Clinic opened in 1980, Jean Purdy hired two laboratory assistants who worked as quality control technicians. They handled the Petri dishes, completed the paperwork, and witnessed procedures. The clinical embryologists, on the other hand, were charged with the handling of gametes (including sperm preparation) and embryos and communication with patients. The pioneering embryologists were often involved in optimizing follicular recruitment protocols and timing of egg retrievals. By 1983, when Louise Brown turned 5 years, worldwide fewer than 100 embryologists were involved in clinical work. They experienced a very different work environment than exists at present—so much was unknown and there was little guidance. At present, aspiring embryologists can participate in Master of Embryology programs, and they can get training in specific technologies and techniques through courses, which provide hands-on experience. Embryologists and trainees can reinforce and expand their theoretical knowledge through a rich literature of thousands of articles, dozens of textbooks and “how-to” books, and attend one of the many specialized workshops organized annually around the world. They can watch videos on the Internet or participate in web-based journal clubs and discussion groups. In some countries, embryology directors and managers must attain postgraduate credits to keep qualification standards and their laboratories must be audited and certified. These new opportunities have provided for an expansion of the workforce and the possibility to more appropriately staff ART laboratories. Proper staffing itself is a significant contributor to improved safety and better outcomes (7).

### **The expanding horizons of IVF: from partial zona dissection and subzonal insertion of sperm to intracytoplasmic sperm injection, slow freezing to vitrification, morphology to morphokinetics and genetics**

During the years, the IVF laboratory has incorporated many transformative technologies that have continued to be refined and perfected. Most important have been oocyte and embryo cryopreservation; assisted fertilization for treatment of male factor infertility; genetic diagnosis of embryos before transfer; and development of new embryo selection methodologies and platforms, including embryo morphokinetics using time-lapse microscopy.

**Cryopreservation.** Chris Polge and co-workers were the first to deep-freeze mammalian spermatozoa in 1949. Human spermatozoa were frozen in Iowa (USA) a few years later, by Raymond Bunge and Jerome Sherman. In 1971, David Whittingham, Stanley Leibo, and Peter Mazur changed the field of embryology by freezing cleavage-stage mouse embryos (8). In the early 1980s, in relatively quick succession, the human embryo was cryopreserved at all embryonic stages from the zygote to the hatched blastocyst, with minor adaptations, but survival rates remained <80% for many years. The effort was well founded in science, as basic scientists working with rodents and farm animals had already mastered the technology years earlier. The past 10 years have seen a dramatic improvement in oocyte and embryo cryopreservation with the introduction/application of vitrification. With survival rates of nearly 100%, treatment approaches that involve cryopreservation of all embryos and oocytes, have become more viable, revealing clinical advantages of delayed transfer in natural rather than stimulated cycles.

**Assisted fertilization.** Micromanipulation to assist fertilization was first applied successfully in 1986 in the mouse through zona drilling before insemination. During this procedure, an acidified Tyrode's solution was expelled onto the zona by a micromanipulator to cause focal zona dissolution and allowing spermatozoa easier access to the oolemma (9). In 1988, babies were born to couples with male factor infertility after application of a mechanical form of zona drilling (10). These were the first pregnancies in the human established with the micromanipulator as a surgical tool. That same year, teams in Singapore and Rome (Italy) reported on injection of spermatozoa into the perivitelline space. Although both methods improved the prospects for treatment of more extreme forms of male factor infertility, fertilization rates were low due to the absence of a quick block to polyspermy on the membrane level. However, once monospermic fertilization was achieved, implantation rates were as high or higher than after standard insemination. Assisted fertilization improved dramatically with the introduction of intracytoplasmic sperm injection (ICSI) by a team in Brussels (Belgium) (11). One important factor that led to this success was the design of the injection tool (P. Devroey, personal communication). This needle was very thin, sharp, and straight unlike the tools that were developed earlier. The design allowed non-traumatic piercing of the membrane and placement of spermatozoa in the cytoplasm with precision. It was quickly demonstrated that fertilization rates were as high as conventional IVF even in the most severe of male factor cases. At present, ICSI is being used with increasing frequency, in some cases replacing standard insemination altogether. Although successful in terms of pregnancy, live birth rates, the wisdom of nondiscriminant application of ICSI when it is not indicated continues to be debated.

**Preimplantation genetic testing.** Successful genetic diagnosis of single gene defects through blastomere biopsy, or preimplantation genetic testing (PGT), was first reported by Alan Handyside (12) and the team at Hammersmith Hospital in London in 1989. Basic science had again led the way in this case, after Richard Gardner, while in the Cambridge lab-

oratories of Bob Edwards, showed in 1968 that trophectoderm biopsy and sexing of the rabbit embryo was compatible with obtaining live offspring (13). The development of PCR by Gary Mullis and his team in the 1980s were crucial to the success of rapid single cell sequencing, which was a prerequisite for PGT (14). The discovery of the high incidence of chromosomal anomalies in biopsied embryonic cells led to the approach of testing of embryos and selective transfer of only euploid embryos, a variation on PGT, now dubbed PGT-A or PGT for aneuploidy (15). The efficacy of PGT-A (in all its variations) has been the subject of debate for >20 years. Embryo biopsy has shown that manipulation of embryos at a cellular level is not always without harm and optimization of the techniques is much needed. Perhaps this can be achieved using a noninvasive biopsy-free approach? There are also concerns about the high frequency of mosaicism, but PGT has been of tremendous benefit to couples at risk for transmitting genetic disease.

**Embryo selection technologies.** One of the remaining challenges in the ART laboratory is the development of efficacious (and affordable) embryo selection methods, which would help facilitate routine single ET in all patient groups. Early observers of embryo development in vitro were surprised by the apparent variation, not just between patients, but among embryos of the same patient. In the mouse, small aberrations from the morphological norm were known to significantly reduce implantation, but in the human, embryo morphology and rate of development were only loosely correlated with outcome. The search for important characteristics that could predict implantation has brought under examination many aspects of gamete and embryo development in culture and complicated algorithms have been developed. However, after 30 years, not a single common morphological marker has been identified that can predict the future success of an embryo with certainty. The recent development of time-lapse microscopy has made permanent record-keeping a reality, but reliable embryo selection using a single parameter or algorithm applicable to all patients remains elusive.

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## DEVELOPMENT OF IN VITRO FERTILIZATION CULTURE MEDIA AND THE IMPORTANCE OF BLASTOCYST TRANSFER

David K. Gardner, D.Phil.

### Deliberations on culturing the human embryo

Consideration of a cell's physiology is a logical starting point when trying to culture it. This premise is challenging when considering the preimplantation human embryo, whose physiology and metabolism changes dramatically from

fertilization to implantation. The fertilized oocyte and cleavage-stage embryo are relatively quiescent, exhibit limited oxidative capacity, and use carboxylic acids predominantly. In contrast, the blastocyst is one of the most active tissues in the body, characterized by exponential growth, expansion of a blastocoel, increased oxygen use and oxidative metabolism, a large demand for glucose (largely to support biosynthetic requirements), and a somewhat paradoxical production of lactate through aerobic glycolysis (thought to be key in embryonic signaling with the endometrium) (Fig. 1). The dynamics and complexities of preimplantation embryo physiology, therefore, help to explain the long and complex road to blastocyst transfer.

### Four decades of media in human in vitro fertilization

Since the birth of Louise Brown in 1978, conditions used to support development of the preimplantation human embryo in the in vitro fertilization (IVF) laboratory have changed considerably. During the early years of human IVF, there was no specialized laboratory equipment and no embryo-specific media for the task in hand. Consequently, culture technologies were largely borrowed from tissue culture laboratories, which included the use of “big-box” styled incubators, designed to maintain carbon dioxide levels to buffer the bicarbonate-based culture media. Before the 1990s the media used in human IVF had two distinct categories. Tissue culture media, such as Eagle’s minimal essential medium (also called Dulbecco’s minimum essential medium [DMEM]) and Ham’s F-10 (16), which were nutrient rich, containing high concentrations of glucose, amino acids, vitamins, nucleotides, and trace elements required to maintain somatic cell lines in vitro. The other type of media used was simple balanced salt solutions such as Earle’s (16). Such media lacked amino acids and vitamins, and were really just salt solutions supplemented with the carbohydrates pyruvate, lactate, and glucose. Typically both types of media were supplemented with serum, either the patient’s own or from fetal cord blood. These types of media were not specifically designed to support the human embryo in culture, and although they could keep the cleavage-stage embryo alive, they could not readily support the development of viable human blastocysts. Consequently, transfer of embryos at the pronucleate oocyte and cleavage-stage embryo to the uterus (asynchronously) was adopted out of necessity. Resultant implantation rates using this approach were low, typically 10%–15%, and therefore it became routine to transfer more than one embryo to attain acceptable pregnancy rates (PRs). In 1995, the average number of embryos transferred was four. However, the downside to having to transfer multiple embryos is the finite probability of conceiving with more than one baby. During the 1990s and early 2000s, the medical concerns for both mother and babies as a result of IVF pregnancies established with twins, triplets, or high order multiple gestations were well founded and frequently reviewed (17).

In the mid-1980s, Menezo (18) and Quinn (19) and their colleagues developed two embryo-specific media, B3 and human tubal fluid (HTF), respectively. Menezo’s B3 medium



included nucleotides, vitamins, and amino acids, and showed that serum was not required for culture of the cleavage-stage human embryo, and that serum albumin could be used as the protein source. Quinn’s HTF medium was a modification of the earlier simple embryo culture media, such as Earle’s and T6, and was supplemented with serum. Both media were subsequently used for cleavage-stage embryo culture and transfer. However, although these media were developed with the human embryo in mind, they only supported development of embryos during the cleavage stages, culminating in a PR in the 20% range after the transfer of more than one embryo (18, 19).

Fortuitously, in the late 1980s there was a resurgence of interest in the physiology of the human embryo and the female reproductive tract, which in combination led to the development of serum-free, stage-specific media (16). Analysis of the embryos from several mammalian species determined that the inclusion of serum not only compromised development, physiology, metabolism, and the epigenetic state of the embryo, but was also associated with adverse outcomes after transfer (20). Furthermore, serum introduced variability into the culture system, making it impossible to compare data from patient to patient, and from clinic to clinic, and also prohibiting the analysis of embryo physiology. Another issue raised from the animal literature was of asynchronous embryo transfer (ET) to the uterus. Although the transfer of pronucleate oocytes and cleavage-stage human embryos to the uterus can result in pregnancies, in all mammalian species studied at present, the transfer of cleavage-stage embryos results in seriously compromised transfer outcomes compared with transfer at the blastocyst stage (21). The reason: the environments within the oviduct and uterus differ significantly in terms of nutrient availability, and the presence of a complex mixture of cytokines and growth factors in the uterus (21). Therefore, the transfer of a cleavage-stage embryo to the uterus places it into an environment that is not ideal, and hence its physiology is compromised. As a result, it became imperative to develop serum-free culture systems that could support the development of viable human blastocysts to synchronize the embryo with its in vivo environment.

Analysis of the human oviduct and uterine fluids, at the time when the embryo resides there, revealed distinct differences in the concentrations of carbohydrates. The oviduct is characterized by relatively high levels of pyruvate (0.32 mM) and lactate (10.5 mM), with relatively low levels of glucose (0.5 mM). In contrast, the human uterus contains relatively low concentrations of pyruvate (0.1 mM) and lactate (5.87 mM), but higher levels of glucose (3.15 mM) (22). The significance of these gradients is twofold. First the availability of nutrients reflects the dynamic metabolism associated with the preimplantation period, which progresses from a metabolism based on pyruvate and lactate, to one based on glucose, and second, the actual concentration of nutrients available to the embryo not only affects metabolism but also the epigenome (20). The relative availability of nutrients within the female reproductive tract mirrors precisely the changing requirements of the preimplantation embryo at each stage of development (20).



**FIGURE 1**

Pre Compaction	Post Compaction
	
Resides in the oviduct	Resides in the uterus
One cell type	Two distinct cell types: Inner cell mass & Trophoblast
Individual cells	Transporting epithelium
Maternal genome, in the form of stable mRNA derived from the oocyte	Embryonic genome
Low biosynthetic activity	High biosynthetic activity
Low $QO_2$	High $QO_2$
Metabolism based on low levels of oxidation of pyruvate, and some use of the malate-aspartate shuttle	Metabolism based on both high levels of glucose oxidation and the ability to produce lactate through aerobic glycolysis (the latter being required for signaling and for increased biosynthesis)
Requirement for specific amino acids including alanine, aspartate, glutamate, glycine, proline, serine, and taurine, which act as pH regulators, osmolytes, antioxidants, and chelators	Requirement for a more comprehensive group of amino acids, with optimal development of the inner cell mass requiring essential amino acids
Highest sensitivity to its environment, lacking many mechanisms required to maintain intracellular homeostasis	With the development of a transporting epithelium, the embryo acquires greater resilience
Difficult to attribute viability to morphological features	Ability to more accurately assess embryo viability through the grading of the two-cell types

Characteristics of human preimplantation embryo development.

Forty years of IVF. *Fertil Steril* 2018.

Of physiological significance, the oviduct is characterized by high levels of amino acids not required by somatic cells such as alanine, glycine, serine, proline, and taurine. These amino acids fulfill several key physiological roles during cleavage stages and hence have a significant role in supporting early development in culture (Fig. 1). The inclusion of amino acids results in increased viability and development after transfer in animal models (23). Embryos after the compaction stage have a different amino acid requirement to the cleavage stages, using all amino acids. These and other data lay the foundations for the development of stage-specific sequential media, the

first of which were G1 and G2. Before clinical use, these media underwent extensive animal embryo experimentation and >5,000 embryos (both laboratory and domestic animals) were transferred and fetal development analyzed (23). Having established their efficacy in animal models, the media underwent a pilot clinical study using donated embryos to determine their effectiveness. Once this was established, a pilot transfer study was undertaken to ensure that the pregnancies and resultant children were healthy, followed by a prospective randomized trial, to determine the clinical efficacy of these media. The latter establishing that day 5 transfer was associated with a

significant increase in implantation rate compared with the transfer of cleavage-stage embryos (24). Hence, the introduction of sequential culture media into human IVF followed amongst the most comprehensive development of any new technology introduced into clinical IVF.

Several years after the first clinical use of sequential media, Biggers and Racowsky (25) evaluated whether the mouse embryo culture medium, KSOM(AA), could support human embryo development to the blastocyst stage. This medium was derived using a computer program, which generated successive media formulations based on the response of mouse embryos in culture to form blastocysts. The original simplex optimized medium was termed SOM, and subsequently KSOM (which contains around 10 times more potassium than SOM). This variation was modified by another laboratory to include amino acids, which subsequently became known as a global medium clinically. More recently, the advent of time-lapse microscopy has created demand for uninterrupted culture (although this approach is not considered physiological) (26). In such monophasic media, the embryo is not exposed to physiological nutrient gradients as it develops and differentiates. Although at present data indicate that both media types appear to give similar outcomes with regard to blastocyst formation and pregnancies (27), the long-term outcomes of sequential and monophasic systems await analysis. The composition of human embryo culture media and the development of modern culture systems have recently been extensively reviewed (16)

One needs more than media to grow an embryo

During the past 4 decades our understanding of mammalian embryo physiology has increased significantly, and several factors other than media have been shown to have a profound effect on embryo development, metabolism, epigenetics, and subsequent viability (reviewed in detail in Gardner and Kelley, 2017 [20]). Maintaining a stable temperature and pH has been shown to be key to attain and maintain excellent clinical outcomes (20). However, it is the oxygen concentration that is also crucial in embryo culture. Atmospheric oxygen is 20%, whereas physiological levels in the reproductive tract are around 5%. Compared with physiological levels, atmospheric oxygen is highly detrimental to the embryos of all mammalian species studied, negatively affecting metabolism, the proteome, gene expression, and the epigenome (20). Furthermore, oxygen predisposes the embryo to greater vulnerability from other stresses in the culture system (20). Disappointingly, it was reported that 75% of IVF clinics surveyed use atmospheric oxygen for some part of the embryo culture, whereas a staggering 25% used this high oxygen throughout the entire embryo culture (28). The use of different oxygen concentrations during human embryo culture has made the comparisons of outcomes between clinics extremely difficult.

**Impact of blastocyst transfer.** The physiological and potential advantages of blastocyst transfer are listed in Table 1. All of these, combined with the excellent survivability of the blastocyst after vitrification, underlie the significance and continued increase in the uptake of blastocyst transfer.

TABLE 1

Importance of blastocyst transfer in human in vitro fertilization.
Synchronization of embryonic stage with the uterus, known to compromise pregnancy outcome in animal models
Reduced uterine contractions by day 5, thereby reducing the potential for embryo expulsion
Ability to grade the embryo proper through the quantification of the inner cell mass and trophectoderm, thereby improving embryo selection
Increased implantation rates
Significant reduction in fetal loss during gestation
Reduced time to pregnancy
Facilitated a greater uptake of single embryo transfer worldwide, thereby reducing the complications associated with multiple births
Allowed for the introduction of trophectoderm biopsy for preimplantation genetic screening and diagnosis, resulting in less damage to the embryo than biopsy of the cleavage stages
<i>Forty years of IVF. Fertil Steril 2018.</i>

**Evaluation of clinical data.** Although the advantages of blastocyst transfer have been presented, analysis of the literature reveals a less than conclusive story. A review by Maheshwari et al. (29) concluded that the data to support blastocyst transfer were weak, and although several articles reported good outcomes with blastocyst transfer, this was not always the case. A subsequent analysis of the studies revealed that there were striking differences in the ways in which human embryos had been cultured. In particular, those studies that did not find a benefit of blastocyst transfer had used atmospheric oxygen, whereas a commonality among those studies reporting significant benefits from day 5 transfer had all used 5% oxygen for embryo culture (16). This latter point highlights the difficulties of interpreting meta-analyses, when the studies included are all assumed to have performed IVF in the same way, when in fact this is simply not the case. This clearly underlines the need for a standardization of reporting of methods used in all aspects of the treatment of our patients during clinical studies and trials.

CONCLUDING THOUGHTS

The introduction of blastocyst transfer has been one of the most thoroughly investigated technologies introduced into human IVF, supported by hundreds of publications from several animal species as well as clinical trials. An initial concern regarding day 5 transfers was that the extra 2 days in culture could induce aberrant epigenetic profiles of the embryo, and that we would actually introduce more harm than good. This does not appear to have been the case. Rather, the human embryo is at its greatest sensitivity during the cleavage stages (26), and studies have revealed that any evidence of abnormal imprinting is either induced during oocyte maturation/superovulation, or during the cleavage stages, and are not added to by an additional 48 hours in culture (20). What is evident is that implantation rates have increased, pregnancy losses have decreased (30), and that the time to pregnancy has been greatly reduced, following the introduction of blastocyst transfer. Given the significant emotional stress associated with infertility treatment, and the high dropout rate after a failed transfer, time to pregnancy is a highly relevant issue for our patients.

## THE STORY OF ICSI

Gianpiero D. Palermo, M.D., Ph.D.,  
 Claire L. O'Neill, B.S., Stephen Chow, B.A.,  
 and Zev Rosenwaks, M.D.

### The beginning of IVF

This year we celebrate the 40th anniversary of IVF. Although it was a remarkable achievement, it was accompanied by a frustrating issue—the unexpected occurrence of complete fertilization failure observed 1 day after insemination—a predicament that precludes the chance of rescuing a particular cycle. Even at present, fertilization failure continues to be an unpredictable and significant limitation to IVF success. In the early 1980s, the very pioneers who introduced the world to IVF envisioned applying assisted reproductive technology (ART) to the treatment of male factor infertility (31). The issue was that these male factor cases were characterized by severely compromised semen parameters, and it became obvious that a threshold of about 40% motility and at least 10 million spermatozoa was required to attempt standard in vitro insemination with realistic chances for success.

Nonetheless, even with an adequate number of spermatozoa, fertilization often failed. This failure led to changes in the way semen was prepared, with the goal of enhancing the selection of the most motile spermatozoon by swim-up or density gradient techniques, as well as allowing spermatozoa to capacitate before reaching the egg. The specimen vessel itself, originally a tube, was replaced by a Petri dish to better observe gametic interaction and to discern the number of spermatozoa necessary for insemination in microdrops. This restriction was introduced to increase the intermingling of the two gametes. However, this technique did not successfully address the issue at hand, as it was common to see fertilization rates of barely 50% when mild male factor cases were involved.

The growing interest in the treatment of male factor cases sparked the development of assisted fertilization. In the early 1980s, the first attempts to actively manipulate gametes were made, leading to the development of techniques designed to strip the zona pellucid (ZP), partially digest it, or crack it altogether (32–34). These techniques were merely an attempt to enhance, albeit simplistically, gamete interaction. They were often performed on a fraction of the oocytes, with the remaining ones subjected to standard insemination. Although these attempts modestly increased fertilization rates, in many cases investigators were still plagued by fertilization failure or by an unacceptably high rate of polyspermic fertilization.

### How intracytoplasmic sperm injection came about

In 1988, Gianpiero Palermo began a sabbatical in Brussels at the Academic Hospital of the Dutch-speaking Brussels Free University. The sabbatical was arranged by his mentor at the University of Bari, Vincenzo Traina, and André van Steir-

teghem, who was visiting the university during Palermo's last year of residency in obstetrics and gynecology. Van Steirteghem was familiar with the university because he and other international pioneers of ART, including the Joneses, came to Bari in 1982 for one of the first world congresses on IVF.

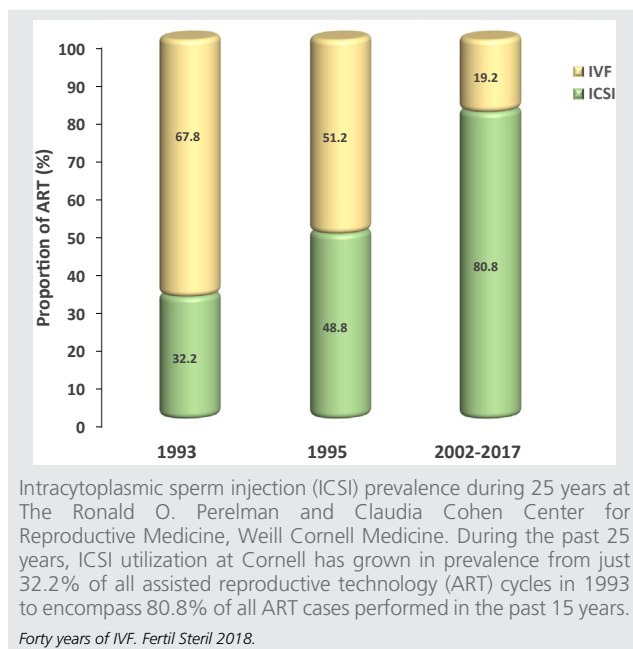
When Palermo arrived in Brussels, he attended a scientific workshop at the Center for Reproductive Medicine at the University Hospital Brussels, where he met key members of the group and familiarized himself with ART and its related challenges. He was immediately fascinated by the work, and he began learning transvaginal ultrasound-guided oocyte retrieval, expanding his knowledge of stimulation protocols, and gaining experience in embryology laboratory techniques. During the course of his research with van Steirteghem, it became clear that the major concern with IVF was the inability to predict fertilization in male factor cases.

Later that year, several articles were published on the use of microinjection procedures, including one on subzonal insemination (SUZI) (35) and another describing a spermatozoon that had been injected directly into the oocyte cytoplasm (36). This micromanipulation technique—the direct injection of a single spermatozoon—was the most elegant, as it empowered the male gamete to be used with an emphasis on the single cell. Palermo began practicing with unfertilized human eggs from cycle attempts, and then proceeded to inject human spermatozoa into hamster eggs. Later, he tried to microinject in the mouse to achieve a live offspring. This was particularly difficult, as the presence of the perforatorium on the mouse sperm nucleus requires a pipette much wider than the actual diameter of the sperm head; in fact, it is even larger than the pipette used for human intracytoplasmic sperm injection (ICSI) (paradoxically, to inject a mouse oocyte that is half the diameter of a human egg).

Prior experimental work involved removing the spermatozoon's flagellum to facilitate the injection, but these attempts failed to achieve an offspring. A few years later, it was confirmed that retaining the flagellum was the key to ICSI success in humans. This was later corroborated by the hypothesis of the paternal inheritance of the human centrosome (37).

Eventually, Palermo devised a subzonal injection—rendered popular by the work of Simon Fishel in Italy (38)—of the mouse egg by inserting a single spermatozoon within the perivitelline space. However, this did not yield consistent fertilization due to the asymmetry of the mouse acrosome, causing sporadic contact between the inner acrosomal membrane and the oolemma. Therefore, he decided to improve the chances of fertilization by enhancing the acrosome reaction (39). This led to the human application of the technique, and several couples were subsequently treated by SUZI (40). The procedure Palermo devised entailed a customized injection within the perivitelline space, a technique that proved auspicious to the development of ICSI (41).

At the time of the development of the procedure in Belgium, the research unit was located a few yards away from the embryology laboratory where the human eggs were retrieved. Oocytes were transported to the research laboratory for micromanipulation in a thermostatic box, and the

**FIGURE 2**

resulting embryos were walked back to embryology laboratory for transfer. Each micromanipulation cycle was stressful and time-consuming; every attempt required transporting the oocytes from the embryology laboratory, preparing the media solutions, sterilizing the glassware, and customizing the microinjection tools. Preparing the media solutions was the most challenging aspect of the entire assisted fertilization protocol, particularly the sperm preparation. It required formulating a method to select spermatozoa, incubate or treat them to enhance the acrosome reaction, and then identify the most appropriate sperm cell for injection. This was in addition to performing cumulus cell removal, injection, and careful culture of the embryo.

Implementing a micromanipulation protocol for use in clinical cases was daunting, not only logistically but also in terms of experimental design. During a visit by Basil Tatzis, he and van Steirteghem suggested that to prove the superiority of micromanipulation, the oocytes should be split between the standard insemination method and the microinjection procedure. Palermo argued against this approach due to reports at the time by Jacques Cohen's group, who performed standard IVF and partial zona dissection within the same oocyte cohort (34). Palermo was concerned that the inevitable replacement of embryos derived from different insemination methods would obscure the accomplishments of the micromanipulation technique. He asked that couples only be referred to him if they had experienced consistent failure after being treated several times in the standard fashion by IVF. He would then use the micromanipulation procedure for the entire cohort of oocytes retrieved.

At first, Palermo only included couples with enough spermatozoa available to rule out protocol issues with the tech-

nique. The subsequent inclusion of couples in which the male partners presented with low sperm concentrations resulted in extremely unpredictable fertilization rates.

During a subzonal injection of three spermatozoa into the perivitelline space within an oolemma dimple, a breach in the membrane allowed a spermatozoon to penetrate the cytoplasm of an oocyte. Palermo told van Steirteghem about this accidental penetration of the spermatozoon during the SUZI procedure. Van Steirteghem said that based on the early experiences in Norfolk, Virginia (USA), injecting into the cytoplasm does not work, and that it should not even be tried. However, Palermo noticed that after continual recurrence of these incidental punctures, fertilization almost always occurred. He decided to perform ICSI on a few oocytes within each SUZI cohort. When these injections resulted in embryos of good quality, they would be selected for transfer. After four pregnancies (42), he and van Steirteghem realized that they were on the verge of something new and promising for the field.

In early 1993, one year after reporting the first ICSI pregnancy in Belgium, Palermo contacted Zev Rosenwaks inquiring about a position in the clinical and research embryology laboratories at Weill Cornell Medical College. Rosenwaks, realizing the revolutionary impact of Palermo's accomplishment, was enthusiastic about recruiting Gianpiero to Cornell.

## Popularity

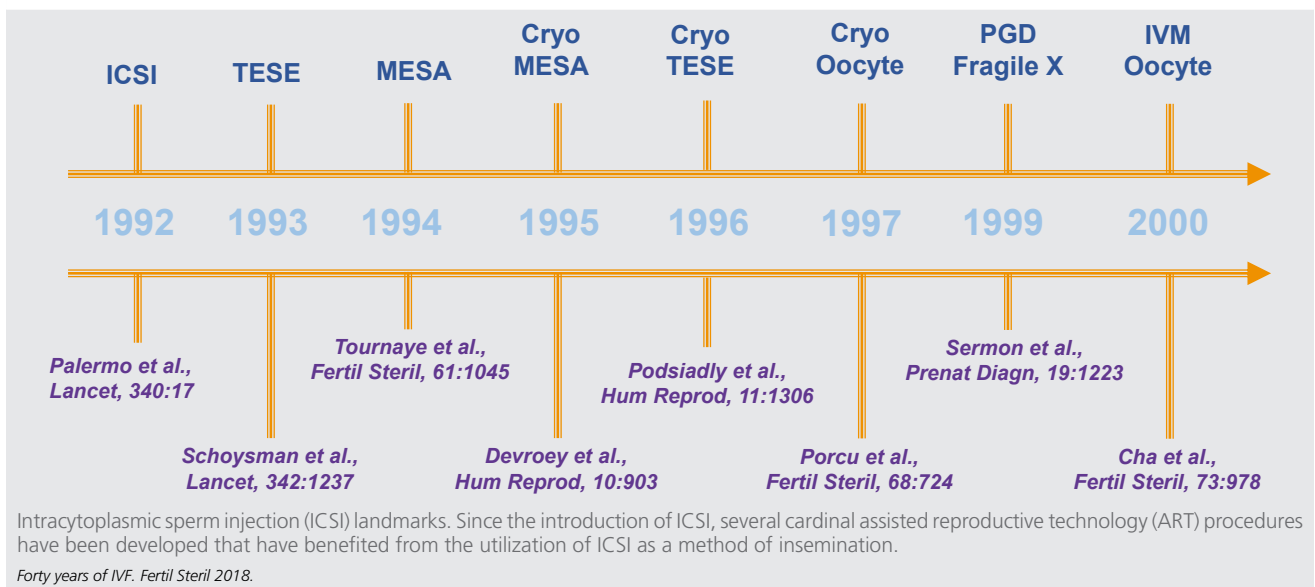
Since its establishment, the use of ICSI in centers worldwide has steadily grown, maintaining a comfortable majority over standard in vitro insemination cycles in many countries, and comprising a virtual totality of all ART cases performed in others. In fact, data published by the International Committee Monitoring Assisted Reproductive Technologies for 2008–2010 indicate that for the 60 countries that reported in those years, 1,980,244 ART cycles were carried out by ICSI, representing 67% of all cases performed, with an equivalent proportion of the 1,144,858 newborns reported resulting from sperm injection cycles (43).

At The Ronald O. Perelman and Claudia Cohen Center for Reproductive Medicine, Weill Cornell Medicine, we have also observed a progressive increase in the use of ICSI since its initial implementation (Fig. 2). During the past 25 years, we have performed 15,137 cycles of standard in vitro insemination, resulting in a fertilization rate of 68.5% (123,007/179,695), a clinical pregnancy rate (PR) (characterized by the presence of a fetal heartbeat) of 42.2% (5,533/13,105), and an ongoing/delivery rate of 34.6% (4,536/13,105).

In terms of ICSI cycles, we have performed 33,006 with ejaculated spermatozoa, resulting in an overall fertilization rate of 75.3% (176,974/234,927), a clinical PR of 37.7% (10,875/28,870), and an ongoing/delivery rate of 32.8% (9,464/28,870) in transferred cycles. The Center for Reproductive Medicine has also treated men with azoospermia, which requires a surgical sperm extraction, in 3,046 cases. The use of ICSI with surgically retrieved sources of spermatozoa has resulted in an overall fertilization rate of 58.4% (15,843/



FIGURE 3



27,137), a clinical PR of 43.2% (1,184/2,740), and an ongoing/delivery rate of 39.1% (1,071/2,740) in transferred cycles.

Although our use of ICSI may seem disproportionate, it reflects the unique nature of our Center. When they come to us, many of our patients have already been classified elsewhere as having severe male factor infertility, and they often have had repeated ART failure. In addition, our close collaboration with the Reproductive Urology Service at Cornell appeals to patients who elect to use ICSI with surgically retrieved spermatozoa.

Although significant advances have been made in treating patients with severe male factor infertility, in which no spermatozoa can be isolated from the ejaculate, occasionally we are unable to retrieve any gametes from men who suffer from maturation arrest or germ cell aplasia. Spermatozoa are not generated within the seminiferous tubule at all in these instances.

It should be noted that many researchers are looking to push these boundaries in the field of male factor infertility (44). Some investigators are attempting to use stem cell therapy and extended germ cell culture to give men with severe male factor infertility the opportunity to have a biological child. With the inevitable arrival of these techniques, including in vitro spermatogenesis and possibly neogametogenesis, the only plausible insemination method for these cells will be ICSI. This suggests that ICSI will remain a pillar of ART treatments for the foreseeable future.

## Outlook

When the first ICSI report was published (42), it was clear that the procedure would revolutionize the treatment of infertility, as it extended our ability to treat patients with male factor infertility. A total of 50% of all infertility, either in combination or alone, is due to male factor infertility. It can be said

that ICSI is as much of a breakthrough as the development of IVF itself.

At present, there is an array of indications for the application of this reliable insemination method (Fig. 3). The ability to pair timed fertilization with the sparing use of spermatozoa also renders ICSI a particularly desirable tool for use in developing technologies. However, we do recognize that there are concerns about its excessive use. At our Center, the decision to use ICSI often begins in the clinic and involves thoroughly counseling patients and obtaining their explicit consent. For the rare cases in which the decision is left to the embryology laboratory, ICSI use is primarily dictated by the characteristics of a suboptimal inseminating specimen. To add an additional layer of objectivity, the decision to perform the procedure is made by the team responsible for standard in vitro insemination rather than by the ICSI team itself. In addition, our clinic often serves as a referral center due to its collaboration with reproductive urologists, who strongly favor the use of ICSI to treat their severe male factor cases.

There are risks involved with ICSI procedures. These techniques bypass the natural selection and, most important, use gametes from men who often have a subtle genetic issue that affects their fertility, which could lead to consequences for their offspring. There is wide agreement in our field for the need to properly evaluate the children conceived by all ART procedures. Although there have been many reassuring studies (45) on the perinatal outcomes of newborns and on the developmental health of young children from ICSI, this anniversary marks the first two and a half decades of using the procedure, culminating in long-awaited initial follow-up reports on adolescents and young adults conceived with ICSI. These studies have provided encouraging results on the medical and reproductive health of the first cohorts to reach reproductive age (45). Continued follow-up reports on the health of ICSI offspring will provide invaluable analysis

on the safety of the procedure as well as insights into any confounders that may affect its outcomes.

At present, ICSI remains the preferred insemination protocol, with some centers using it in >90% of their insemination cycles. It is possible that ICSI will be perceived by future generations as the evolution of standard in vitro insemination.

## OOCYTE CRYOPRESERVATION FOR ELECTIVE FERTILITY PRESERVATION

Ana Cobo, Ph.D.

### Elective fertility preservation

Successful fertility preservation (FP) has revolutionized assisted reproduction practice. Fortunately, we have overcome the challenge of safeguarding reproductive capacity by saving oocytes to acquire future biological offspring. A large population is likely to benefit from this approach, including women about to be treated for cancer or immunological disease, whose reproductive potential may be affected by using gonadotoxic agents (46, 47). The FP is also useful for women with genetic predisposition to premature ovarian reserve depletion. Patients with other disorders, like endometriosis that may compromise future fertility, can also benefit from FP (46).

Safe oocyte storage is now a reality and has resulted from hard work in the cryobiology field for almost three decades. Before oocyte cryopreservation, embryo freezing was the preferred option for FP in postpubertal women. However, the semen sample requirement could be inconvenient for single women, or even for women with partners. Ovarian tissue freezing is also available when oocyte cryopreservation is not a valid alternative, given the need to either initiate cancer treatment immediately or the impossibility of performing ovarian stimulation when hormone-sensitive tumors are present (47).

Thanks to vitrification, oocytes can now be harvested and safely stored to attempt pregnancy in the future when diseases are cured or when circumstances become more favorable (48). A recent meta-analysis (49) showed that vitrification is superior to slow freezing for clinical outcomes and cryosurvival. Although the technology was initially validated large scale in ovum donation programs (50), its efficiency for patients conducting IVF autologous cycles is proven (51–53).

Once this technique became available for medical indications, its use for nonmedical reasons was not long in coming. Therefore, another population is increasingly using FP; it includes women who wish to postpone motherhood and childbirth for no apparent medical reason. These motivations are frequently grouped as “social reasons” or “elective fertility preservation (EFP)” where the main reason is age-related fertility decline (54). At present, many women are dedicated to their careers and delay pregnancy until after younger childbearing years. Women are often forced to choose between motherhood and their professional aspira-

tions and financial security (55). This leads to delayed motherhood, which explains why women visiting reproductive clinics are usually more than in their mid-30s. As a result, they endure repeated failed cycles because of advanced maternal age or search for egg donation programs. Lack of a partner is also a common reason for delaying pregnancy and it affects mainly developed countries, where significantly low birth rates are reported. These social pressures make women decide to “stop” their biological clock by freezing their oocytes. A survey study that analyzed women’s attitudes to EFP shows that a significant proportion of young women would consider safeguarding their reproductive potential, or at least are open to the idea of social oocyte freezing (56, 57). The number of women who decide on elective oocyte freezing has recently grown exponentially (46, 58). In our experience, this figure means a fivefold increase during an 8-year period (58). Undoubtedly, this new field in IVF represents a milestone for not only assisted reproduction workers, but also embodies a means for women’s emancipation (59).

We herein review available evidence for oocyte vitrification efficiency in women who sought EFP. We also briefly overview the vitrification technique.

### The technique

Vitrification is a physical phenomenon that involves the solidification of an aqueous solution without ice. Vitrification circumvents ice formation by the direct conversion from liquid into a vitreous solid. This process is facilitated by applying high cooling rates and increasing viscosity (60), which, in turn, increases the glass transition and results in vitreous solid forms. Factors, other than cryoprotectant concentration and cooling rates, are strongly involved with the efficient vitrification probability. To improve survival Seki and Mazur (61) demonstrated that the warming rate was even more critical than the cooling rate. The technique’s main shortcoming is that it needs a high cryoprotectant concentration, which can damage oocytes and embryos through chemical toxicity and osmotic stress. Improved protocols lower toxicity and support high cooling rates by using very small volumes (62). Traditionally, the devices that use minimum volumes are plunged directly into liquid nitrogen to allow direct contact with samples and are known as open systems. Conversely, closed systems avoid direct contact between samples and liquid nitrogen thanks to their hermetic sealing before vitrification. The main advantage of these systems is that may be safer as they avoid contamination from any infectious agents possibly present in the liquid nitrogen used for vitrification, or from other potentially infected neighbor samples during storage. Open systems have been debated for the hypothetical cross-contamination risk. However, no reports on this matter (63) exist after a 30-year history of cryotransfers in assisted reproduction or after 10 years of experience using open devices for vitrification in IVF clinics worldwide. A variety of vitrification tools and protocols exists on the market, which have been applied with varying degrees of success. Although, open systems have been the preferred oocytes vitrification method, closed

systems were not as successful for oocytes as open devices, especially when oocyte vitrification is applied in routine clinical practice. Closed systems have lately evolved and a recent prospective clinical trial has revealed that oocytes vitrified in hermetic systems show similar clinical efficiency to their sibling fresh oocytes (64). Another randomized sibling-oocyte study compared an open versus closed system and confirmed the efficiency of the latter (65).

### **Efficacy and safety of oocyte cryopreservation for FP**

Data on EFP strategy efficiency are emerging, but are still scarce mainly due to the intrinsic nature of FP. Fertility preservation implies an indeterminate time elapsing from oocyte vitrification until women decide to use them, as they feel ready for motherhood and have overcome the personal drawbacks that initially led them to freeze the oocytes. A study (48) from 2013 provides information on the first babies born after elective freezing of oocytes for nonmedical reasons.

A more recent study (58) provides extensive information on outcomes after EFP, including the profile of the women who had their oocytes vitrified, the rate at which they return to use them, their clinical outcomes, and the probability of having a baby according to the number of used oocytes. Unlike other publications that evaluate the efficiency of egg freezing in different populations, basically in egg donors or poor responders, this study (58) includes the largest series to date from actual FP populations, and provides demographic and clinical data of the women who underwent EFP ( $N = 1,468$ ) and returned to attempt pregnancy ( $N = 137$ ).

As expected, most women who came to vitrify their oocytes were single (75.6%) and professionals with a high level of education (72.8%), which coincide with the essential motivations described for this population (58). Most of these women decided on EFP at advanced age as most (63%) decided FP when they were aged 37–40 years. A non-negligible 16.2% were aged  $\geq 40$  years when they decided on vitrification, and most were aged  $< 30$  years (58). It is striking that the age of the women who underwent EFP matched that of those who traditionally came to IVF clinics to treat infertility problems. This is a contradiction because the fundamentals of FP consist in preserving fertility and not preserving infertility. Consequently, when analyzing the results obtained in this “aged freezers” population, we found that age played a fundamental role in achieving success by this strategy. Not surprisingly, the mean oocyte storage time was short (2.1 years), probably because these women decided on FP at advanced reproductive ages. Thus, they could not “buy” as much time as if they had been younger when deciding on FP.

As expected, age at vitrification strongly impacts the outcome regarding the number of retrieved oocytes and the metaphase II oocytes finally vitrified, the survival of the oocytes, pregnancy and live birth rates. The first effect of age is shown by the many oocytes either retrieved or vitrified in patients aged  $\leq 35$  years compared with patients aged  $> 35$  years (58). The lowest amount of mean retrieved was for patients aged  $\geq 40$  years (5.1; 95% confidence interval:

4.2–6.0) and 3.9 (95% confidence interval: 2.6–5.0) for mean metaphase II oocytes vitrified. This obeys the natural ovarian reserve depletion.

The oocyte survival rate is also age related. Table 2 (58) shows higher survival and live birth rate per patient for the women aged  $\leq 35$  years. That the survival of oocytes is lower in older patients is clearly relevant as these women have fewer oocytes to cope with the whole process, which may undermine cycle prognosis. Accordingly, pregnancy rates (PRs) and cumulative live birth rates (CLBR) worsen as patient's age increases (Table 2) (58). A more stratified analysis showed differences in terms of CLBR between the youngest and oldest women (66.3% [95% confidence interval: 35.8%–97.4%] vs. 13.3% [95% confidence interval: 3.9%–30.5%] with ongoing PRs for patients aged  $\leq 29$  years vs.  $\geq 40$  years, respectively) ( $P < .05$ ).

When preserving fertility, perhaps the first question women ask is how many oocytes they should vitrify to maximize their chances of giving birth. The CLBR calculation per patient according to age shows that age strongly affects outcomes, but neither explains the pace at which rates lower nor considers the number of oocytes needed to have a child. Conversely, calculating survival curves by the Kaplan-Meier approach is a much more accurate method to know the probability of having a baby at any point on the curve according to the number of used oocytes when considering the most powerful confounder (i.e., patient's age). Perceptibly, the more oocytes, the greater the probability, but the relationship is not linear. For women aged  $\leq 35$  years, we observed a huge difference in CLBR when using only five (15.4%) versus eight oocytes (40.8%), which is an 8.4% increase in CLBR per additional oocyte. However, if aged  $\geq 36$  years, the increase in CLBR is considerably less marked when using the same number of oocytes (from 5.1% CLBR using 5 oocytes to 19.9% with 8; a 4.9% increase in CLBR). The success rate in the younger group ( $\leq 35$  years) was twice that achieved in the older group ( $\geq 36$  years; 60.5% vs. 29.7%, respectively) when using 10 oocytes. With 15 oocytes, CLBR continues to increase in the  $\leq 35$ -year group, but the women aged  $\geq 36$  years reach the plateau for the same number of oocytes. Thus, success at this point is independent of the number of used oocytes. Accordingly, we suggest vitrifying 8–10 metaphase II oocytes to obtain reasonable success rates. In women  $> 36$  years, the number of oocytes need to be individualized, along with the possibility of offering preimplantation genetic screening. This leads to a debate about cost-effectiveness as recent data reveal that egg banking for FP is more cost-effective in women aged  $< 38$  years (66, 67).

Although both arguments are valid, we believe it necessary to provide very young and older women with adequate information. The former should be enlightened about them being less likely to use their stored oocytes in the future as natural conception is more probable. Although older women are more likely to use their vitrified oocytes, they should be accurately informed about their fewer reproductive chances. As we revealed, some women who did EFP aged  $< 40$  years gave birth, which makes it very difficult to set upper limits to apply the strategy.

TABLE 2

## Clinical outcome according to age at vitrification.

Variable	Oocyte donation	Poor responder, ≤35	Poor responder, > 35	Elective FP, ≤35	Elective FP, > 35
No. of patients	15,899*	316	648	101	425
No. of cycles	18,579	332	680	108	504
Age, mean ± SD	25.3 ± 2.2 <sup>a</sup> ** (25.2–25.3)	33.3 ± 1.4 <sup>b</sup> (33.2–33.5)	38.6 ± 1.5 <sup>c</sup> (38.5–38.7)	32.5 ± 2.8 <sup>b</sup> (31.9–33.1)	38.7 ± 1.0 <sup>c</sup> (38.6–38.8)
No. of inseminated oocytes, mean ± SD	11.4 ± 2.1 <sup>a</sup> (11.3–11.4)	7.5 ± 4.2 <sup>b</sup> (7.3–7.7)	6.8 ± 1.5 <sup>c</sup> (6.7–6.9)	10.3 ± 3.7 <sup>d</sup> (10.2–10.4)	8.6 ± 1.4 <sup>e</sup> (8.5–8.7)
Survival rate	92.3% <sup>a</sup> (92.2–93.4)	83.6% <sup>b, d</sup> (81.7–85.6)	84.9% <sup>b</sup> (83.6–86.2)	94.7% <sup>a</sup> (93.3–96.1)	82.2% <sup>c, d</sup> (81.0–83.4)
Clinical pregnancy rate	59.3% <sup>a</sup> (58.6–60.0)	38.7% <sup>b</sup> (32.5–44.9)	30.6% <sup>c</sup> (27.1–34.1)	63.3% <sup>a</sup> (53.9–72.7)	33.9% <sup>c</sup> (29.4–38.4)
No. of live births	11,445	164	208	67	122
CLBR/cycle	61.6% <sup>a</sup> (60.9–62.3)	50.4% <sup>b</sup> (44.9–55.8)	30.6% <sup>c</sup> (27.1–34.0)	62.0% <sup>a</sup> (52.9–71.2)	24.4% <sup>c</sup> (20.7–28.2)
CLBR/patient	71.9% <sup>a</sup> (71.2–72.6)	51.8% <sup>b</sup> (42.3–57.3)	32.1% <sup>c</sup> (28.5–35.7)	66.3% <sup>a</sup> (57.1–75.5)	28.7% <sup>c</sup> (24.4–33.0)

Note: CLBR = cumulative live birth rate; FP = fertility preservation, SD = standard deviation. Numbers in parentheses are 95% confidence intervals. Different superscripts indicate statistical differences ( $P < .05$ ).

\*Recipients.

\*\*Donors age.

Forty years of IVF. Fertil Steril 2018.

We believe that our findings (58) are most revealing when considering the very relevant fact that data were obtained from analyzing the results in women who actually did EFP. Other studies have addressed the factors that may influence EFP outcome through oocyte vitrification and have predicted birth probabilities by studying other populations using vitrified, or even fresh, oocytes. A recent study (68) focused on developing a model to predict the likelihood of live birth according to age in EFP, but using fresh oocytes cycles. Live birth predictions were provided by considering the proportion of euploid blastocysts and the number of oocytes needed to generate them according to age. A meta-analysis (69) with 10 studies of oocyte cryopreservation by slow freezing/vitrification indicated fewer probabilities of giving birth as age increased, irrespective of the technique used, but better outcomes with vitrification. In it, rates gradually lowered between the ages of 25 and >40 years, with a cutoff point between success and failure in live birth set at 36 years. However, it also confirmed that a child can be achieved even at 42 or 44 years of age (69). Cil et al. (69) tabulated the live birth probabilities for 25- to 42-year-olds according to the number of oocytes thawed, injected, or to embryos transferred.

Undoubtedly these tools can be useful, especially for EFP, as casuistry is very small. However, they can lead to inaccuracy as they assume that all populations behave similarly in survival and clinical outcome terms. Conversely, we found relevant differences between infertile young women and young women undergoing EFP. Table 3 shows the data of three populations using vitrified oocytes (i.e., recipients of donors' oocytes, poor responders, and EFP patients). The survival of oocytes, number of inseminated oocytes, and clinical PRs and live birth rates are provided. Data on oocyte donation cycles ( $N = 15,899$ ; 18,579 cycles) and poor responders ( $N = 964$ ; 1,012 cycles) were collected from 2012–2017, and the data from actual EFP patients cover the past 10 years ( $N = 526$ ; 612 cycles). The results were calculated according to age ( $\leq 35$  and  $\geq 36$  years) in poor responders and EFP. In the ovum donation group, donor age was tabulated ( $25.3 \pm 3.2$  years) and clinical data were obtained from recipients (mean age,  $41.5 \pm 3.2$  years). We may reasonably expect

comparable results in matching age groups. It is striking that survival and clinical outcomes (clinical and live birth rates) were worse for poor responders ( $\leq 35$  years) than the oocyte donation and young EFP  $\leq 35$  years groups (Table 3). The young EFP group mirrored the outcomes of the donor oocyte group. With a similar number of inseminated oocytes ( $11.4 \pm 2.1$  and  $10.3 \pm 3.7$ ), clinical PR (59.3% and 63.3%) and live birth (71.9% and 66.3%) rates were comparable ( $P > .05$ ) for oocyte donors and EFP  $\leq 35$  years, respectively. Survival rates were similar for donor oocytes (92.3%) and EFP  $\leq 35$  years (94.7%). Noticeably, survival in poor responders was comparable between young and older women, but clinical outcome was significantly higher for patients aged  $\leq 35$  years. As expected, the results were comparable between old ( $\geq 36$  years) poor responders and EFP patients of the same age, with fewer oocytes inseminated in poor responders compared with EFP groups but, as expected, clinical outcomes were higher for younger poor responders (CLBR, 50.4% vs. 30.6% for  $\leq 35$  and  $\geq 36$  years, respectively). The behavior of the young poor responder group could be explained by them being infertile patients with compromised ovarian reserve and a good likelihood of having compromised oocyte quality. Therefore, they have fewer and lower-quality oocytes despite being young, which are responsible for the impaired results. Hence, the overall picture in Table 3 shows that differential outcomes exist according to age and populations. The young EFP group mirrors the outcomes achieved in the gold standard group (i.e., donors oocytes' reinforce our recommendation that women considering EFP would be better off deciding to do this at a younger age).

In conclusion, the efficiency of oocyte vitrification to safeguard fertility is currently a consolidated option. However, we believe that it is mandatory to explain to women who seek EFP that oocyte cryostorage is not an insurance policy to secure future motherhood, but a means to increase their chances of having a biological child. These chances depend on age and the number of stored oocytes. The number of vitrified oocytes should be adjusted according to patient's age to increase the probability of having a child, irrespective of oocytes coming from one stimulation cycle or more. In women, undergoing EFP should be encouraged



TABLE 3

Survival and clinical outcome in three populations.

Outcome variable	Oocyte donation	Poor responders		Poor responders		Elective fertility preservation patients		Elective fertility preservation patients	
		95% CI	≤35 y	95% CI	≥36 y	95% CI	≤35 y	95% CI	≥36 y
No. of patients	15,899*	NA	316	NA	648	NA	101	425	NA
No. of cycles	18,579	NA	332	NA	680	NA	108	504	NA
Mean age (y)	25.3 ± 2.2 <sup>a†</sup>	(25.2–25.3)	33.3 ± 1.4 <sup>b</sup>	(33.2–33.5)	38.6 ± 1.5 <sup>c</sup>	(38.5–38.7)	32.5 ± 2.8 <sup>b</sup>	38.7 ± 1.0 <sup>c</sup>	(38.6–38.8)
(mean ± SD)									
No. of oocytes inseminated	212,620 (11.4 ± 2.1) <sup>a</sup>	(11.3–11.4)	2,438 (7.5 ± 4.2) <sup>b</sup>	(7.3–7.7)	4,403 (6.8 ± 1.5) <sup>c</sup>	(6.7–6.9)	1,087 (10.3 ± 3.7) <sup>d</sup>	4,445 (8.6 ± 1.4) <sup>e</sup>	(8.5–8.7)
(mean ± SD)									
Survival rate (%)	92.3 <sup>a</sup>	(92.2–93.4)	83.6 <sup>b,d</sup>	(81.7–85.6)	84.9 <sup>b</sup>	(83.6–86.2)	94.7 <sup>a</sup>	82.2 <sup>c,d</sup>	(81.0–83.4)
Clinical pregnancy rate (%)	59.3 <sup>a</sup>	(58.6–60.0)	38.7 <sup>b</sup>	(32.5–44.9)	30.6 <sup>c</sup>	(27.1–34.1)	63.3 <sup>a</sup>	33.9 <sup>c</sup>	(29.4–38.4)
CLBR/cycle (%)	11,445 (61.6) <sup>a</sup>	(60.9–62.3)	164 (50.4) <sup>b</sup>	(44.9–55.8)	208 (30.6) <sup>c</sup>	(27.1–34.0)	67 (62.0) <sup>a</sup>	122 (24.4) <sup>c</sup>	(20.7–28.2)
CLBR/patient (%)	11,445 (71.9) <sup>a</sup>	(71.2–72.6)	164 (51.8) <sup>b</sup>	(42.3–57.3)	208 (32.1) <sup>c</sup>	(28.5–35.7)	67 (66.3) <sup>a</sup>	122 (28.7) <sup>c</sup>	(24.4–33.0)

Note: CLBR = cumulative live birth rate; CI = confidence interval; NA = not available. Superscript letters indicate statistical differences ( $P > .05$ ).

\* Recipients.

† Donor age.

Forty years of IVF. *Fertil Steril* 2018.

to decide on this option when aged <35 years because of greater biological efficiency.

## THE OFTEN-OVERLOOKED EMBRYO TRANSFER

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Assisted reproductive technology (ART) has seen several advancements in the laboratory to improve embryo development, including custom embryo culture platforms, incubators, and culture media. Integration of genetics with blastocyst biopsy and preimplantation genetic diagnosis/preimplantation genetic screening have helped improve embryo selection. Technological advancements have also benefited the clinical side of ART, with implementation and improvements in ultrasound, refinement of gonadotropins, and hormone stimulation with a variety of stimulation protocols customized to the individual patient.

One area that seems to receive less attention, but is arguably as important as any step in the IVF process, is ET. Even with the best embryos, without an atraumatic transfer to the correct location inside the uterine cavity, production of a live birth is impossible.

In comparison to other advances in the IVF laboratory and clinic, relatively few advances have occurred with ET. Production of specialized catheters have likely helped ease the process of ET. Improvements in ultrasound have also likely aided placement of the embryo into the uterus. However, when controlling for embryo quality and other variables, the physician has a dramatic impact on the success of the ET procedure (70). Physician technique and even response in the face of unforeseen occurrences during ET are key factors in success. Examples of difficult transfers, retained embryos, malfunctioning syringes, or other mishaps can be recounted; many with happy endings due to the actions of the doctor. Use of an ET training simulator (71) and development of a simulator by the American Society for Reproductive Medicine (ASRM) may further aid physician training in this delicate process. Critical aspects required for optimized ET are discussed.

### Key considerations for successful ET

**Catheter/embryo placement.** Proper placement of the catheter tip is an important variable affecting ET outcome. Although avoidance of touching the fundus when performing a transfer is widely accepted as a critical component, the ideal location of catheter/embryo placement is less clear. Several studies have determined that the distance from the catheter tip to the fundus was a variable affecting outcome. When the catheter tip was 5–27 mm from the fundus, pregnancy rates (PRs) were higher than when the catheter was closer. Furthermore, ectopic pregnancy rates (EPRs) were lower with this lower cavity transfer (72). The position of the air bubble transferred at the time of ET and its relation to PR has shown similar results.

The recent ASRM Practice Committee Guidelines for performing ET indicates that there is fair evidence from randomized controlled trials and cohort studies to indicate that catheter placement impacts implantation and pregnancy (73). Proper catheter location at the time of transfer can be summarized as follows. Embryos placed too high in the cavity may increase the probability of endometrial trauma and may induce uterine contractions with potential adverse effects. Upper or midcavity transfers, >10 mm from the fundus, appear to optimize implantation by avoiding the lower cavity where implantation is compromised or cervical implantation can occur and avoids issues associated with transfer too close to the fundus.

**Ultrasound.** In a retrospective comparison (74), 4,807 ETs were examined noting the degree of difficulty. Easy or intermediate transfers resulted in a 1.7-fold higher PR than difficult transfers ( $P < .0001$ , 95% confidence interval: 1.3–2.2). In a meta-analysis (75), it was found that ultrasound guidance lowered the incidence of difficult ETs with an odds ratio of 0.55. Thus, avoiding difficult ET is important to optimize clinical outcomes and ultrasound guidance appears to be key tool in achieving this goal.

Contamination of the catheter with blood may be a marker for difficult ET and has also been linked to poor ET outcomes, although with mixed data. When retrospectively assessing outcomes, various studies have shown higher success rates when no blood was noted on the catheter compared with transfer with blood present.

Ultrasound has many benefits related to ET, such as lowering the incidence of difficult transfers, confirming catheter placement in the correct part of the fundal cavity, minimizing contamination of the catheter tip with blood and mucous, and decreasing the chance of traumatizing the fundus and stimulating uterine contractions. Compared with “clinical touch,” several randomized controlled trials and meta-analyses have confirmed significant improvement in clinical PRs with ultrasound guidance. The ASRM practice guidelines for ET find evidence to recommend the use of ultrasound during ET to improve clinical PRs and live birth rates (73). Transabdominal and transvaginal ultrasound appear to be similarly effective in terms of pregnancy outcome (76).

**Catheter types.** Several catheters are available for use during ET. Catheters may vary in packaging, length, diameter, and echogenic visibility. However, classification generally depends on two categories: soft and stiff.

Soft catheters may follow the contour of the endometrial cavity more easily and thereby result in less risk of endometrial trauma or plugging the catheter tip. The negative aspect of soft catheters is that they are more difficult to insert and pass. Nevertheless, a meta-analysis (77) revealed improved outcomes (odds ratio: 1.34) favoring soft catheters versus stiff ones. In some cases, insertion of the catheter is difficult due to cervical stenosis. Different approaches have been undertaken to alleviate this issue, including cervical dilation at the time of retrieval.

However, this has been found to lower subsequent PRs. Dilation has also been accomplished several weeks before ET and has been found to improve outcomes. A malleable stylet device can be used with some soft catheters to negotiate a difficult internal os. Use of this device compared with a soft catheter alone did not decrease clinical PRs or implantation rates. When using the malleable stylet, it is important to leave the outer sheath just proximal to the internal os.

**Catheter loading.** Loading of preimplantation embryos into the transfer catheter is a critical component of the ET process with various considerations. As a result, types of catheters and syringes, and how these items are handled are important considerations

Although various studies examined the different types of ET catheters, relatively few publications discuss syringes used for ET. Three main options exist for transfer syringes. These include 1-mL glass Hamilton syringes, 1-mL rubber-free syringes, and 1-mL syringes with rubber stoppers. No clear advantage of one syringe over another has been demonstrated, although key factors should be considered before selection of a particular device. Considerations often include the preference for amount of resistance when depressing the plunger and the type of plunger with regard to toxicity concerns. Rubber plungers have historically been associated with increased risk of toxicity. Although glass Hamilton syringes have been used historically, these reusable devices require sterilization between cases and can wear out over time. Regardless of the syringe type used, an appropriate bioassay is recommended before clinical implementation to rule out embryo toxicity.

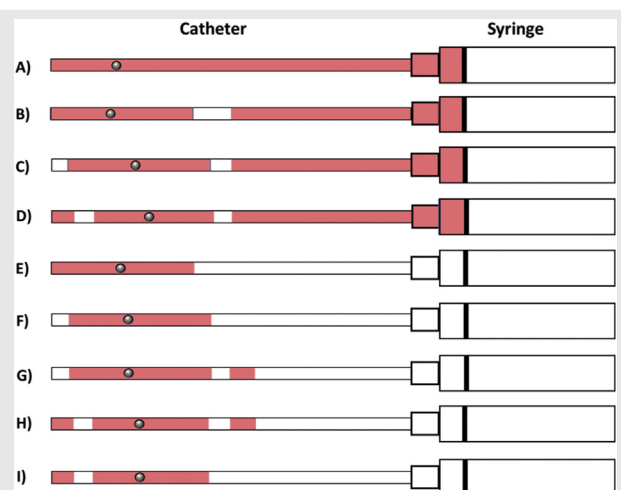
Rinsing of the catheter before loading embryos for transfer is often recommended to remove any substances or debris that may be present inside the catheter. This prevents their deposition into the uterus.

Once catheter type and syringe type are selected, and catheters are prepped, various methods of loading embryos are available. Much debate exists over the impact of air in the ET catheter. Air bubbles can help with ultrasound visualization, and may play a role in proper placement of the embryo (78). Concern exists with possible migration of embryos caused by air bubbles in the uterus after transfer, although this concern may be unfounded (79). Various studies, including a meta-analysis (80), revealed no clear advantage of the fluid-air catheter loading method versus models using only fluid in terms of pregnancy, implantation, miscarriage, EP, or live birth (Fig. 4).

Volume of media in the catheter may be an important factor when using air bubbles. With day 2 or 3 ET, using more fluid flanked by bubbles yielded superior pregnancy and implantation than smaller fluid volumes (81, 82).

**Transfer medium.** In early reports of factors associated with high PRs, use of 50% patient serum in the transfer medium was noted to yield higher outcomes than 10% serum (83). It was assumed that this may be due to

FIGURE 4



Common methods of ET catheter loading. Syringe and catheter types may vary. No clear advantage exists of one loading technique over another. Careful considerations should be given to the size of air bubbles, the amount and type of medium used for ET. Shading indicates fluid; white indicates air. Gray circles represent embryos.

Forty years of IVF. *Fertil Steril* 2018.

increased protein content and resulting higher colloid osmotic pressure of the transfer medium being more similar to uterine fluid and the resulting impact on embryo migration in the uterus.

Subsequent studies have identified that supplementation of the medium used for ET may impact outcomes, possibly by impact of the uterine environment. The ET medium containing 0.5 mg/mL of hyaluronan was shown to benefit mouse implantation and fetal development compared with transfer medium without hyaluronan, possibly by facilitation of rapid diffusion of catheter contents to the uterine lumen due to fluid characteristics. A Cochrane review (84) of 16 studies indicates that moderate quality data exist to indicate that use of adherence compounds like hyaluronan in ET medium for humans can improve clinical pregnancy and live birth. However, conflicting data exist and it is unclear whether the use of hyaluronan with ET medium is beneficial versus the use of a transfer medium with an elevated protein content.

Although not necessarily placed into the uterus at the same time as the embryo, infusion of hCG into the uterine cavity immediately before ET has been reported to improve outcomes. Subsequent prospective randomized controlled trials failed to find any benefit. A Cochrane review (85) of 12 trials found that due to variability in the data, current findings do not support the use of hCG infusion into the uterine cavity before ET. Factors, such as timing and amount of hCG, may be important considerations.

**Transfer velocity.** The speed of the embryo-fluid injection into the uterus could also be a variable in ET outcome.

In a study by Grygoruk et al. (86), a mock transfer was accomplished using mouse embryos with either a fast injection or a slow injection speed. Embryos in both groups were compared with control embryos where no ET was accomplished. The percentage of embryos that were shrunk and/or collapsed increased in the rapid injection group. In addition, rates of apoptosis in the embryos of the rapid injection group were increased. Thus, embryo trauma may be related to the rapidity of injection and care should be taken to inject the fluid as slowly as possible. In addition, in vitro modeling suggests that rapid injection may lead to embryo placement issues and possibly promote EPs (78).

**Timing.** The timing surrounding ET may also be involved in success. Matorras et al. (87) demonstrated that the interval between catheter loading and the discharge of the embryos into the cavity affected IVF outcomes. When this delay was >120 seconds, there was a decrease in PRs from 31.6% to 19.1% and a decrease in implantation rate from 15.9% to 9.4%. This may be related to how long the embryos are outside the incubator and experiencing environmental stress. The delay in injection might be a surrogate marker as well for the difficulty of ET, as these variables were not separated. Nevertheless, minimizing the time between loading and transfer would seem to be appropriate. The influence of the time interval before withdrawal of the catheter after ET has also been investigated, but no differences in clinical outcome have been seen.

The decision of when to transfer embryos is also an important factor in the performance in the procedure. There is growing evidence of impaired endometrial receptivity during fresh IVF cycles due to the controlled ovarian hyperstimulation (COH) medications and the altered hormonal stimulation of the uterine lining. Shapiro et al. (88) were one of the first to demonstrate that frozen ET cycles significantly improved ongoing PRs and implantation rates compared with fresh transfers. There has also been more recent evidence of improved obstetric outcomes with frozen versus fresh transfers.

**Progesterone.** Fresh ET occurs after COH. The combination of GnRH agonist and follicle aspiration, which can deplete granulosa cells (GCs), results in a P deficiency in the luteal phase. The role of P supplementation in fresh ET has been extensively studied. A large meta-analysis by van der Linden et al. (89) found that comparing P with no treatment, its administration for luteal phase support significantly increased the live birth rate (odds ratio: 2.95).

Improvements in cryopreservation and the expanded use of chromosomal screening of embryos at the blastocyst stage have led to an increased use of frozen ETs. The two main approaches to preparing the endometrium for frozen ET include the natural cycle or an estrogen (E)/P-supplemented (programmed) cycle. A meta-analysis (90) of 20 studies found no differences in clinical PRs or live birth rates between natural frozen ET cycles and programmed cycles.

TABLE 4

## Elements for successful ET.

## Goal

Easy, atraumatic transfer  
 Proper placement  
 Minimize embryo stress  
 Negotiate a difficult/stenotic cervix  
 Optimize implantation, minimize contractions

*Forty years of IVF. Fertil Steril 2018.*

## Key approaches

Trial transfer, ultrasound, soft catheter  
 Inject slowly 1.5 cm from fundus, confirmed by ultrasound  
 Minimize transfer time, catheter loading, control temperature/pH, careful injection  
 Before cycle luminaria or dilation, malleable stylet, ultrasound  
 Blastocyst transfer, frozen ET, avoid trauma to cervix or fundus

With regard to programmed cycles, the main variables are the timing of the start of P and the route of administration. A Cochrane Database Review (91) of prospective randomized trials concluded that transferring blastocysts on the 5th or 6th day of P results in a significantly higher PR (odds ratio: 1.87; 95% confidence interval: 1.13–3.08) than if P was started a day earlier (day 4) or a day later (day 7). This study also found that vaginal and IM P resulted in equivalent clinical PRs (odds ratio: 1.07; 95% confidence interval: 0.61–1.89), although more recent assessments may contradict this finding.

**Endometrial injury.** A Cochrane review (92) of 14 randomized controlled trials indicates that moderate quality evidence indicates that endometrial injury performed between day 7 of the previous cycle and day 7 of the ET cycle is associated with an improvement in live birth rates and clinical PRs in women with more than two prior failed ETs. There is no evidence of an effect on miscarriage, multiple pregnancy, or bleeding. Importantly, endometrial injury on the day of oocyte retrieval is associated with a reduction of clinical rates and ongoing PRs and may also reinforce the importance of an atraumatic ET and avoidance of endometrial injury close to the time of ET. Although current evidence suggests some benefit of endometrial injury, additional studies are required with stringent controls and improved patient stratification.

**Bedrest.** After ET, bedrest has been a controversial subject, with some investigators recommending extended bedrest and others, virtually none. Recent studies (93, 94), including a meta-analysis, have failed to demonstrate a clear improvement in ongoing pregnancy with bedrest after ET and some researchers have indicated a possible a detrimental effect of bedrest. Thus, although individualizing recommendations for patients' preferences and anxiety, bedrest after ET has no proven benefit.

In conclusion, attention to detail and adherence to key factors are critical to optimize transfer of a preimplantation embryo to the uterus (Table 4). The performance of an atraumatic ET is essential to IVF success. Ultrasound guidance appears to be one adjunct that maximizes the chance for a successful ET. The delivery of embryos 1.0–2.0 cm from the fundus using a soft catheter yields optimal implantation rates. Catheter loading is important, and although air bubbles are useful, no single technique appears to be superior to another. The inclusion of hyaluronan in

the ET media appears to have benefit for implantation. Soft catheters appear to improve outcomes compared with stiff catheters. There is no evidence that bedrest significantly improves pregnancy or live birth. Due to the adverse effects of COH on the endometrium, frozen ETs have demonstrated improved PRs as well as better obstetric outcomes.

## TOWARD SINGLE EMBRYO TRANSFER

René Frydman, M.D.

At inception of assisted reproductive technology, around 1980, each pioneer IVF center used the natural cycle as the standard protocol. Progression of the dominant follicle was followed by daily measurements of LH in plasma or urine for identifying the preovulatory LH surge. Oocyte retrieval was performed by laparoscopy, which succeeded in barely 60%–70% of cases.

To solve this problem and increase the IVF yield, ovarian stimulation (OS) was proposed, using clomiphene citrate first and then hMG. Later, the availability of GnRH agonists allowed us to prevent the risk of premature ovulation, which greatly improved the physician's flexibility for managing OS.

Before embryo freezing became available—in 1984—all or nearly all embryos obtained through OS were transferred to maximize the then dismal pregnancy rates. Failures still outnumbered successes, yet multiple pregnancy nonetheless at times occurred, particularly in younger women. The majority of infertility doctors, often not being involved in obstetrical care, tended to be oblivious of the complications emanating from multiple pregnancies. Therefore, it logically was the pediatricians who first blew the whistle about this novel epidemic of multiple pregnancies, accompanied at times by severe prematurity and catastrophic complications, such as mental retardation. Consequently, following Alan Trounson's and Linda Moorh's pioneering work in embryo freezing, the majority of teams elected to freeze excess embryos. Still, most physicians transferred two or three embryos to optimize pregnancy rates.

Numerous meetings took place to alert IVF practitioners to this growing concern and to attempt to curb the



incidence of twin and high-order multiple pregnancies. A randomized trial published by Jan Gerris's group (95) described the characteristics of high-quality embryos as having four to five blastomeres on day 2 and at least seven on day 3 after insemination and <20% of anucleated fragments. Embryos that fulfilled these criteria had implantation rates of 42.3%.

These data were confirmed a year later by a publication showing that elective single ET (eSET) on day 3 markedly reduced the twinning rate (96). Two years later, it was suggested that eSET can be proposed for women <36 years of age at the first or second treatment cycle when a top-quality embryo is available (97).

In 2000, the European Society of Human Reproduction and Embryology declared that a twin pregnancy rate of  $\geq 25\%$  is unacceptable. The target objective was a twin pregnancy rate of approximately 10% and no triple pregnancy. "Get the number of babies you want, but one at a time" was the motto.

Sweden, Belgium, and the United Kingdom updated their legislation to impose eSET on all women except those who had suffered two failed assisted reproductive technology cycles or were over 39 years of age. The policy is thus based on the biologist's ability to identify a top-quality embryo.

Different culture media were proposed to facilitate the development of embryos to the blastocyst stage. The idea

was to select out embryos that had a better chance of implanting by eliminating those that arrested on day 3. Classification of blastocysts offers an opportunity for the biologist to select out the best ones. More recently, effort has been devoted to using time-lapse images of embryo development, although we are still awaiting the final proof of efficacy of this approach.

A different approach has consisted of looking at the genetic composition of the developing embryo through preimplantation genetic testing for aneuploidy. Although extremely promising, this approach is still being tested for the risk of damage caused to the developing embryo. Alternate options are similarly being pursued for validating noninvasive genetic testing. More recently, the mitochondrial endowment of the embryo has also been looked at, but this is still controversial.

In conclusion, promoting and identifying embryos with high developmental potential are primary goals for the years to come. Without ignoring the endometrium, it appears that fostering the achievement of top-quality embryos should help avoid unrelenting OS strategies leading to the freezing of excessive numbers of embryos that may never be used. The eSET objective is dependent on our ability to identify top-quality embryos that have high implantation and developmental potential.

## TECHNIQUES DEVELOPED FROM IVF

### IVF FROM INCUBATION TO INJECTION

**Lauren A. Bishop, M.D., Davora Aharon, M.D., Catherine Gordon, M.D., Erika New, M.D., and Alan Decherney, M.D.**

Tremendous advancements have been made in the field of assisted reproductive technology (ART) over the past 40 years. Laboratory techniques have evolved, allowing for far greater precision and improvements in patient care as well as outcomes. Culture media have improved, enabling fertilization to occur in vitro and the transfer of embryos to occur at the blastocyst stage. The treatment of male factor infertility has evolved, with much higher fertilization rates seen with the development of intracytoplasmic sperm injection (ICSI). Patients are also exposed to fewer operating risks as we have transitioned from laparoscopic to transvaginal procedures. Here, we will discuss the necessary steps along the way to the current state of IVF.

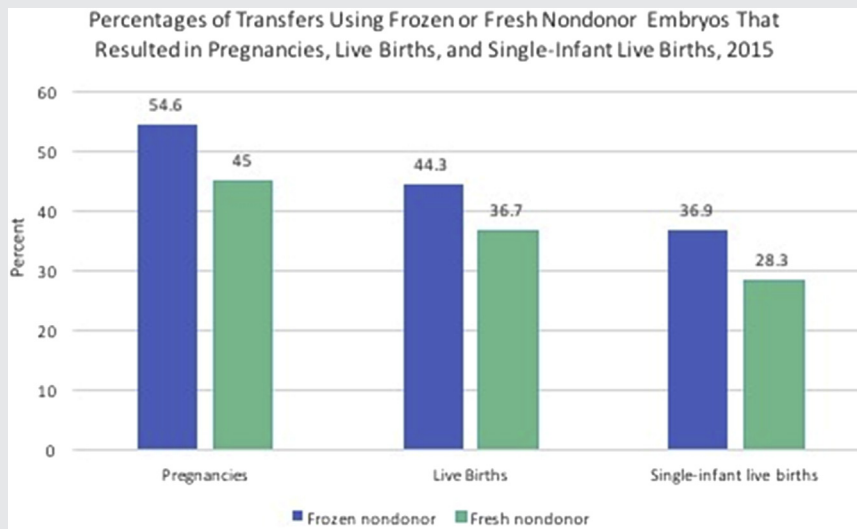
### GAMETE INTRAFALLOPIAN TRANSFER

Gamete intrafallopian transfer (GIFT) is a fertility technique in which both oocytes and sperm are placed directly into the fal-

lopian tube, allowing for IVF to occur. The technique was shown to be successful in primates in 1980 and was first offered to humans at a religious-affiliated hospital in Dayton, Ohio, in 1983 as an alternative to IVF (98). Shortly thereafter, the first successful pregnancy conceived through GIFT occurred in 1984.

Patients treated using GIFT underwent routine ovarian stimulation, and oocyte retrieval was performed by laparoscopy or minilaparotomy. Postretrieval, up to four mature oocytes were drawn into a catheter followed by sperm, while allowing for air space between the two media to ensure fertilization did not occur until the media were placed in the fallopian tube (98). The catheter was then laparoscopically passed through the fimbriae and advanced to the ampullary portion of the fallopian tube where the gametes were injected (98). Meirow and Schenker demonstrated the passage of four oocytes resulted in a 28% pregnancy rate (99). Transferring additional oocytes did not increase pregnancy rates, only the risk of multiples up to 31% (99).

In 1995, GIFT was performed in 13.5% of ART cycles worldwide (99). Despite the manipulation of the fallopian tube that occurs with catheterization, ectopic pregnancy rates were similar between GIFT and IVF, 5.5% and 5.54%, respectively (99). Benefits of GIFT included decreased cost and a more physiologic environment for fertilization. It was also an option for those with religious beliefs, which precluded

**FIGURE 5**

Percentages of transfers using frozen or fresh nondonor embryos that resulted in pregnancies, live births, and single-infant live births, 2015. Reproduced from Centers for Disease Control and Prevention (103).

Forty years of IVF. *Fertil Steril* 2018.

them from using IVF. However, if pregnancy was not achieved, patients would require additional stimulated cycles, thus increasing the overall cost compared with IVF cycles with embryo vitrification. In addition, GIFT required patency of at least one fallopian tube and adequate sperm and posed additional risks associated with surgery under general anesthesia (99).

Initially, pregnancy rates with GIFT exceeded those of IVF, with a pregnancy rate of 33.9% with GIFT compared with 19.1% with IVF (100). With advancements in IVF, subsequent randomized trials showed the clinical pregnancy rate after GIFT was only 34%, compared with 50% after IVF (101). While GIFT is infrequently used today, it is a unique technology that still may be requested by patients, particularly those with religious objections to IVF.

### ZYGOTE INTRAFALLOPIAN TRANSFER

To circumvent concerns of poor fertilization with GIFT, a procedure known as zygote intrafallopian transfer (ZIFT) was introduced in the late 1980s. Oocytes were retrieved laparoscopically or transvaginally, fertilized, and laparoscopically transferred directly into the fallopian tube. This technique allowed providers to confirm fertilization while allowing early development and embryonic transport via the fallopian tube.

ZIFT was limited by the need for an invasive laparoscopic procedure, imposing the risks associated with general anesthesia and abdominal surgery as well as additional costs. As advancements were made in ultrasound, transvaginal follicle aspiration became the foremost

technique used for oocyte retrieval, allowing for a more minimally invasive approach. Per the 1994 Society for Assisted Reproductive Technology data, GIFT and ZIFT had higher pregnancy rates than IVF (28.7% and 29.1% vs. 21.1% per retrieval) (102). However, given the minimally invasive approach of IVF, 78% of practitioners preferred this method over tubal transfer of gametes or zygotes. By 2015, with improvements in IVF technology, IVF accounted for over 99% of ART procedures as the live birth rate per transfer improved to 36.7% for fresh nondonor embryos and 44.3% for frozen nondonor embryos (Fig. 5) (103).

Current practice generally reserves the use of ZIFT for difficult ETs due to cervical stenosis or patients with recurrent implantation failure (104). Results have been inconsistent on the impact of ZIFT for implantation failure. Older studies have demonstrated significantly higher pregnancy rates in recurrent implantation failure patients using ZIFT compared with IVF (104), however, IVF techniques have improved since that time. While ZIFT currently accounts for <1% of all ART conventionally performed, it may still have a role in a select patient population.

### SUBZONAL INSEMINATION

Micromanipulation techniques including partial zona dissection (PZD) and subzonal insemination (SUZI) were developed in the 1980s to enhance the success of IVF in couples with male factor infertility.

PZD facilitates sperm entry into the oocyte by creating one or more holes in the zona pellucida. The hole was created mechanically with a needle or chemically using

acidic Tyrode's solution (105). Results of PZD were initially promising in cases of male factor infertility, with fertilization rates of 68% compared with 33% with conventional insemination (105). However, the results were inconsistent, and overall fertilization rates were low in patients without prior IVF cycles. PZD resulted in a rate of polyspermy of up to 57%, as the number of sperm entering the perivitelline space could not be controlled (105, 106).

SUZI was developed as a more direct method of fertilization. SUZI consists of inserting one or more spermatozoa directly under the zona pellucida into the perivitelline space (107, 108). This enabled the treatment of severe male factor infertility, as well as decreased rates of polyspermy due to control over the number of spermatozoa injected (108). It has been demonstrated that up to four spermatozoa could be injected without increasing the rate of polyspermy, while injection of 5–10 sperm was associated with a 50% rate of polyspermy (106). Initial studies reported fertilization rates of 25%–71% with this technique (107). While this presented an option for men with severe infertility who failed IVF, overall clinical pregnancy rates remained low, at only 2.9% of embryos transferred (109).

PZD and SUZI presented innovative potential solutions to male factor infertility, however, their clinical application was limited due to overall low success rates and elevated rates of polyspermy. The advent of ICSI presented a technique with improved outcomes, with the first live birth using this procedure documented by Palermo et al. in 1992 (110). A randomized comparison found that ICSI doubled the fertilization rate compared with SUZI (33% vs. 16%) and generated embryos in 83% compared with 50% of cycles (111). ICSI has become the standard of care for patients with male factor infertility, with fertilization rates of 60%–70%, similar to rates of conventional insemination in men with normal semen parameters (112). The use of ICSI in IVF cycles has increased from 36.4% in 1996 to 76.2% in 2012 among fresh IVF cycles and in 93.3% of cycles with male factor infertility (113).

Despite the current lack of clinical use of PZD and SUZI, these innovations were critical to the development of ICSI, a technique whose use is now widespread and crucial to the success of IVF in couples with male factor infertility.

IVF has evolved dramatically over the past 40 years. Although some of these pioneering techniques are rarely used in current practice, each has been pivotal in historic advancements in the field.

## IN VITRO MATURATION OF OOCYTES

Seang Lin Tan, M.D.

In vitro fertilization (IVF) is very successful, with cumulative live birth rates exceeding spontaneous conception in fertile women (114). However, few people are aware that in vitro maturation (IVM) of oocytes had in fact been re-

searched long before clinical IVF was established. IVM was first observed in rabbit oocytes by Pincus and Enzmann in 1935. Edwards focused on achieving meiotic maturation of animal and human oocytes and then achieving fertilization. Edwards remained an advocate of IVM for the rest of his life. When the two of us founded the London Women's Clinic with Howard Jacobs and Stuart Campbell, I recall him telling me that "If given a choice, no patient would prefer to use hormonal medications," and he was a driving force behind the formation of the International Society of In Vitro Maturation of Oocytes.

### DEFINITIONS

Today what actually constitutes IVM is controversial. Some believe that stimulation of any kind, especially hCG priming, negates the use of the term IVM. I think from a pragmatic clinical perspective any egg collection cycle, when the majority of the oocytes are expected to be at the germinal vesicle (GV) stage, could be named an IVM cycle. For research purposes, IVM can be broken down into hormonally stimulated or unstimulated IVM (115).

### HISTORY

Initial successful IVM cycles were reported by Cha in Korea and Trounson in Australia in early 1990s. However, because maturation and pregnancy rates through the 1990s were relatively low, IVM was rarely undertaken. In the mid-1990s, I recruited Ri Cheng Chian, who promised to produce a functional IVM program if I could secure him \$500,000 funding. We started the program within a year. We were fortunate, and our first series of 25 patients had a maturation rate of 84%, fertilization rate of 87%, and live birth rate of 40% (116). While the norm was collecting immature oocytes without any hormonal priming, I had decided to give hCG 10,000 IU when the biggest follicle was <10 mm to time egg collection (116), because Willis et al. had observed that granulosa cells responded to LH once follicles reached 9–10 mm in ovulatory women with normal or polycystic ovaries (PCOs), but anovulatory women with PCOs responded to LH even when follicles were as small as 4 mm (117).

Our randomized controlled trial (118) showed that after hCG priming, the eggs were all collected at the GV stage, and after 24 hours the maturation rate to the metaphase II (MII) stage was almost 75% in women given hCG compared with 5% in women who were not given hCG. By 48 hours of maturation, rates were 84% versus 69% in hCG and control groups, respectively. These maturation rates have never been exceeded since (118), although Sanchez et al. have achieved maturation rates approaching these with preincubation culture using C-type natriuretic peptide (119). We preferred intracytoplasmic sperm injection (ICSI) for fertilization as we believed that the zona was harder in women with IVM, although subsequent studies have suggested that perhaps conventional IVF may suffice if semen analysis is normal. From the earliest days it was quite clear that synchronization of oocyte development and the endometrium was critical and we used estrogen from the time of egg collection to accelerate

endometrial growth and P from the time of fertilization of the embryos.

Fetal outcomes of IVM pregnancies are as good, and the incidence of congenital malformations is no different from that of pregnancies derived from IVF and spontaneous pregnancies in healthy women (120).

One secret that I realize years later accounts for our excellent results at the time was the meticulous nature of checking the GV eggs several times a day. I recall Chian would email me every couple of hours after egg retrieval to tell me when the eggs had reached MII, and he would do ICSI soon after even if it was the middle of night. The only other embryologist who did this was Hai Ying Chen, and again we had extremely high results. Most embryologists check the eggs only once daily and fertilize them when they are at the MII stage. This way, for instance, GV oocytes collected on a Monday morning would be noted to be at the MII stage on Tuesday morning and ICSI would be performed. It is possible that the eggs had become MII stage by Monday night, but they are injected with sperm many hours later, which could hamper their potential. Going forward, it could prove useful to culture GV eggs in time-lapse imaging machines so that the precise moment of becoming MII can be determined and fertilization can be done soon after. In practice, it is easier to schedule IVM egg collections in the early afternoon.

While IVM was almost exclusively reserved for women with PCO or PCO syndrome (PCOS), over the next few years, indications broadened to include poor responders who declined egg donation, over-responders to ovarian stimulation for IVF (converting to IVM with early trigger and collection if over-response risking ovarian hyperstimulation syndrome [OHSS] was evident after a few days of stimulation), egg donors who declined to have hormonal stimulation, and those who had failed egg maturation in regular IVF (121). One interesting indication was patients who had unexpectedly repeated poor embryo quality for no obvious reason. In fact, our very first successful IVM patient was a reproductive endocrinology and infertility fellow in her early 30s. She produced almost all poor-quality embryos in three IVF cycles with different stimulation protocols. In her first IVM cycle she produced a large number of good-quality embryos and had a baby. Later on, she again produced almost all poor-quality embryos in several top IVF programs in the Middle East and Europe. She returned to Montreal, had good-quality embryos with IVM, and had a second baby. Since then we have had a number of patients like her, and this shows that there are a select group of patients who for some unexplained reason do not do well with any stimulation protocol but are successful with IVM only.

## CURRENT IVM PROTOCOL

We induce withdrawal bleed, measure the antral follicle count, and repeat a second ultrasound scan when we estimate the largest follicle to be 12–14 mm; then we give hCG 10,000 IU and perform oocyte retrieval 38 hours later. If the patient is anovulatory, we give FSH 150 IU on days 4, 6, and 8. To synchronize the development of the eggs and endometrium, we usually start administering  $E_2$  in a dose of 2 mg 3 times a

day from the time of egg collection and P from the time of fertilization of the embryos. The dosage of estrogen depends on the endometrial thickness (if the endometrial thickness is <6 mm, 10–12 mg of  $E_2$  is given, if endometrial thickness is between 6 and 8 mm, 8–10 mg of  $E_2$  is given in divided doses). Although fresh ET can be done on day 3 or 5, recent data suggest frozen ET in an artificial cycle is better. Walls et al. (122) have shown that although the live birth rate per cycle started with fresh ET is lower with IVM than with IVF, the live birth rate per cycle with frozen embryos with IVM or IVF is comparable at over 30% per cycle (122).

In the last few years there have been a number of new techniques of performing IVM egg collection. Dahan et al. has described “rapid pass” technique, performed by passing a 19-gauge single lumen oocyte aspiration needle through the ovarian stroma, with constant suction (7.5 kPa) applied (123). This technique involves collecting the visible follicles first; the oocyte collection needle is then passed repeatedly through the ovarian stroma, while suction is applied constantly for 3–5 minutes. In this case, the stroma may appear devoid of follicles on ultrasound. Suction is maintained while moving the needle through the long axis of the ovary, while repeatedly changing the vertical path of collection to have the needle tip pass through a significant proportion of the ovarian stroma. After 3–5 minutes of constant suction, the needle is removed from the ovary and flushed with heparinized saline to clear any possible blood clots. Using this technique, as many as 125 oocytes have been collected in a single cycle (123). However, many of the oocytes retrieved were extremely immature and did not mature well by conventional IVM techniques. Recent advances by Evelyn Telfer and colleagues in Edinburgh in the in vitro growth of primordial follicles will hopefully improve this in the future (124).

Another innovation has been the introduction of a 19-G single-channel pseudo double lumen needle by Steiner and Tan, which has been shown in some studies to obtain superior oocyte retrieval results (125).

## POTENTIAL APPLICATIONS OF IVM

### IVM in the era of antagonist protocol with agonist trigger

Many IVF doctors feel that with GnRH agonist trigger in GnRH antagonist cycles, there is no risk of OHSS and, therefore, no need for IVM. In fact, although the risk of OHSS is significantly reduced, it is still present. Table 5 summarizes a few recent studies showing that early severe OHSS can occur if there is agonist trigger with low-dose hCG (1,500 IU) rescue, if there is agonist trigger with high-dose estrogen and P supplementation for fresh ET, or even if there is agonist trigger and a freeze all embryos policy. Only IVM can avoid OHSS totally.

### IVM instead of natural cycle IVF

Natural cycle IVF involves no ovarian stimulation and trigger at 18-mm follicle but is associated with 30% premature LH surge and ovulation. Modified natural cycle IVF involves starting daily GnRH antagonist and FSH injections once a follicle reaches 14 mm, until hCG trigger is given when the



TABLE 5

## Summary of recent studies.

First author and reference number	Title	Results
Seyhan (126)	Severe early OHSS following GnRH agonist trigger with the addition of 1,500 IU hCG	23 cycles GnRHa trigger + 1,500 IU hCG 35 hours later, luteal support, fresh ET; severe early onset OHSS: 21.7% (5/23).
Iliodromiti (127)	Consistent high clinical pregnancy rates and low OHSS rats in high-risk patients after GnRHa triggering and modified luteal support: a retrospective multicentre study	275 GnRHa triggered cycles + 1,500 IU hCG luteal phase. OHSS: total, 4.4%. Early onset, 2.6%: one severe 0.72%; four moderate 1.5%; two mild 2.2%. Late onset, 1.8%: one severe and four mild.
Iliodromiti (128)	Impact of GnRH agonist triggering and intensive luteal steroid support on live-birth rates and OHSS: a retrospective cohort study	363 GnRHa trigger + intensive luteal support, and fresh ET (Vietnamese population). One late onset severe OHSS case; two early onset mild/moderate OHSS cases with freeze all.
Fatemi (129)	Severe OHSS after GnRH agonist trigger and “Freeze all” approach in GnRH antagonist protocol	GnRH trigger no luteal hCG, freeze all; two cases of severe OHSS.
Gurbuz (130)	GnRH agonist and freeze all strategy does not prevent severe OHSS: a report of three cases	GnRHa trigger, no luteal hCG, freeze all: three cases of severe OHSS.
Ling (131)	GnRH agonist trigger and ovarian hyperstimulation syndrome: relook at “freeze-all strategy”	Severe OHSS reported in PCOS young woman 12 hours after oocyte pickup.

Forty years of IVF. Fertil Steril 2018.

leading follicle is 18 mm. Hence, there are typically three FSH and three GnRH antagonist injections, and generally one MII oocyte is retrieved with 15%–20% clinical pregnancy rate per cycle started. In stimulated IVM, three injections of FSH are given on days 4, 6, and 8, and hCG is given when the leading follicle is 12–14 mm with no need for GnRH antagonist administration. Several MII and many GV oocytes can be obtained to generate multiple blastocysts so that in a good IVM program, a clinical pregnancy rate/cycle started of 45%–50% can be obtained in women up to 37 years of age. Hence, I believe that IVM should replace natural cycle or modified natural cycle IVF.

### IVM instead of FSH/IUI

The first line of treatment in many fertility programs is FSH stimulation combined with IUI. This treatment requires approximately eight to 10 daily injections of FSH and has a pregnancy rate of 15%–20% and a multiple pregnancy rate of 30% and costs about \$2,000 in North America for medications and IUI. In comparison, IVM requires one (hCG) to four injections (FSH days 4, 6, and 8 and hCG), followed by egg collection when the biggest follicle is 10–14 mm followed by ET and will yield a 45%–50% pregnancy rate and a 5%–10% multiple pregnancy rate and costs \$4,000 with no risk of OHSS. Therefore, especially in countries where patients begin attempting fertility treatment when they are relatively young, IVM may in fact replace FSH and IUI as the first-line infertility treatment. Hatirnaz et al. have achieved a 35% live birth rate/cycle with elective single ET (132), and unpublished data suggest similar results with letrozole-stimulated IVM cycles.

### IVM and fertility preservation

When presented with an oncology patient wanting fertility preservation, we determine whether ovarian stimulation can be safely performed and how long the patient can

wait before the start of chemotherapy (Fig. 6) (133). If hormone stimulation is not contraindicated and chemotherapy can wait, we will perform ovarian stimulation followed by mature egg collection. If, however, hormone stimulation is contraindicated or there is no time, then IVM with or without ovarian tissue freezing are the options. I saw a stage 2 estrogen receptor breast cancer patient on a Wednesday afternoon clinic who needed to start chemotherapy a week later. We gave her hCG 10,000 IU that evening, retrieved 19 GV eggs on Friday, matured 17 of them by Sunday, and vitrified 17 MII oocytes Sunday evening (134). We were able to preserve her fertility without any ovarian stimulation, surgery, or delay of chemotherapy. The use of IVM also allows multiple egg collections to be performed at any phase of the menstrual cycle including the luteal phase (135).

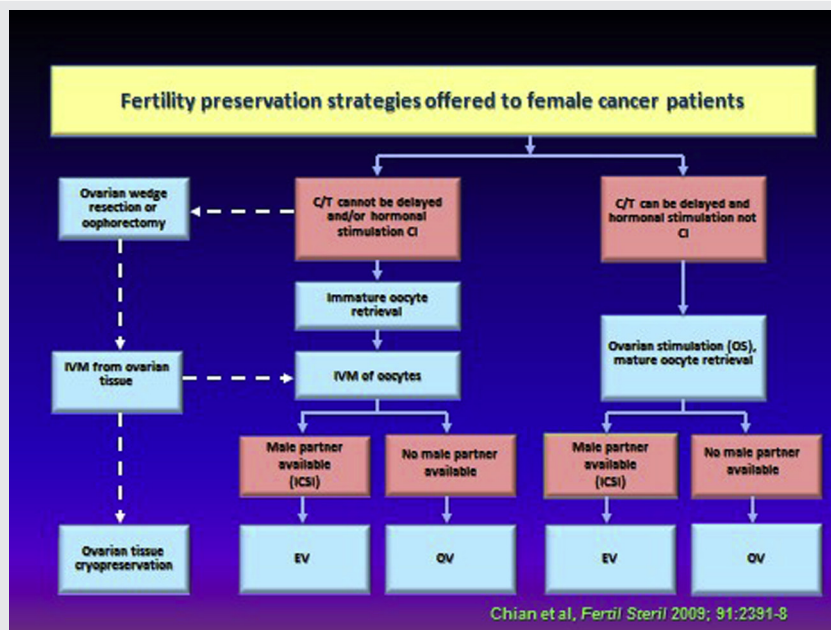
## POTENTIAL FUTURE APPLICATIONS

### GV nuclear transfer to overcome age-related infertility

GV nuclear transfer is a potential future use of IVM. Because aneuploidy probably occurs with meiotic division of the oocyte, it is plausible that if GV nuclear transfer is undertaken with a woman in her late 40s to an enucleated oocyte from a young egg donor, with electrofusion, followed by IVM and preimplantation genetic screening, it may be possible to avoid egg donation by making the older woman's eggs better quality.

### Ethnic differences in IVM outcome

One unexpected finding in the last couple of years is that we have observed much better results with IVM in some ethnic groups than in others. I had always been surprised by the initial results from some groups in California showing that IVF results were lower in women of Chinese origin compared with Caucasians. It led me to believe

**FIGURE 6**

Preservation strategies offered to female cancer patients.

Forty years of IVF. *Fertil Steril* 2018.

that perhaps because the protocols that we use are almost all derived from research in Western Europe or North America, we just assume that they are equally applicable to all age groups and all ethnic origins. Just like we realize today that patients with the same type and stage of cancer may respond quite differently to different treatment protocols, it may well be that this applies equally to reproduction. Because of our long history of the use of IVM, we had the pleasure of working, training, and collaborating with IVM centers around the world. It often appears that centers in Vietnam, China, Japan, and Korea are able to get better results than many more established centers in Western Europe and in North America. Therefore, I am now planning to set up a chain of centers worldwide in which the same protocols can be used for both IVF and IVM, with people of different backgrounds with the same standard operating procedures and culture media. Combined with genetic screening of embryos in appropriate cases, all the data can be subjected to deep learning and algorithms for artificial intelligence to try to tease out the role of ethnic origin in results of fertility treatment of IVF and IVM. This perhaps could lead to paradigm changes in the coming years.

### IVM for social egg freezing

A major issue with reproduction nowadays is the low fertility rate below replacement levels in developed countries, largely as a result of delayed childbearing. If a man has cancer, there is no debate that he should freeze sperm before chemotherapy. Women do not need chemotherapy to lose their fertility. Fertility invariably rapidly declines with advancing age. It

would therefore seem sensible that every woman should consider freezing eggs when they are young to preserve fertility. In practice, many women are unaware of this rapid decline of fertility (136), which requires public education. At other times, they feel it would not affect them but the fact is that even happily married women with children face a high risk of divorce and may need to start a second family in their later years so that social egg freezing makes sense. The final impediments are cost and need for ovarian stimulation. The latter two concerns can be overcome by IVM social egg freezing. Although current results are suboptimal (133), research by Moawad et al. (137, 138) will hopefully improve success rates in the future, so that every young woman can easily undertake multiple IVM egg freezing cycles to create a personal egg bank for herself.

### CONCLUSIONS

Although IVM was first introduced as a method to avoid OHSS in women with PCO/PCOS, the use of IVM has evolved but is nevertheless useful in women at very high risk of OHSS, those who wish to avoid hormonal stimulation completely, some women who repeatedly produce poor-quality embryos with regular IVF for no obvious reason, and poor responders who do not wish to undertake egg donation. IVM should be considered whenever natural cycle or modified cycle IVF is deemed indicated. IVM is useful for fertility preservation when there is a contraindication to the use of hormonal stimulation or when there is extreme urgency, and IVM may be deemed an alternative to FSH IUI especially in young patients. Potential applications of IVM in the future include

GV nuclear transfer to overcome age-related infertility and IVM for social egg freezing.

## OOCYTE DONATION

**Richard J. Paulson, M.D., M.S.**

Oocyte donation was the natural consequence of the technique of IVF. Once it became commonplace to observe oocytes in a laboratory dish, it did not require a large leap of the imagination to realize that human eggs could be donated, in a manner analogous to that of sperm. In the early 1980s, at a time when only a handful of successful pregnancies with IVF had been achieved, several groups began experimenting with egg donation. It was thought to be a logical method of achieving pregnancy in women with premature ovarian failure as well as in those who wished to avoid passing on to their children a heritable disease.

Since all oocyte retrievals at that time required laparoscopy, a relatively traumatic procedure, initial attempts at oocyte donation involved eggs obtained at the time of IVF performed on infertile women who were themselves undergoing fertility treatment. Since embryo cryopreservation was also in its infancy, fertilized eggs obtained at the time of IVF were either transferred to the uterus or discarded. Therefore, when a larger number of eggs was obtained, one or more could reasonably be donated to a potential recipient without compromising the success of the treatment of the potential donor. It was this set of circumstances that led to the first successful clinical pregnancy after egg donation (139). A woman undergoing IVF in Australia had five oocytes retrieved and elected to donate one of those oocytes to another infertile woman, who was ovulating naturally at the same time. The oocyte successfully fertilized, was transferred into the synchronously developing endometrium of the recipient, and successfully implanted. Unfortunately, this pregnancy ended in a miscarriage. Shortly after this proof of concept, the same group of investigators reported a successful term pregnancy after the transfer of an embryo derived from a donated egg. In this case, the recipient had premature ovarian failure and the endometrium had been prepared with exogenous estrogen and P (140). This pregnancy, achieved in late 1983, proved to be a landmark event. The success of this technique demonstrated several basic physiologic principles. First, there did not appear to be a prohibitive immune reaction on the part of the recipient of the donated oocyte. Second, the recipient's uterus could be prepared for implantation with the use of exogenous estrogen and P. The new procedure appeared to be a viable option for women who needed a donated egg to conceive. However, a major barrier to the widespread use of egg donation by IVF at that time was the need for general anesthesia and laparoscopy to obtain the eggs from the donors. This relatively invasive step discouraged the use of designated egg donors, such as sisters wishing to donate eggs to their infertile sisters. Less invasive options for obtaining eggs began to be explored.

The nonsurgical transfer of embryos had previously been reported in several mammalian species, with the first report (in a rabbit model) appearing in 1890 (141). The method relied

on natural ovulation and fertilization in the donor. The developing embryo was then flushed from the uterus of the donor before implantation and then transferred to the uterus of the recipient (142). In the early 1980s, a group of investigators led by John Buster at Harbor-UCLA attempted to apply this technique to humans (143). The concept was not complicated, even if the actual practice proved challenging: normally ovulatory women under the age of 35 were selected to be egg donors. At the time of spontaneous ovulation, they were inseminated with the sperm of the husband of the infertile recipient. The uterus of the donor was then flushed sequentially on post-LH surge days 4, 5, and 6 in an effort to recover the fertilized blastocyst. The recovered conceptus was then transferred to the uterus of the infertile woman, whose ovulation was monitored and thus known to be synchronous with the ovulation of the donor. Endometria of recipients with ovarian failure were prepared with exogenous estrogen and P. Several pregnancies were achieved in this manner, by both the group at Harbor-UCLA (144) and a group in Italy (145).

The clinical efficiency of uterine flushing as a treatment of infertility remained low, since nonstimulated natural ovulatory cycles were being used. The logical step of adding controlled ovarian stimulation of the donors to the process was subsequently investigated by two groups in the late 1980s. Unfortunately, ovarian stimulation did not produce the hoped-for success; rather than increasing the yield of recovered blastocysts, the number of recovered embryos recovered actually decreased (146). Moreover, some donors now experienced retained pregnancies, as the uterine flushing procedure appeared to become less efficient as a result of the stimulation (147). The reasons for this lack of success are still not clear, because further attempts at uterine lavage were abandoned as a new method of oocyte retrieval came into use that relied on the rapidly advancing technology of transvaginal ultrasound.

Transvaginal ultrasound-guided follicle aspiration revolutionized oocyte donation. Egg retrievals could, in most instances, be accomplished in an office setting under conscious sedation. Egg donors no longer needed to undergo insemination with sperm or risk a subsequent retained pregnancy. Controlled ovarian stimulation could be used, thus increasing the yield and efficiency of the process. Whereas previous egg donation attempts used "spare oocytes" donated by infertile women, egg donation now resembled standard IVF. This change in the process made meaningful comparisons between the two techniques possible. It became clear that oocytes obtained from young, fertile donors resulted in much better IVF outcomes than those obtained with oocytes obtained from older, infertile patients (148). It also became possible, for the first time, to compare pregnancy outcomes in younger versus older recipients. Since quality of oocytes could now be controlled, the effect of the age of the uterus on pregnancy success could be estimated. As is now well known, no decrease in pregnancy rates was observed with the increasing age of the recipient, even beyond the age of 40 years (149). The observation of this unanticipated physiologic principle opened the doors to oocyte donation to women of advanced reproductive age and made pregnancy possible

for thousands of women who were previously thought to be hopelessly infertile. Subsequent studies demonstrated that pregnancies could be achieved even in women who were beyond the age of natural menopause (150). Obstetrical risks are higher for older women, but pregnancy rates with eggs donated by young women remain high, regardless of the age of the recipient.

Since recipients often had premature ovarian failure, and thus were not normally cycling, endometrial receptivity had to be induced with the use of exogenous hormones. A variety of doses and routes of administration of estrogen as well as P were tried. After some 30 years of experience, there is still not one “ideal” regimen that works better than others (151). It does appear that P has to be administered either by the IM or the transvaginal route, as oral, rectal, and transdermal routes are associated with excessive metabolism and inadequate bioavailability. Since estrogen is required in far lower doses than those of P, virtually all routes have been reported to be successful, including oral, transdermal, IM, and vaginal. One of the unanticipated findings that resulted directly from the exogenous administration of steroids via the vaginal route was the discovery of the apparent selective uptake of steroids from the upper vaginal canal directly into the endometrium. It had been known for quite some time that steroids are absorbed readily through the vaginal epithelium, but when endometrial biopsies were performed, endometrial tissue levels of both estrogen and P were found to be far higher after vaginal than after IM administration (152). Therefore, vaginal administration of steroids for the purpose of endometrial development is a highly efficient process. The physiological reasons for this “uterine first-pass effect” are only speculative at this time.

Since the timing of administration of both estrogen and P could now be controlled, it also became possible to define a timing window, during which embryo implantation would best take place. The “window of implantation” was a novel concept (142, 153) that has continued to be investigated to the present day; a full understanding and description have not yet been achieved. It does seem fairly clear that to prepare the endometrium for implantation, adequate estrogen pretreatment or “priming” must take place to induce sufficient P receptors in the endometrium. After this pretreatment period, adequate P signaling is required to set in motion the endometrial changes that eventually result in endometrial receptivity during the implantation window. Optimal timing of the initiation of P relative to the time of egg retrieval continues to be debated, likely because the window of implantation is fairly wide, and many other confounding factors influence embryo implantation (151).

In the early 2000s, techniques of oocyte cryopreservation advanced to the point where reliable results could be obtained with elective oocyte cryopreservation and subsequent thawing (154). This was an important advance in assisted reproduction, primarily for women who wished to preserve their fertility when faced with imminent ovarian failure, such as occurs as a side effect of cancer therapy. Other women chose to preserve their oocytes as a method of mitigating the age-related decline in reproductive potential. In the context of oocyte donation, oocyte cryopreservation made possible the

establishment of oocyte banks, which provided frozen donor oocytes to recipients in a manner analogous to that of frozen sperm in sperm banks. Oocyte banks brought with them a new set of issues that are, as of this writing, still unresolved. Previously, there was little motivation to obtain more oocytes from a single stimulation of a donor than was reasonably needed to produce a pregnancy. However, with egg banks, every oocyte obtained from a donor has very real commercial value. Oocyte donors are producing ever more oocytes from stimulations that are designed to maximize oocyte yield. How this gradual change in donor stimulation is going to translate into pregnancy outcomes and risks to the donors remains to be seen. However, at present, it does appear that success rates with frozen eggs are similar to those with fresh eggs.

In the future, it may be possible to generate human oocytes from stem cells. Both sperm and oocytes have been successfully generated from induced pluripotent stem cells in the laboratory in nonhuman mammalian species, leading to live births. It seems likely that this technique, best described as “in vitro gametogenesis,” will be applicable to humans as well (155). The biological details of such processes are still incompletely understood, but it seems plausible that in vitro-generated oocytes might well be able to circumvent the age-related decline in reproductive potential, in addition to providing gametes to those patients without gonads. When this occurs, oocyte donation from one woman to another will likely stop being used as a method of overcoming infertility. In vitro-generated oocytes will allow women to reproduce with their own genetics at a time when their own in vivo-generated oocytes are no longer functional. Analogously, fertility preservation by cryopreservation of gametes may be obviated by this technology. Since preimplantation genetic testing allows those patients who carry heritable diseases to avoid passing these to the next generation, oocyte donation is also unlikely to be needed for this indication in the future.

At present, however, oocyte donation remains an important component of the treatment of infertility. It remains an essential treatment for women whose ovarian function is lost to premature ovarian failure, as a result of gonadotoxic treatment for malignancies or simply as the result of reproductive aging. When, in the future, oocyte donation becomes simply a part of the history of assisted reproduction, many of its lessons will remain, including the induction of endometrial receptivity, understanding of embryo-endometrial synchrony, and hormonal support of early pregnancy.

## HISTORY OF THE USE OF GESTATIONAL CARRIERS IN THE UNITED STATES

James M. Goldfarb, M.D., M.B.A.

### INITIAL GESTATIONAL CARRIER CASE

The first birth using a gestational carrier occurred in April 1986 (156). The biological parents had a long history of



infertility. The wife had lost both of her fallopian tubes to ectopic pregnancies. In 1981, the couple went to Bourn Hall in England to attempt IVF. At that time, Bourn Hall had the most experience in the world with IVF. The couple did conceive with IVF at Bourn Hall. However, at 22 weeks of gestation, the wife's uterus ruptured. The uterine rupture caused the baby to die and the wife to undergo a hysterectomy. The husband, a New Jersey cardiologist, realized that his sperm and his wife's eggs were obviously able to conceive a pregnancy. In 1984, he and his wife inquired of several fertility centers that had successful IVF programs at that time whether it would be feasible to have embryos conceived with their sperm and eggs implanted into another woman's uterus. My team at the Mount Sinai Medical Center in Cleveland, Ohio, found the proposal to be very interesting and challenging. We contacted the couple and told them we were, from a scientific standpoint, very interested in their proposal. However, we were concerned about the ethical and legal issues. We did offer the couple the opportunity to come to Mount Sinai to further evaluate their proposal. The couple had consulted a lawyer and brought a large amount of legal documentation to Mount Sinai. The couple met with the Mount Sinai Ethics Committee and the Mount Sinai Institutional Research Board. After long deliberations, the Ethics Committee and the Institutional Review Board both approved the proposal for this one case.

The logistics of synchronizing the cycles of the biological mother and the gestational carrier were much more complex at that time than they are now. GnRH agonists were not being used nor was programmed endometrial stimulation with estrogen and progesterone. In addition, embryo freezing was not at all efficacious at the time. Thus, we had to hope that the timing of the biological mother's egg retrieval would be somewhat synchronous with the spontaneous ovulation of the gestational carrier. The first two attempted cycles had to be canceled because the cycles were not synchronous. The gestational carrier became discouraged and was not willing to attempt a third cycle. A second gestational carrier was chosen, and the first cycle with this carrier was sufficiently synchronized to proceed and was successful. The baby was born in April 1986 and made history as not only the first baby born by a gestational carrier but also as the first baby to be legally handed over to a non-birth mother without having to be adopted. During the pregnancy, the biological parents had obtained a court order declaring them the legal parents and stating that their names should be on the birth certificate. The baby was featured on the cover of *Life* magazine in April 1987, in honor of her first birthday. She graduated from Emory University in 2008 and currently lives in New York City where she got married in 2016. Attending her wedding was one of the highlights of my career.

## INDICATIONS FOR USAGE OF GESTATIONAL CARRIERS

The initial, and still classic, indication for use of a gestational carrier is a woman with no uterus, either because she has had

a hysterectomy or because she was born without a uterus. With time the indications for use of a gestational carrier greatly expanded to include

1. Uterus that is compromised enough to clearly impair implantation.
2. Medical disease that would be dangerously exacerbated by a pregnancy.
3. Failed IVF. Initially this did not seem to be an indication for the use of a gestational carrier, but with time it has been shown to be efficacious for selected women with failure of implantation.
4. Recurrent miscarriage especially if there is an uncorrectable uterine issue, but it may also be efficacious in certain cases of unexplained recurrent miscarriages.
5. Poor obstetric history including most commonly recurrent premature deliveries.
6. Same-sex male couples/single men.

## USE OF GESTATIONAL CARRIERS IN THE UNITED STATES

The use of gestational carriers in the United States has greatly increased through the years, numerically and also proportionally, compared with nongestational carrier cycles. According to the Centers for Disease Control and Prevention, in 1999 there were 727 gestational carrier cycles performed in the United States, which accounted for 1% of IVF cycles in the United States. In 2013, there were 3,432 gestational carrier cycles, which accounted for 2.5% of IVF cycles performed in the United States. Between 1999 and 2013, the number of clinics performing gestational carrier cycles increased from 167 (45%) to 324 (69%). Because many countries do not allow the use of gestational carriers, patients outside of the United States elect to come to the United States in order to use a gestational carrier. The percent of gestational carrier cycles used by non-U.S. residents has fluctuated over the years. From 1999 through 2005 the percentage of non-U.S. residents using gestational carriers decreased from 9.5% to 3.0%. Then from 2005 to 2013 the percentage increased from 3.0% to 18.5% (157).

Over the years the percentage of gestational carriers using donor eggs has increased. Currently slightly more than half of gestational carrier cycles use donor eggs. Although the relative contribution is not reported, clearly one of the reasons for the increase in the use of egg donors in gestational carrier cycles is the increased number of same-sex male couples and single males desiring to have a family.

As embryo cryopreservation has become so much more efficacious, the majority of the gestational carrier cycles use frozen embryos. This negates the need to coordinate the carrier and the biological mother's cycles. The use of frozen embryos is also useful in cases in which there is concern about obtaining transferable embryos from the biological parents. In this case, one can determine whether there will be transferable embryos before the biological parents incur the expense (especially legal expense) of finalizing a gestational carrier.

The use of gestational carriers is much more complex than the use of egg donors. The American Society for Reproductive Medicine (ASRM) has established recommendations

for practices using gestational carriers (158). While one can never completely eliminate contentious issues with gestational carriers, it seems that most of the publicized problems in the United States are due to not conforming to the recommendations of the ASRM and/or delivering in states that are not friendly to the use of gestational carriers. There are at least three states (New York, New Jersey, and Michigan) that declare paid gestational carrier contracts to be nonenforceable and/or illegal. Other states have many restrictions including supporting gestational carrier procedures only if the intended parents' gametes are used. In contrast, states such as California consistently uphold intended parents' rights to parenthood regardless of whether they use their own genetic material or donated gametes. An example of a case that became extremely contentious involved a same-sex male couple who used an anonymous egg donor and the sister of one of the men as a gestational carrier. Toward the end of the pregnancy the carrier stated she wanted custody of the twin boys. The ASRM guidelines recommend psychological counseling of the gestational carrier and independent legal counsel for the carrier and the biological parents and that the carrier has had at least one birth. In this case, none of these recommendations were followed and, in addition, the baby was delivered in New Jersey. Needless to say, this resulted in prolonged legal proceedings and in somewhat of a joint custody arrangement.

Even more so than with many other reproductive technologies, the use of gestational carriers has raised multiple psychological and ethical issues. The details of these issues are beyond the scope of this article but include reproductive autonomy, commodifying women, commodifying children, coercion, exploitation, and impact on the family.

## CHOOSING A GESTATIONAL CARRIER

Choosing a gestational carrier is much more difficult than choosing an egg donor, particularly an anonymous egg donor, whom the infertile couple will never meet or have an ongoing relationship with. About two-thirds of our couples/individuals use an agency or social media to find a gestational carrier. The other third use a gestational carrier they know. The known carriers are often relatives, but our patients have used a variety of other sources, such as friends and neighbors. One of our patients had two daughters carried by two different gestational carriers. She was very open about her use of gestational carriers and mentioned her use to the checkout woman at the grocery store. The woman said she would love to be a gestational carrier. Shortly thereafter, my patient decided to have another child and took the checkout woman up on her offer. Another of my patients had previously donated eggs to her sister and then later found that she would need to use a gestational carrier. Her sister to whom she had donated eggs became her gestational carrier.

As mentioned previously it is imperative to follow the ASRM guidelines when gestational carriers are being used. It is particularly important to make sure there is no coercion when known carriers are being used. For example, a mother could put pressure on a daughter to carry a pregnancy for another daughter who needs a carrier. It is imperative to meet with the proposed carrier alone and to make sure she

is not being coerced. I always tell the proposed carrier that if she feels she is being coerced I will say there is a medical reason she cannot be a carrier.

## WHAT HAVE WE LEARNED

Clearly the management of the gestational carrier treatment cycle has become much simpler over the years since the first successful cycle in 1985. The major reasons are the use of programmed endometrial stimulation for the carrier and the shift to the use of frozen embryos in gestational carrier cycles. It has also been clearly shown that nonmedical problems with use of gestational carriers can be minimized by strict adherence to the ASRM guidelines.

The use of gestational carriers has also enhanced knowledge in other areas of reproductive endocrinology. At the beginning of this decade it began to be questioned whether for many (or possibly all) patients going through IVF it may be preferable to freeze all embryos and transfer them in a later programmed cycle. Results of gestational carrier cycles were helpful in providing insight into this question. The 2010–2011 Society for Assisted Reproductive Technology data showed that up to age 40 years, the age-adjusted success rates for gestational carrier cycles were 7%–8% higher than for fresh non-gestational carrier cycles. In contrast, when success rates were compared between gestational carriers and infertile women using thawed embryos derived from egg donors, the success rate of gestational carriers was only 1%–2% more than that of infertile women (159). These data supported the rather new postulation that the endometrium in programmed frozen transfer cycles is preferable to that in a fresh IVF cycle.

Woo et al. reported on perinatal outcomes in the same women after natural conception versus a subsequent gestational carrier cycle. They showed increased negative perinatal outcomes in the offspring from the gestational carrier cycles. They suggest assisted reproductive procedures may affect embryo quality, despite a healthy uterine environment (160). A recent article by Murugappan et al. (161) found, as have others, that the live birth rate across all IVF types was higher for gestational carriers. They also found the use of a gestational carrier resulted in higher birth weights among autologous fresh transfers and donor fresh and cryopreserved transfers. We recently compared outcomes of pregnancies when embryos derived from donor eggs were transferred to a gestational carrier or to an intended parent. As with autologous eggs, the use of donor eggs had a higher live birth rate when transferred to a gestational carrier. In addition, the incidence of prematurity and low birth weights was significantly lower in gestational carriers (unpublished data).

## THE FUTURE

It certainly seems the trend toward increased use of gestational carriers will continue. The main limitation on the degree of growth is the extremely high cost involved with the use of a gestational carrier. The recent success of uterine transplantation offers an alternative to gestational carrier in the subset of cases where the woman has no uterus or a nonfunctional uterus (161). Currently, the medical complexity and the cost of uterine transplantation will greatly limit its use. However,

as with other disrupting technologies, the process may, with time, become more efficacious and efficient, thus providing a more realistic alternative to a subset of women for whom now the only alternative is a gestational carrier.

## UTERUS TRANSPLANTATION

Mats Brännström M.D., Ph.D.

Absolute uterine factor infertility (AUI) was untreatable, until 2014, when proof of concept was established with the birth of a healthy baby boy after uterus transplantation (UTx) (162, 163). Women with AUI have either congenital/surgical uterine nonappearance or any anomaly (functional/anatomical) that hinders implantation or continued pregnancy. The AUI prevalence is around 20,000 women of fertile age in a population of 100 million (164). Gestational surrogacy is an alternative, but not a treatment, to achieve genetic motherhood and legal motherhood (after adoption from surrogate). The surrogacy procedure is nonapproved in most countries due to ethical, religious, and/or legal reasons.

An uterine allograft is a type of vascularized composite allograft transplantation, like the face and hand. However, the extraordinariness of UTx, compared with transplantation of the hand and face, is that live donors (LDs) can also be used. Moreover, also unlike solid organ transplants, the blood flow of a uterine graft will be shared by two individuals (mother and fetus), and the success of UTx is not demonstrated within days but after more than a year after transplantation, with the possible delivery of a healthy child. After the woman has delivered the number of children she wishes, hysterectomy should be conducted. Thus, immunosuppression (IS) is only temporary, and this will considerably reduce the risk for IS-associated side effects, such as nephrotoxicity, diabetes, and certain malignancies.

The Swedish team began research preparations in the UTx area in 1999, initially with experiments in the mouse. This resulted in the first live births, in any species, being reported in the mouse in 2003 (165). Later our preparations for clinical UTx involved four more animal species, including nonhuman primates, and preclinical human studies. Our evolution of this new procedure, from basic animal studies toward clinical application, follows the IDEAL recommendations for the introduction of surgical innovations (166).

## ANIMAL RESEARCH

Several animal models have been used in preclinical UTx research. UTx was then customized with respect to the size of the vasculature and the opportunity to accomplish vascular connections. In the rodent models, the deceased donor (DD) concept was exclusively used since parts of the aorta or common iliac arteries were harvested alongside the uterine graft, to gain large enough vessels to master vascular anastomoses. Moreover, the availability of inbred mouse and rat strains made it possible to perform syngeneic transplantation, with no need for IS. This allowed us to study the effects of transplantation surgery in isolation, without the added effects of IS. In the larger animal models (mostly sheep and nonhuman

primates), both the DD and LD concepts were evaluated. The LD concept was evaluated in animals both by autologous UTx, with no need for IS, and by allogeneic UTx.

The definition of a successful UTx procedure is whether it can render the recipient fertile to deliver a healthy offspring. This issue has been evaluated in several animal species as described below.

### Fertility after UTx in rodents

The mouse UTx model was heterotopically transplanted with the uterine graft and with blood flow through end-to-side caval-caval and aortic-aortic vascular anastomoses (165). The original demonstration of embryo implantation in any UTx setting was in the mouse after ET. Pregnancy rate per uterus was equal in the transplanted uteri as compared with the native control uteri with offspring of normal weight (165). The growth trajectory up to adulthood followed the typical curves, and normal fertility was observed. No studies exist on fertility after allogeneic UTx in the mouse.

In the rat, with larger blood vessel size, the uterus was placed orthotopically and with anastomoses performed end-to-side on the common iliacs. In syngeneic UTx, between inbred Lewis rats, the pregnancy rate was similar in UTx animals as in controls, and there was no difference in the number of pups per pregnancy (167). The growth trajectory was similar in offspring from animals of the UTx group and the sham-operated control group. The first ever report of fertility after allogeneic UTx was a study exploring this in the rat with discordance between two major histocompatibility sites and tacrolimus IS to prevent rejection (168). Pregnancy rates were similar in the UTx group and in the control group. A follow-up study, also with tacrolimus IS, involved Lewis donors and Piebald-Virol-Glaxo recipients (169). The pregnancy rate was somewhat lower in the UTx group, as compared with the two sham-operated control groups. Birth weights of UTx offspring and growth trajectories of the pups until postnatal week 16 were unaffected in comparison with controls (169). These data indicated for the first time that allogeneic UTx may be regarded as safe in terms of perinatal outcome, at least in a rodent species.

### Fertility after UTx in sheep

Fertility in the sheep was first demonstrated after autologous UTx with uterine-tubal-ovarian transplantation and end-to-side vascular anastomoses of the uterine artery, utero-ovarian vein, and ovarian artery, including an aortic patch, to the external iliacs (170). After spontaneous mating, offspring of normal sizes were delivered by cesarean section, around 2 weeks before term (170).

The allogeneic sheep UTx model involved surgery with hysterectomy including short vascular pedicles of the uterine artery and vein with two identical operations done in parallel to shift the uteri between outbred sheep (171). Anastomosis was by bilateral end-to-end anastomosis of the uterine arteries and veins. ET was performed in five ewes, and three became pregnant. One pregnancy resulted in a live birth via cesarean section, and the other two in ectopic pregnancy and miscarriage (171). The live birth is so far the only live birth from a large animal undergoing allogeneic UTx.



## Fertility after UTx in nonhuman primate

The only offspring after UTx in a nonhuman primate species was after autologous UTx in the cynomolgus macaque (172). The uterus was retrieved with the uterine artery and the deep uterine vein, which after back table flushing were anastomosed bilaterally end-to-side to the external iliacs. The total surgical duration was 13.5 hours. Pregnancy after mating occurred after three menstrual bleedings. The pregnancy was uneventful until day 143, when partial placental abruption occurred and a cesarean section was performed with delivery of a live offspring but with fetal respiratory distress.

## THE STORY BEHIND THE SWEDISH UTx TRIAL

The concept of UTx was presented to me in 1998, when I did a fellowship in gynecologic oncology in Adelaide, Australia. I had moved back from Sweden to Australia, where I had, in the early 1990s, done a 3-year period of postdoctoral research in ovarian physiology and ovulation in the group of Rob Norman and Colin Matthews. After obstetrics and gynecology resident training in Sweden, I was ready to move back down under for some more years of training, but now on the clinical side. My intention was to do a fellowship in reproductive medicine in Adelaide. However, an Australian citizen already occupied that position and I was instead offered a training position in gynecologic oncology. That subspecialty had never been in my thoughts, since I had long research experience in a field strongly related to reproductive medicine. However, I came to like the extensive surgery of gynecological malignancies, and my comprehensive experience in rodents was beneficial to quickly incorporate the tricks of the trade of oncology surgery into my surgical arsenal. I worked intensively with my resident doctor Ash Hanafy, and after some months we took care of large parts of the surgery, while the chief surgeon was busy with administrative tasks. In October 1998, we had an unusual preoperative consultation with a charismatic woman in her mid-20s. She had cervical cancer stage 1b and was planned to undergo a standard radical hysterectomy with lymph node dissection the following week. We told her about the consequences of infertility due to the planned hysterectomy but that her ovaries would be preserved. With her solution-oriented mind she quickly responded with a way to solve this. Her solution was to transplant the uterus from her mother to treat her approaching surgery-induced AUFI. I was bewildered by her suggestion of this concept that I had never heard about. An important activity for young doctors in Australia on Fridays is the after-work pub to debrief the clinical ups and downs during the week that had passed. The debriefing at the pub this afternoon was all about the suggestion of a restoration of fertility by UTx in a woman having undergone hysterectomy or being born without a uterus. The concept got clearer with further discussions. My unique combination of a solid background in infertility-related research and the recently acquired skills in advanced pelvic surgery opened my eyes to the achievability of UTx as an infertility treatment. The morning after, a PubMed search made me aware that research on this topic had actually been done, mainly in dogs, in the 1960s and 1970s, but then the research was aimed

to develop treatment for tubal factor infertility, with a transplantation of the oviducts possibly simplified by inclusion of the uterus in the graft as well. The experiments were at a time before effective IS was introduced, and with the introduction of IVF in 1978 the research activities in this field ceased.

As stated in the section above, we initiated our animal-based research on my return to Sweden in 1999 and it took 15 years until clinical proof of concept. This is a similar time span as for IVF development, from when Edwards started his first attempt to mature human oocytes to the birth of Louise Brown. Concerning both IVF and UTx, the research-based approach and the long duration for preparations before clinical introduction have probably been key factors to success.

## THE FIRST EIGHT BABIES IN THE WORLD—ALL FROM THE SWEDISH TRIAL

Nine LD UTx procedures were performed in Sweden in 2013, with eight recipients having congenital uterine agenesis and one having undergone radical hysterectomy due to cervical cancer 7 years before the trial (173). Broad medical and psychological examinations were done on recipients and donors before UTx, and partners of recipients underwent psychological screening as well. The donors were related (mostly mothers) in all but one case, where a close family friend volunteered for donation. Five out of nine donors were postmenopausal at donation, with two of the donors being above 60 years of age. Importantly, all donors had previous uncomplicated pregnancies with live births. Uterus recovery, including dissection of the uterus with bilaterally vascular pedicles comprising segments of the internal iliac arteries and veins, lasted for 10.5–13 hours. The perioperative and postoperative outcomes of the donors were favorable, and the hospital stays were <1 week. All donors were in good psychological and medical health at follow-up 1 year after surgery (174).

After graft procurement in the donor and back table preparation, the chilled and flushed uterus was positioned inside the pelvis of the recipient after dissection of the external iliacs. Anastomosis was by bilateral end-to-side anastomoses to the external iliacs of the uterine pedicles that included uterine vessels and the anterior iliac arteries as well as patches/segments of the internal iliac veins. Surgical duration was 4–5 hours, and the hospital stay was 4–9 days. The IS treatment was conventional induction IS with maintenance of tacrolimus and mycophenolate mofetil, the latter being discontinued after 8 months if no or only one rejection episode had occurred during this period but replaced with azathioprine in patients with multiple rejection episodes. The 6-month outcome was that seven of nine uteri were still in place, with one removed because of thrombotic occlusion of the uterine vessels and the other due to persistent intrauterine infection with abscess formation (174).

Single ETs were performed from around 12 months after UTx. The fifth woman to undergo UTx in the Swedish trial became pregnant at her first ET with a cleavage-stage embryo, and subsequently the first live birth after UTx took place in Sweden, on September 4, 2014 (163).

In this case, a rejection episode at gestational week 18 was diagnosed, which was effectively reversed by an intermittent increase in corticosteroids. The pregnancy was then



uneventful, and she worked full time until gestational week 31+5 days when the woman was admitted to the hospital due to preeclampsia. During the following morning, a cesarean section was performed, and a healthy boy (1,775 g; -11%) was delivered. The second UTx baby (175) was delivered in November 2014 by cesarean section around gestational week 35. The baby was of normal (+4%) weight. The uniqueness of this case was that the recipient received the uterus donation from her own mother, so the uterus bridged three generations. These two children as well as the six children who were delivered in 2014–2017 are healthy. The take-home baby rate among the seven UTx women who have undergone ET attempts of this Swedish trial is now 86%, and the clinical pregnancy rate is 100%, with one recipient having had a miscarriage after clinical pregnancy.

The Swedish group is underway to complete a second UTx study, including 10 LD UTx attempts and with the donor surgery being performed through robotic-assisted laparoscopy to render the procedure more minimally invasive and to possibly decrease the surgical duration.

### THE NINTH UTx BABY— THE FIRST IN THE UNITED STATES

The ninth UTx baby worldwide was delivered in Dallas, in December 2017. The UTx procedure was an LD procedure from a fully altruistic donor and followed the initial three surgically failed attempts in September 2016 (176).

### THE TENTH UTx BABY— THE FIRST FROM A DECEASED DONOR

The first successful DD UTx attempt in the world was performed in Sao Paulo, Brazil, in September 2016 (177). The procurement was from a young brain-dead donor, and this procedure was purposely prolonged to avoid vascular leakage at flushing on the back table and after reinitiating blood flow in the recipient. The birth of a healthy baby was announced in the media in mid-December 2017. This birth is a milestone in UTx, since it is the first proof of concept of DD UTx.

### CONCLUSION AND FUTURE DIRECTIONS

UTx is the first available treatment for AUFI. As a consequence of meticulous research and preparations, the initial clinical introduction of UTx in 2013 resulted in several births that occurred during 2014–2017. UTx should stay at an experimental stage for several years; this will allow time to optimize the procedure further and to ensure that the procedure is safe with respect to long-term medical and psychological effects, which in the setting of LD UTx include the effects on the donor, recipient, partner of recipient, and future children.

UTx may in the future also come to include nontraditional patient groups such as male-to-female transsexuals and women with androgen insensitivity syndrome. These two groups would also need oocyte donation to accomplish pregnancy after combined IVF and UTx procedure.

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## FERTILITY PRESERVATION IN WOMEN FOR MEDICAL REASONS

Jacques Donnez, M.D., Ph.D.,  
Sherman Silber, M.D., and  
Marie-Madeleine Dolmans, M.D., Ph.D.

Enthusiasm for fertility preservation in single women with cancer began with the classic report by Gosden et al. in 1994 of successful pregnancies in sheep from transplantation of cryopreserved ovary tissue slices. In Europe, the program of ovarian tissue freezing in the field of fertility preservation started in 1996, following a symposium organized in Brussels by Johan Smits, Bob Edwards, David Baird, and Jacques Donnez.

In 2004, Donnez et al. reported the first successful frozen ovarian tissue transplantation, followed later by Meirow et al. in Israel. In the United States, Silber reported in 2005 the first successful fresh ovarian tissue transplantation between monozygotic twins, and 2 years later the first successful frozen ovarian transplants.

Around the same time of these first successes with frozen ovarian tissue, oocyte freezing with vitrification was introduced from Japan and Spain. In the majority of centers, patients have access to oocyte vitrification or ovarian tissue freezing. The indications of course depend on the disease, the age, the timing, and the expertise of the center.

Oocyte cryopreservation by means of vitrification has become the standard method to preserve fertility in women with benign diseases, those seeking fertility preservation for personal reasons, and women with cancer if treatment can be safely postponed (178). Ovarian tissue cryopreservation is specifically indicated for adolescents and women who require immediate cancer treatment (178, 179). The present review focuses on the indications for these two techniques of fertility preservation and the results obtained.

Fresh tissue transplantation in women with premature ovarian insufficiency (POI) will also be addressed in this paper, allowing us to define characteristic differences between fresh and frozen-thawed ovarian tissue reimplantation.

### INDICATIONS FOR FERTILITY PRESERVATION (SUPPLEMENTAL TABLE 1)

#### Malignant diseases

Fertility preservation remains a challenge, particularly in case of breast cancer and hematologic malignancies (Hodgkin lymphoma, non-Hodgkin lymphoma, and leukemia), which constitute the most frequent indications for fertility preservation (178). Chemotherapy (especially with cytotoxic alkylating agents), radiotherapy, surgery, or a combination of these treatments can induce POI (178–181) because the

ovaries are very sensitive not only to cytotoxic drugs, but also to radiation exposure of 5–10 Gy in the pelvic area.

The likelihood that POI will develop after therapy is related to the ovarian reserve, which can vary enormously from one woman to the next (180, 181). Therefore, giving a patient or his/her parents an accurate assessment on the risk of infertility is difficult, because how a disease will develop cannot be predicted (179).

### Benign conditions

Certain benign conditions carry the risk of POI, so fertility preservation should be offered in these instances as well. Indeed, many autoimmune and hematologic disorders require chemotherapy, radiotherapy, or both, and sometimes even bone marrow transplantation. Other conditions that can impair future fertility include bilateral ovarian tumors, severe or recurrent ovarian endometriosis, and recurrent ovarian torsion. Ovarian endometriomas typically result in a reduced ovarian reserve (182). Local intraovarian inflammation induced by the presence of endometriomas triggers follicle “burnout” characterized by activated follicle recruitment with subsequent atresia. There is also increasing evidence that surgery may cause considerable damage to the ovarian reserve. In case of recurrence after surgery, fertility preservation is strongly recommended (178). A family history of POI and Turner syndrome are further indications.

### Age-related fertility decline

The largest group of women seeking fertility preservation includes those who wish to postpone childbearing for various personal reasons and the biggest threat to their fertility is age. This point will be discussed elsewhere in this supplement.

### OOCYTE CRYOPRESERVATION (FIG. 7)

Data from a very recent review (183) suggest that oocyte vitrification-warming is superior to slow-freezing-thawing in terms of clinical outcomes.

In case of fertility preservation for benign indications or social reasons, oocyte cryopreservation is clearly the highest-yield strategy (183, 184). Moreover, it gives women the possibility of reproductive autonomy. Cobo et al. (184) recently reported the outcomes of 137 women returning to use their oocytes that had been previously vitrified for nononcologic reasons. In their series, the authors observed a huge difference in cumulative live birth rates (CLBRs) when only five oocytes were used (15.4%), compared with eight (40.8%) or ten (60.5%) (Supplemental Fig. 1) if subjects were  $\leq 35$  years of age. In women aged  $>35$  years, the respective CLBRs were 5.1%, 19.9%, and 29.7%. Therefore, women should be encouraged to freeze their eggs at a younger age to give themselves the best chance of having a biologic child.

We should point out, however, that the excellent results achieved by acclaimed centers around the world cannot be generalized and used by less experienced centers to counsel candidates for oocyte cryopreservation (178).

When contemplating fertility preservation in women affected by cancer, there are five important points to bear in mind if oocyte vitrification is to be carried out (178):

- 1) To allow time for oocyte vitrification, chemotherapy needs to be delayed by at least 10–12 days.
- 2) The subject must be postpubertal.
- 3) Specific controlled ovarian stimulation protocols should be applied according to the steroid sensitivity of the cancer in question.
- 4) Information on oocyte quality in women with cancer is lacking.
- 5) The excellent results obtained in egg donation programs cannot be extrapolated to women treated for cancer. Cobo et al. reported lower live birth rates after oocyte vitrification in the population of women with cancer (184).

### OVARIAN TISSUE CRYOPRESERVATION (FIG. 7)

In prepubertal girls and women who cannot delay the start of chemotherapy, cryopreservation of ovarian tissue (Fig. 7) is the only option for fertility preservation (178). However, strict selection criteria need to be applied. As comprehensively discussed in one of our recent papers (178), the most important are age  $<35$  years, a  $\geq 50\%$  risk of POI, and a realistic chance of surviving for 5 years.

### Effect of ovarian biopsies on hormone production

Obtaining multiple biopsy samples from one ovary has not been shown to compromise future hormone production (178), whereas removal of a single ovary may shorten the time to menopause by 1–2 years.

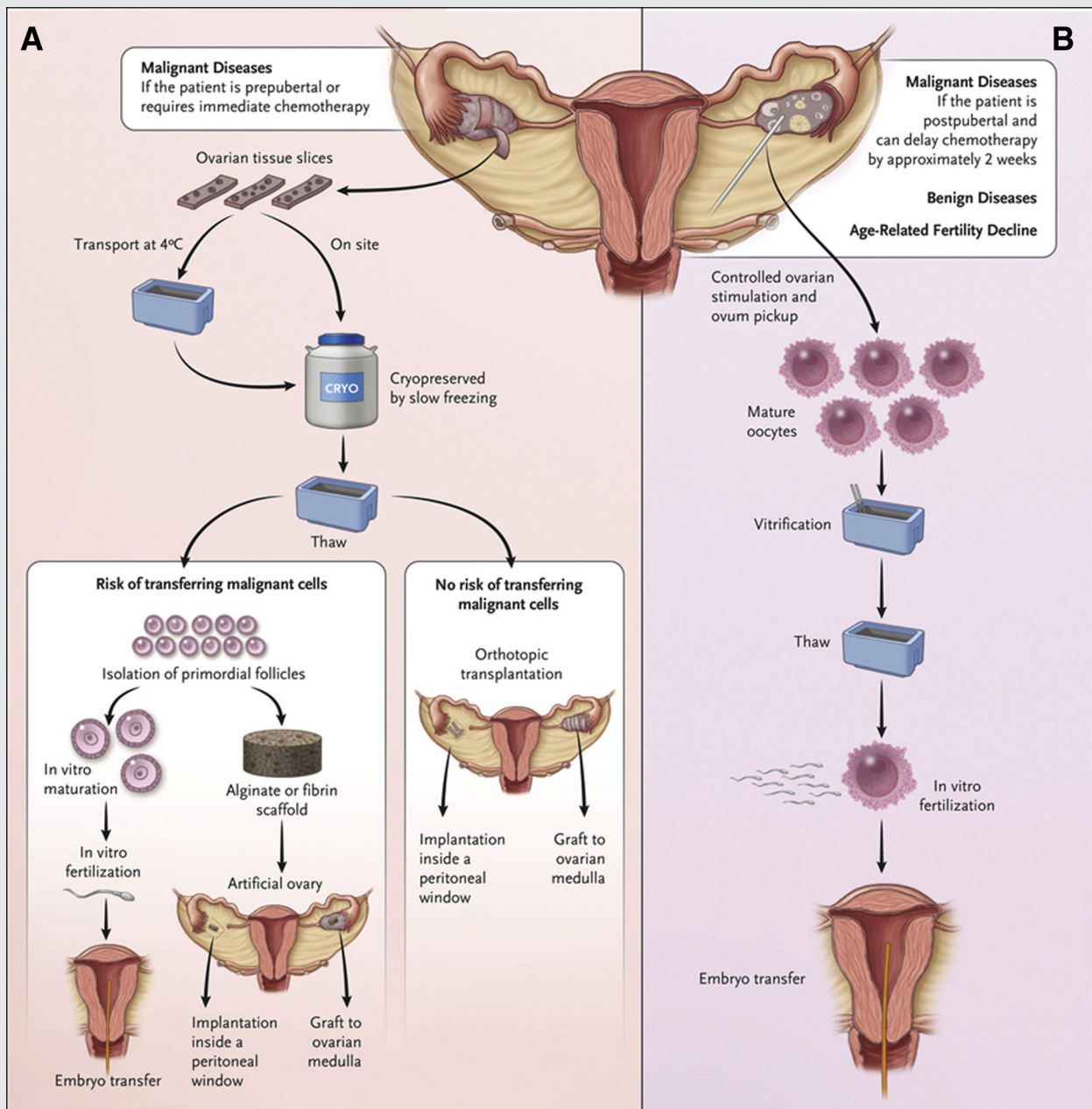
### Techniques of cryopreservation

Initiated by Gosden, the slow-freezing procedure has been widely applied in a clinical context since 1996. Some authors favor vitrification (185), but a great majority of centers still prefer the slow-freezing technique because more than 95% of live births have been achieved after reimplantation of slow-frozen-thawed ovarian fragments (178, 179). On the other hand, there is no evidence that vitrification of ovarian tissue is superior to slow freezing, because vitrification has resulted in only two live births so far.

### Reimplantation of ovarian tissue: pregnancy and live birth rates

After reimplantation of ovarian tissue in the pelvic cavity (Fig. 7), ovarian activity is restored in more than 95% of cases (178). The mean duration of ovarian function after reimplantation is 4–5 years, but it can persist for up to 7 years, depending on follicular density at the time of ovarian tissue cryopreservation. The first pregnancy issuing from this procedure was reported in 2004. Pregnancy and live birth rates have continued to climb steadily, showing an exponential increase (Fig. 8). Indeed, taking into account the latest published series (186–189), the number of live births as of June 2017 exceeded 130 (178).

FIGURE 7



1) If the patient is prepubertal or requires immediate chemotherapy: Ovarian tissue is removed in the form of multiple biopsies (or an entire organ) and cut into cortical strips. The tissue is then cryopreserved by slow freezing on site (or transported to a processing site at a temperature of 4°C). After thawing:

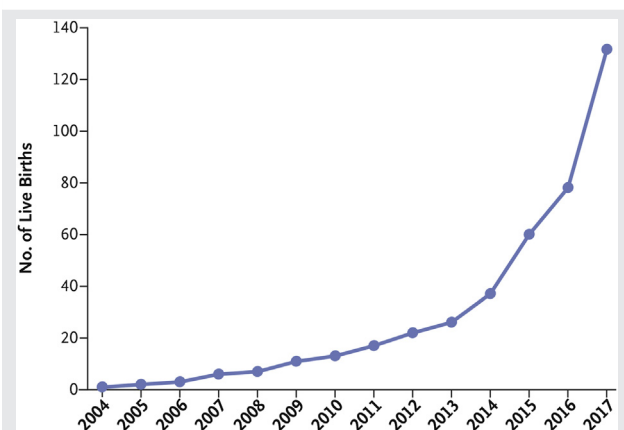
- If there is no risk of transmitting malignant cells, the ovarian tissue can be grafted to the ovarian medulla (in the presence of at least one ovary) or reimplanted inside a specially created peritoneal window.
- If there is a risk of transmitting malignant cells, ovarian follicles can be isolated and grown in vitro to obtain mature eggs, which can then be fertilized and transferred to the uterine cavity. Isolated follicles may be placed inside a scaffold (alginate or fibrin), creating an "artificial ovary" that can be grafted to the ovarian medulla or peritoneal window.

2) If the patient is postpubertal and can delay chemotherapy by ~2 weeks: Mature oocytes are removed after ovarian stimulation and vitrified on site. After thawing, they are inseminated and, in the form of embryos, transferred to the uterine cavity.

3) The combined technique also can be applied, involving ovarian tissue cryopreservation followed by controlled ovarian stimulation and vitrification of oocytes. This combined technique theoretically yields a 50%–60% chance of obtaining a live birth. (From Donnez J, Dolmans M, N Engl J Med 2017;377:1657–65.)

Forty years of IVF. Fertil Steril 2018.



**FIGURE 8**

Since the first pregnancy reported in 2004, the number of live births has reached more than 130, showing a logarithmic increase over the past 2 years and highlighting the need to move from experimental studies to open clinical application (from Donnez J, Dolmans M, N Engl J Med 2017;377:1657–65).

Forty years of IVF. Fertil Steril 2018.

Because the number of reimplantations performed worldwide (the true denominator) is not known, data collection was based on patients from five major centers ( $N = 111$ ), yielding a pregnancy rate of 29% and a live birth rate of 23%. These rates were subsequently confirmed in a series of 74 women (FertiPROTEKT network), with pregnancy and live birth rates of 33% and 25%, respectively (190).

### Personal series

In our series of 22 women undergoing ovarian tissue reimplantation, the live birth rate was 41% (9/22), with a total of 15 live births (178). One woman in our series delivered three times, making her one of two patients worldwide to experience three pregnancies and births resulting from a single ovarian tissue reimplantation procedure.

To improve outcomes, we must address issues such as follicle loss after reimplantation due to ischemia and follicular activation (burnout). One approach involves enhancement of graft revascularization by delivering both angiogenic and antiapoptotic factors (178); another seeks to boost neovascularization using of adipose tissue-derived stem cells, as recently demonstrated in an experimental model by our group.

Transplanting ovarian tissue to heterotopic sites remains somewhat questionable, however, if fertility restoration is the goal.

### Combined technique: ovarian tissue cryopreservation followed by immediate oocyte vitrification (Fig. 7)

It was recently demonstrated that ovarian tissue cryopreservation, followed immediately by ovarian stimulation and oocyte retrieval (with a view to vitrifying mature oocytes),

does not impair oocyte number or quality (183). By combining vitrification of oocytes and cryopreservation of ovarian tissue in patients with cancer, a live birth rate of 50%–60% might conceivably be obtained (Fig. 7). The combined technique thus increases the efficacy of the procedure and gives young cancer patients a greater chance of success.

Vitrification of oocytes for age-related fertility decline yields a CLBR of 60.5% among healthy women <36 years of age (184). In women with cancer, the figure drops to 34% (191), probably because of inferior oocyte quality in women affected by the disease (178).

We therefore suggest that this combined technique be offered to postpubertal patients at high risk of POI, as long as chemotherapy can be delayed without jeopardizing cancer treatment, to maximize their chances of achieving pregnancy (178).

### FRESH OVARIAN TISSUE TRANSPLANTATION

There are actually very few indications for fresh ovarian tissue transplantation. One previous indication is monozygotic twins discordant for ovarian failure (192), and another is allografting from a related or unrelated donor who had previously been a bone marrow donor to the patient (193). A third indication involves women in POI whose religion does not allow egg donation but would tolerate an ovary allotransplant. The latter would require immunosuppression similar to that of any other solid organ transplant.

### Between monozygotic twins

The first successful fresh ovarian tissue transplant in humans occurred in 2005 between identical twins, one of whom had POI and the other healthy and fertile. Grafting of ovarian cortex was the technique used (192).

There are a variety of techniques that have been used for transplantation of ovarian tissue, but successful approaches all adhere to the same basic surgical principles (Supplemental Fig. 2). Recipients of fresh ovarian cortical tissue have “dead” ovarian cortex resected to expose medullary tissue, and hemostasis is obtained microscurgically with the use of microbipolar forceps. The ovarian cortical graft is attached with the use of interrupted 9-0 nylon sutures under optical magnification. The medullary bed is also sutured to the undersurface of the cortical graft to maintain close tissue approximation. This approach avoids microhematoma formation between the transplanted cortex and underlying medulla which, just as with skin grafts, would cause the overlying grafted tissue to die. Donnez and Dolmans use a laparoscopic approach, but the basic microsurgical principles remain the same (182).

All of Silber's series cases were successful ( $N = 9$ ), in that all of them restored normal hormone function (Supplemental Fig. 3). Among those nine patients, seven conceived, leading to 14 pregnancies and 11 healthy births. These patients all favored spontaneous pregnancy over in vitro fertilization and egg donation, and wished to accomplish this in a one-time procedure without ovarian hyperstimulation. Studies do not show unilateral oophorectomy to be detrimental to fertility or with any impact on age of menopause, and Silber



felt comfortable allowing the donation of an ovary from sister to sister.

The recipients continued to cycle from 2 to more than 8 years. Even those few with a low ovarian reserve conceived from the fresh transplant within 2 years, and then again after transplantation of residual frozen tissue. Normal menstrual cycles resumed within 4–5 months, and day 3 FSH levels returned to normal by 4.5 months in all cases. As already noted, in Silber's series a total of 11 healthy babies were born after the nine fresh tissue transplantations.

In the series by Donnez et al. (193), two other live birth were documented after allografting of ovarian cortex between monozygotic twins with Turner syndrome (45,XO) and discordant ovarian function.

### Allografting between two genetically different sisters (Supplemental Fig. 4)

The first allograft of ovarian cortex between two genetically different sisters was described in 2007 (194), and the first series was published in 2010 by Donnez et al. (195) Three women, aged 20, 15, and 12 years, underwent chemotherapy and total irradiation before bone marrow transplantation (BMT), the donor in each case being their HLA-compatible sis-

ters. Years later, HLA group analysis revealed complete chimerism, and ovarian allografting was performed, with the ovarian tissue donor being the sister who had already donated bone marrow. The technique can be seen in Supplemental Figure 4 and was described by Donnez et al. (195). No immunosuppressive therapy was administered. No signs of rejection was observed. Restoration of ovarian function occurred in all three cases (195).

The first live birth to occur after ovarian tissue transplantation between two genetically different sisters was reported in 2011. Because this is an acceptable practice in monozygotic twins, there is no apparent reason not to apply it in genetically different sisters. This is especially true if one of the sisters previously received bone marrow from the other, leading to complete chimerism (HLA compatibility) and obviating the need for immunosuppressive treatment. This approach allows for natural conception, which could be important on moral, ethical, or religious grounds.

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## GENETICS

### REPRODUCTIVE GENETICS PARALLELING ASSISTED REPRODUCTIVE TECHNOLOGY

Joe Leigh Simpson, M.D.

Human in vitro fertilization (IVF) trailed human genetics in its “coming out” by only a few years in the 1960s and 1970s. Both fields shared at the time a common fundamental lack of scientific knowledge and withering criticism concerning untoward consequences of their “premature” clinical application.

For genetics, the prevailing opinion in the 1950s was that genes played pivotal roles in relatively few medical disorders. A few inborn errors of metabolism were known, as were several famous monogenic disorders (e.g., hemophilia A, sickle cell anemia). But there was little appreciation for the etiologic breadth that single-gene disorders would eventually play in diagnosis and clinical care. Sometimes it was even stated that humans were too complex for a single gene to alter the human phenotype with any frequency. Genetic textbooks often resorted to botanical examples. A taint of eugenics overlay the field of human genetics. This author distinctly recalls his first meeting at the American Society of Human Genetics (1967) when still a medical student. He was advised by a well-meaning and well-known geneticist that physicians should avoid human genetics because charges of eugenics could never be erased. The game changer was prenatal genetic diagnosis which began in the late 1960s just after it was

shown possible to culture amniotic fluid cells (1966). Yet even into the 1970s, clinical application was neither widely used nor universally considered to be salutary.

Pioneers in IVF recall similar experience, as recounted elsewhere in this volume. Like genetics, IVF and its biologic requisites was similarly plagued by insufficient knowledge. Our pioneers were confronted with opprobrium for their attempted “unethical” clinical application.

In 2018, we celebrate parallel success of both IVF and reproductive genetics. We owe this to visionary leaders in both fields who stepped forward not only to advance the science, but also to construct the requisite infrastructure for collaboration and ethical oversight. In this communication we recapitulate how progress in reproductive genetics paralleled that of assisted reproductive technology (ART), sometimes in advance and sometimes following.

### REPRODUCTIVE GENES AND DISORDERS OF SEX DIFFERENTIATION

Not until 1956 did we learn that humans had 46 chromosomes. Before this, the diploid number was thought to be 48, determined on the basis of cross-sectional analysis of testicular tissue. Tissue culture was only in its infancy when Tjio and Levan (196) generated a karyotype from lymphocytes, providing the correct answer. A lesson to be repeated is that new technology proves to be the “game changer.” This sequence would recur over the next decades for both reproductive genetics and IVF (soon to take on the moniker of “ART”).

Soon after Tjio and Levan's discovery, autosomal trisomies (of 13, 18, and 21) and sex chromosomal abnormalities were recognized. Gynecologists learned that Turner syndrome was due to monosomy X. By extension, many reasoned that so was all ovarian failure. It was assumed that ovarian failure equated to presence of monosomy X cells (somewhere), even if lymphocytes were nonmosaic 46,XX. Mutant genes were not considered to be significant causes of ovarian pathology until much later. Now we realize that monogenic causes are far more common in adults with premature ovarian failure than 45,X/46,XX mosaicism. The concept of genetic heterogeneity is now well accepted.

In premature ovarian failure (POF)/premature ovarian insufficiency (POI) the sentinel molecular example occurred in 1994, when Aittomäki and de la Chapelle (197, 198) in Finland applied nascent molecular sequencing to confirm autosomal recessive etiology for XX gonadal dysgenesis. The explanation was a nucleotide missense mutation in exon 7 of the *FSHR* gene, resulting in valine to alanine (Val566Ala). Many other genes were later shown to be pivotal for human oogenesis, initially leveraging knowledge from murine knock-out models. Exploiting the homologous human genes, investigators identified single-gene perturbations responsible for POI (see Qin et al. for updated tabulations [199, 200]). Approximately 25% of POF/POI cases result from a single-gene mutation. Increasingly, POF/POI genes are being shown to involve mechanisms less familiar to reproductive endocrinologists, such as chromosomal breakage or DNA repair.

Similar single-gene advances relevant to ART are lagging. But one can expect genetic heterogeneity. One likely example would be variable response to ovulation-stimulating agents. When inevitably shown, this could be exploited to minimize ovarian hyperstimulation syndrome. Heterogeneity could similarly be predicted for endometrial receptivity. Altered endometrial gene expression has already been observed, and clinically applicable predictive genes are expected. Another recent example are perturbations that exist in zona pellucida genes (201).

## MISCARRIAGES AND CHROMOSOMAL ABNORMALITIES

Miscarriage is common and of importance both in reproductive genetics and in ART. Increasing scientific explanations for miscarriage have resulted in a trajectory similar to monogenic etiology for POF. Initially, chromosomal abnormalities were not envisioned as a common cause for miscarriage. Teratogens were thought to be more likely, or trauma. However, cytogenetic studies in the 1960s soon revealed that at least one-half of all first-trimester miscarriages were due to aneuploidy or polyploidy. Chromosomes involved included not just those causing standard autosomal trisomies (i.e., 13, 18, and 21), but every chromosome. The most common was trisomy 16, responsible for 25% of aneuploid miscarriages or 10%–12% of all miscarriages. Triploidy and tetraploidy were also frequent. When ART later became feasible, the inverse relationship between ART success and miscarriage became obvious. Explanations for low ART success in women

of age  $\geq 35$  years became clear. Reproductive genetics and ART both benefited from early application of cytogenetic technology.

Technologic advances in cytogenetics have since led us further. Preimplantation genetic testing for aneuploidy (PGT-A) is now widely applied to increase the likelihood that an embryo transfer will be successful. One could logically transfer only euploid embryo(s), eschewing transfer of morphologically normal yet actually genetically abnormal aneuploid embryos. The technology could not rely on the karyotype of a single cell, but it was initially possible with fluorescent in situ hybridization (FISH) with chromosome-specific probes. Initially, only five to nine chromosomes could be interrogated, but by 2010 new genomic technologies (array CGH or now next-generation sequencing) allowed all 24 chromosomes to be assessed. Given this, trophoctoderm biopsy and PGT-A have been shown to benefit women of age 35–40 years (202, 203). With the use of PGT-A, pregnancy rates are increased by 20%. Miscarriage rates decrease, being equal in 35–40-year-old women to those of younger women (204). The well-known decrease in ART success is delayed if only euploid embryos are transferred.

## GENETICS OF GYNECOLOGIC DISORDERS: PEDIGREES AND THE PATH TO SEQUENCING

The ability of reproductive genetics to reveal the breadth of genes influencing gynecologic disorders was inchoate for a considerable length of time. A genetic basis of skeletal and connective tissue disorders (e.g., achondroplasia, Marfan syndrome) had been recognized much earlier. Rarity of reproductive geneticists was one explanation for limited progress. Another was the unavoidably reduced number of familial aggregates, given that only women could be affected. Yet, even in the 1960s attention was called to familial aggregates of endometriosis, müllerian aplasia (MA), incomplete müllerian fusion, and polycystic ovarian syndrome (PCOS) (205). Reports were sparse, data were not population based, and causative genes could barely be hypothesized. By the 1980s useful information was being generated. For example, recurrence risks for first-degree relatives in endometriosis proved to be 5%–7%, indicating polygenic etiology (206).

Traversing the gap to the present required the next quantum of technologic progress, namely facile DNA gene sequencing. When the human genome was sequenced, the “normal” DNA sequence allowed one to interrogate regions of individual genes. Gene associations according to agnostic whole genome approaches identified unexpected genes associated with PCOS or POF/POI, after which perturbed sequences could be found (207, 208). Genes responsible for endometriosis and uterine anomalies were identified. Studying outcome of surrogate pregnancies with the use of oocytes of women with MA revealed no uterine anomalies in 18 female offspring. Autosomal dominant etiology, therefore, was not a common explanation (209).

In ART, determining single genes of relevance is in its infancy. Few monogenic phenomena are known, but we should anticipate that we can expect to find these in lethal euploid embryos, oocyte receptivity, and endometrial receptivity.

## SEQUENCING THE EMBRYO BEFORE TRANSFER

If over the past 40 years there has been a field whose velocity of progress is greater than IVF, it must be DNA sequencing. Initially a nucleotide sequence could be deduced only laboriously and retrospectively on the basis of amino acid sequence. Sickle cell anemia was known to be the result of glutamic acid replacing valine in codon 6. The DNA sequence could thus be deduced: adenine in lieu of thymine. Direct nucleotide sequencing for prenatal genetic diagnosis had to await technological advances. Development of polymerase chain reaction technology allowed one to amplify DNA in sufficient quantity to perform sequencing. Restriction-fragment-length polymorphisms could be exploited to cut DNA differentially. One could now distinguish normal from mutant DNA sequences on the basis of fetal DNA fragment lengths and thus distinguish normal from affected. Prenatal diagnosis for  $\beta$ -thalassemia by means of amniocentesis became possible. But sequencing the entire human genome was awaited, often with societal trepidation.

Sequencing the human genome had been a dream of geneticists forever, but for eons the dream was considered to be quixotic, even wasteful. It was initially difficult to imagine how to proceed. One needed to accumulate innumerable small DNA sequences and place them in proper order. A laborious strategy was devised based on identifying fragments in which nucleotides at the end of one fragment corresponded in sequence to those at the proximal end of a second sequence. The two fragments were therefore overlapping and must be sequential (consecutive). This approach led to the sequenced human genome in 2000 and again more completely in 2006 (210, 211).

Sequencing the human genome generated shock and embarrassment to some. We humans did not have the expected hundreds of thousands of protein-coding genes, but only some 21,000. Actually this made it easier to construct a useful reference source from which to search for perturbations in genes (whole exome sequencing [WES]). Existence of a reference source also made it possible to match an unknown DNA sequence with its chromosomal origin. More efficient methods were naturally developed, namely, amplifying and sequencing many DNA fragments concurrently. Chromosome location of a tested sequence could quickly be determined with the use of the encyclopedic “normal DNA” (computer) reference book. That a sample was different from expectations could be determined. One could also use whole-genome sequencing (WGS) for the 98.5% noncoding regions. At present, presence of rare disorders can be detected by sequencing an affected cohort in the nursery. Yield varies but may be sufficiently high (10%–15%) to be applied to routine clinical use. Sequencing is a first-call approach in many clinical circumstances, replacing the current odyssey of laboratory and imaging tests and reducing hospitalization length.

Soon all of us will be sequenced! Ergo, so should embryos before transfer for ART. In miscarriages, 50% are chromosomally abnormal, but what explains the loss of euploid embryos? Actually, only one-fourth of these are morpholog-

ically normal (212). The other three-fourths of euploid embryos (3/8 of all miscarriages) are either growth-retarded or morphologically abnormal according to embryoscopy (211). Etiology plausibly involves single-gene or polygenic perturbation, given that these genetic etiologies account for 80% of the 2%–3% of infants with congenital anomalies. A further reason is that only one-third of protein-coding genes have a known function; many of the unknown two-thirds may be pivotal for embryonic differentiation. Application of WES and WGS for embryo evaluation seems obvious.

What will, then, be the final outcome of genetic testing for ART? I predict that genetic testing—WES, WGS, targeted panels—will become as routine as PGT-A appears to be approaching. PGT-A plus sequencing already significantly increases the number of embryos that should not be transferred. ART transfer after targeted testing or WES will result in 80% success.

## PREIMPLANTATION GENETIC DIAGNOSIS OF MONOGENIC DISEASE AND HUMAN LEUKOCYTE ANTIGEN MATCHING

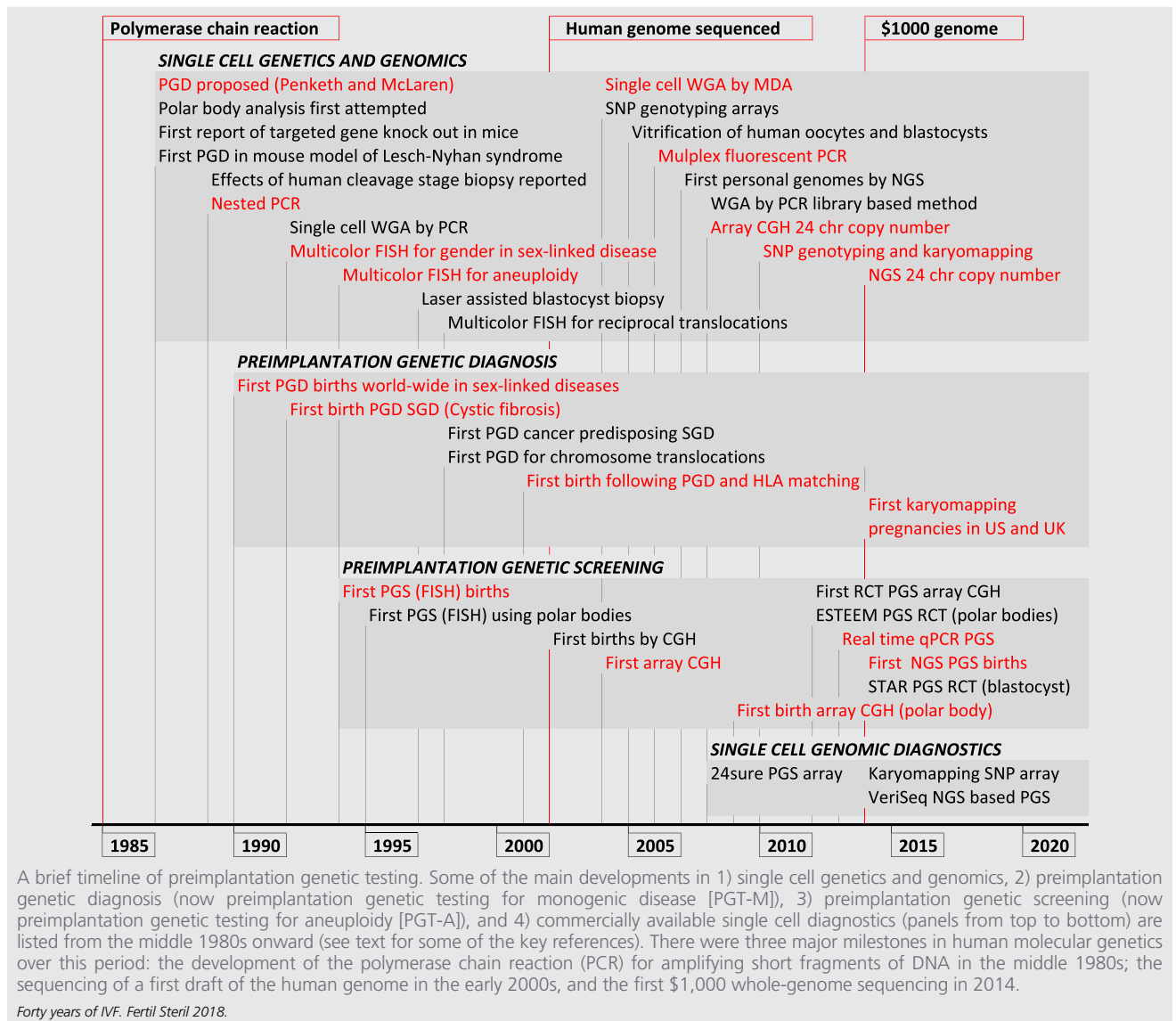
Alan H. Handyside, Ph.D.

As we celebrate the past 40 years of IVF since the birth of Louise Brown in 1978, it is remarkable to reflect that already by the mid-1980s, fewer than 10 years later, genetic testing of human preimplantation embryos for inherited diseases was being proposed (213). The need to offer at-risk couples an alternative to invasive prenatal testing, often resulting in repeated terminations of affected pregnancies, and allow them to start a pregnancy “knowing it was unaffected” had been identified. IVF pregnancy rates had improved. The first papers on the use of the polymerase chain reaction (PCR) to amplify short fragments of DNA from relatively small numbers of cells had been published. For the first time, it was possible to contemplate identifying mutations at preimplantation stages when successive cleavage divisions of the one-celled zygote, fertilized and cultured in vitro, reaches a maximum of a few hundred cells. Then in 1989, building on decades of work which had established the mutations in only a handful of single-gene defects, for example, various hemoglobinopathies, the first of a series of ground-breaking studies in various prevalent inherited diseases identified the common  $\Delta F508$  mutation causing cystic fibrosis by means of rapidly improving sequencing methods (214). The stage was set for the development of biopsy methods to safely remove cells from embryos after IVF and harness the power of PCR to diagnose affected embryos before selecting unaffected embryos for transfer, a process that became known as preimplantation genetic diagnosis (PGD) (Fig. 9).

## PRE- OR POSTCONCEPTION EMBRYO BIOPSY?

Verlinsky and Pergament, collaborating at the time primarily on the establishment of chorion villus biopsy for prenatal diagnosis, were the first to propose PGD by “embryonic”

FIGURE 9



biopsy in 1984 and published an abstract the following year in which they described bisecting three pronucleate triploid embryos at the 4-cell stage and measuring enzyme activity with the use of one-half of the embryo as a model for diagnosing inborn errors of metabolism. Verlinsky and others were later to become the pioneers of polar body biopsy, initially removing only the first polar body for genetic testing, to diagnose if the corresponding fertilized oocyte had inherited the normal or mutated maternal gene copy (allele), though no pregnancy was established after their first clinical attempt (215). An advantage of this preconception approach was that it avoided manipulation of fertilized embryos, which some considered to be unethical and avoided possible damage to the embryo itself. However, the limitations, including recombination in the first meiotic division, which prevents diagnosis in a significant proportion of oocytes, were quickly

recognized. They subsequently developed a strategy of biopsying both first and second polar bodies and if necessary following up with a cleavage-stage blastomere biopsy to avoid the limitations and include detection of the paternal allele. This approach was clinically more successful and over the following decade, Verlinsky et al. published many protocols for a range of single-gene defects.

Based on preliminary work in a mouse model of the X-linked disease Lesch-Nyhan syndrome, our approach was to focus on cleavage-stage biopsy at the 6- to 10-cell stage early on the third day after conception. In the late 1980s, IVF culture media were based on those optimized for mammalian cell lines and supplemented with heat-inactivated maternal serum. This supported development to the blastocyst stage. However, pregnancy rates after transfer of blastocysts were very low. So despite the potential advantage of removing



several cells from the outer trophectoderm layer of the blastocyst for genetic testing, cleavage-stage biopsy was the only practical option for clinical application. To minimize damage to the embryo only a single cell, or exceptionally two cells, were removed for genetic testing. The challenge therefore was to detect the mutation status of the embryo from the single biopsied cell and to do this in only a few hours so that one or more unaffected embryos could be selected for transfer later the same day.

Cleavage-stage biopsy became the predominant method for PGD and continued to be so until clinics increasingly began to switch to blastocyst biopsy over the past 5–10 years. Now blastocyst culture is routine and pregnancy rates per transfer are higher than with cleavage-stage embryos. The original method for biopsying herniating trophectoderm cells was simply to excise them with a needle rubbed against the holding pipette, which damaged cells in the process. The development of noncontact infrared lasers for both zona drilling and biopsy was a major step forward (216); collateral damage is minimized and blastocysts generally re-expand quickly. The ability to cryopreserve embryos at the blastocyst stage with very high survival rates by means of rapid cooling or vitrification, and even revitrify biopsied blastocysts if necessary, has also removed the need for rapid analysis before transfer. This then allows biopsy samples to be transported to genetic testing labs with no constraints on the time required for analysis.

### AMPLIFY ONCE, AMPLIFY TWICE

PCR is a powerful method for amplifying specific short fragments of DNA defined by pairs of oligonucleotide primers, with the use of a heat stable DNA polymerase and cycling the temperature of the reaction between denaturation, annealing, and extension temperatures. The amplification is exponential and typically can generate  $10^6$  copies of the original template. However, because single cells have only ~5–6 pg of DNA, amplifying the DNA fragment sufficiently to detect on electrophoresis gels, for example, is still a challenge regardless of the number of thermocycles.

One answer is to amplify a target sequence in a highly repetitive region of the genome. This enabled a DNA repeat specific to the Y chromosome and present in thousands of copies, to be amplified from single cells. This allows the sex of the embryo to be identified by the presence or absence of the amplified product detected on acrylamide gels, with amplification and electrophoresis taking only 4–6 hours, allowing transfer of unaffected embryos later on the same day as the biopsy. In X-linked recessive diseases (caused by gene defects on the female X chromosome), for example, Duchenne muscular dystrophy, male carriers are affected because the Y chromosome does not have a copy of the gene, whereas female carriers are unaffected but are at risk of having affected male children. Although second best to diagnosing the gene defect itself, which allows affected carrier and unaffected males to be distinguished, therefore, we were able to offer couples at risk of various X-linked diseases PGD by cleavage-stage biopsy, sex identification, and selection of female embryos for transfer. Clinical PGD cycles were started in late 1989, and we reported the first pregnancies in the

world in 1990 (217). The first two were twin pregnancies and both delivered in July of that year. Unfortunately, one of the second set of twins was stillborn.

Although we had confirmed that the sex of the embryos selected for transfer had been accurately identified by means of chorion villus sampling and karyotyping later in pregnancy, a subsequent singleton pregnancy was male. This was the first misdiagnosis after PGD and highlighted the precariousness of basing a diagnosis on the result from a single cell. In this case, presumably the misdiagnosis was caused by failure of amplification. Complete failure of amplification, failure to amplify one of the two parental alleles at random (allele drop out [ADO]), and susceptibility to contamination, particularly product contamination, all remain problematic for single-cell amplification.

For amplification of unique target sequences from a single cell, for example, to detect a mutation or a DNA marker, one round of PCR amplification is not sufficient; partial amplification and then a second full amplification of the product is required. Holding and Monk, working with mouse embryos, were the first to publish success with two rounds of amplification, using “nested” primers in which the second pair of primers recognizes sequences in the initial PCR product (218). Meanwhile, we succeeded in amplifying a short fragment including the common cystic fibrosis deletion from single human oocytes (219), and this work eventually led to the first live birth following PGD of cystic fibrosis in 1992 (220).

Later in the 1990s and early 2000s, several hundred such PCR-based single-cell protocols were published in a range of monogenic diseases. Notably in 1998, the first PGD for a single-gene defect predisposing to cancer was reported, in this case a mutation in the *APC* gene predisposing to familial polyposis coli (221). Over this period, there were technologic advances, including capillary sequencing, which enabled the detection of short PCR products labeled with fluorescent nucleotides. This made it possible to accurately determine the length of the amplified products, including highly polymorphic short tandem repeats (STRs), and the combination of STR markers flanking the affected gene together with the mutation site itself became the criterion standard for PGD protocols, reducing the possibility of errors from ADO at any single locus (222).

The use of fluorescent PCR and capillary sequencing also facilitated the use of highly multiplexed PCR reactions, generating tens of PCR products from single cells. For a number of blood-related inherited conditions, particularly hemoglobinopathies such as  $\beta$ -thalassaemia, this then enabled not only the single-gene defect itself to be diagnosed, but also markers across the human leukocyte antigen (HLA) on the short arm of chromosome 6 could be examined and tissue matched to an existing affected child in the family. If an unaffected and matched embryo could be identified, this opened up the possibility of isolating HLA-matched cord blood stem cells for transplantation to the affected child. The first birth after PGD of  $\beta$ -thalassaemia and HLA matching was reported in 2001 (223), and since then an increasing number of sick children have had stem cell transplantation and been cured of their disease (224).

## THE GENOMIC REVOLUTION

PCR involves cycling the temperature from low annealing temperatures to high denaturing temperatures to separate the products in each cycle. This requires the use of a heat-stable DNA polymerase, Taq polymerase, isolated from a thermophilic bacterium. In the early 2000s, a DNA polymerase,  $\phi$ 29, with the ability to unravel double-stranded DNA, was isolated from a bacteriophage. This polymerase makes it possible to amplify DNA at a relatively low and constant temperature, so-called isothermal multiple displacement amplification (MDA). When used with single cells, MDA produces micrograms of DNA product, for the first time similar to the amount in a prenatal chorion villus sample, and it was possible to envisage using genomic technologies at the single-cell level (Robinson et al., 2003).

Multiplex fluorescent PCR protocols for PGD require labor-intensive optimization of family- and disease-specific tests, which limit access and often delay treatment. One improvement was the introduction of disease-specific panels of markers informative for a majority of couples at risk of a particular disease, so-called preimplantation genetic haplotyping (225). With MDA, however, sufficient DNA is available for analysis with genome-wide platforms. One of the first platforms to become available were single-nucleotide polymorphism (SNP) genotyping microarrays. With these SNP microarrays, it is possible to genotype hundreds of thousands of these markers across each of the 23 pairs of chromosomes in a single test. SNPs are variations in the DNA sequence at specific positions which mostly do not affect gene function and where the variant nucleotide is present in a significant proportion of the general population. Thus, biallelic SNPs in which the sequence varies between two nucleotides, generically referred to as A and B, can be used as ubiquitous genetic markers with an average one informative SNP in every 3 kb.

STR markers are highly polymorphic, and in some cases there can be a different number of repeats on each of the four parental chromosomes. Biallelic SNP markers can only distinguish two out of the four. However, by determining the genotypes of the parents and working out which of the AB alleles at each position is present on individual chromosomes (phasing) with the use of, for example, an existing child, four different sets of genome-wide SNP markers can be identified. Karyomapping uses a bead array to genotype 300,000 SNPs genome wide, with mendelian analysis and an algorithm to avoid errors caused by ADO (226, 227). The main advantage of karyomapping is that any single-gene defect (or combination of defects) within the regions covered by the SNP markers can be diagnosed in one universal test, based on the SNP markers present around the affected gene(s). HLA matching, for example, simply involves examining the SNP markers on chromosome 6. Finally, because there are markers for each parental chromosome, meiotic trisomies, monosomies, and subchromosomal deletions can be identified. Combining PGD by means of karyomapping with aneuploidy testing has improved clinical pregnancy and live birth rates and has now replaced the use of STR markers in all but exceptional cases.

## PROSPECTS FOR PGD IN THE COMING DECADES

The main development, which is already taking place, is the replacement of targeted sequencing with the use of SNP microarrays with next-generation sequencing (NGS). In 2014, the first \$1,000 whole-genome sequencing was achieved, and the cost of sequencing continues to fall. Several early reports have demonstrated the use of NGS-based testing for PGD, including the first demonstration of combined chromosome copy number analysis and targeted haplotyping for mutation and marker analysis (228). At present, the cost per sample of using NGS is greater than microarrays, and the challenge will be to bring the cost down for patients.

Recently, there have been breakthroughs in both gene therapy for  $\beta$ -thalassemia (229) and in genome editing of human embryos (230).  $\beta$ -Thalassemia is the most prevalent inherited disorder worldwide, and a potential cure with the use of autologous blood cells is enormously significant. However, for most other single-gene defects affecting multiple cell types throughout the body, gene therapy may offer only a partial cure. The efficiency of genome editing with the use of CRISPR-Cas9 has yet to be demonstrated definitively, and the risk of any off-target editing remains a barrier for clinical application. Because single-gene defects, whatever their inheritance patterns, are present typically in only one-half of the gametes of the carrier parent, selection of unaffected embryos following PGD is likely to remain the simplest and safest approach to preventing the birth of affected children. For couples, PGD is therefore likely to remain an important alternative to prenatal diagnosis, particularly when combined with aneuploidy testing to identify nonviable genomically unbalanced embryos.

## EVOLUTION OF PREIMPLANTATION GENETIC SCREENING

Santiago Munné, Ph.D.

Preimplantation genetic screening (PGS) was first clinically applied (231) 25 years ago and was based on the observation that many human embryos, contrary to most other species studied, are chromosomally abnormal. Embryos with numeric chromosome abnormalities in all of their cells (aneuploid, haploid, or polyploid) seldom implant, and if they do the vast majority miscarry, with a few exceptions (trisomies for chromosomes 13, 18, and 21 and gonosomes). Therefore, we hypothesized that the selection of euploid embryos for transfer should improve implantation rates and reduce miscarriage rates, improving ongoing pregnancy rates per transfer (231). Assuming no embryo damage from the sampling or biopsy of the embryo, no errors of diagnosis, and no self-correction of abnormalities, single-embryo transfers of all-euploid embryos should provide an ongoing pregnancy rate per cycle similar to the cumulative rate obtained by replacing all undiagnosed embryos one by one. As I will discuss, currently PGS improves implantation rates, reduces miscarriage rates, and improves ongoing pregnancy rates per transfer (232–236) (Fig. 10) but it does not yet achieve the last

FIGURE 10

	Yang et al <sup>235</sup>	Scott et al <sup>236</sup>	Forman et al <sup>237</sup>	Rubio et al <sup>238</sup>	Munné et al <sup>239</sup>
<b>N</b>	<b>103</b>	<b>155</b>	<b>175</b>	<b>205</b>	<b>678</b>
<b>Study type</b>	Prospective, 2 sites	Prospective, 1 site	Noninferiority, single center	Prospective, 4 sites	Prospective, 34 sites
<b>Female age</b>	< 35 yrs	< 43 yrs	< 43 yrs	38 to 41 yrs	<41 yrs
<b>Biopsy day / Transfer type</b>	5 / Fresh	5 / Fresh	5 / Fresh /frozen	3 / Fresh*	5 / Frozen
<b>PGS method</b>	aCGH	qPCR	RT-PCR	aCGH	NGS
<b>Pregnancy Outcome over control</b>	OPR / transfer significantly improved	OPR /transfer significantly improved	Same OPR by replacing 1 euploid than 2 untested embryos	OPR /transfer significantly improved	OPR /transfer significantly improved in the 35-40 year old group

Summary of CRT on PGS v2. \*Frozen for subsequent cycles. 1. Yang et al., *Mol Cytogenet* 2012;5:24; 2. Scott et al., *Fertil Steril* 2013;100:697–703; 3. Forman et al., *Fertil Steril* 2013;100:100–7.e1; 4. Rubio et al., *Fertil Steril* 2017;107:1122–9. 5. Munné et al., *ASRM* 2017.

Forty years of IVF. *Fertil Steril* 2018.

premise, owing to either embryo damage during biopsy or technical shortcomings, although the gap has been narrowing.

Without embryo damage, technical error, or self-correction of abnormalities, one would expect that the more chromosome abnormalities, the better the selection of embryos. The frequency of meiotic errors resulting in aneuploid oocytes and embryos increases with advancing maternal age, but it does not seem to be related to cohort size (237). In that study (237), blastocysts analyzed by means of array comparative genome hybridization (aCGH) showed similar euploidy rates regardless of whether the patient produced fewer than 5 or more than 10 blastocysts, with a range of 70%–67% in egg donors, 53%–69% in patients <35 years old, 49%–51% in patients 35–39 years old, 34%–41% in patients 40–42 years old, and 17% in patients >42 years old. These findings have been confirmed later in much larger data sets with the use of either aCGH or next-generation sequencing (NGS) (Fig. 11). However, what does change with cohort size is the chance of a patient having at least one euploid embryo. To have a  $\geq 95\%$  chance of finding a euploid embryo, four blastocysts need to be produced in egg donors, five to seven in patients <35 years old, the same for patients 35–39 years old, and more than ten for patients 40–42 years old. However, if a euploid blastocyst is replaced, we demonstrated (238) that euploid embryos implant equally well at any age, at least up to 42 years. This finding supports the original hypothesis that the major cause of decline in embryonic potential with advancing maternal age is chromosome abnormalities, and that if euploid embryos are produced and replaced, the maternal age effect on implantation mostly disappears.

But how was PGS developed? As with many scientific developments, it involved a confluence of disconnected fields, adding another step to previous ones, and lots of serendipity. In this case, I had just finished my Ph.D. and, after asking for a

job with the fathers of preimplantation genetic diagnosis (PGD) (Alan Handyside, Yuri Verlinsky, Buck Strom, Mark Hughes, Jacques Cohen, and Jamie Grifo), which all rejected me, I went anyway to visit Cornell because Jacques said “but you can visit us anytime.” So the week after his rejection I drove from Pittsburgh to visit. The following week, Henry Malter, another pioneer in micromanipulation, quit his job and I was hired (first lucky event). My work was going to be on antioxidants (good Lord, I was not even taking vitamins then). Luckily, Jamie Grifo’s team was sexing embryos with probes that required antibodies. A bad fixation guaranteed super-bad results and I was able to improve the fixation and was recruited to the team. Through a second serendipity I met Ulli Weier, who was testing the first fluorescently labeled probes. It was not commercially available yet, and I was lucky to be the first to try them in human blastomeres, thereby being able to perform the first PGS clinical cases with the use of five chromosome probes and achieving the first PGS baby (231). But Cornell lacked a ready source of embryos for research at the time, and my third stroke of luck, Luca Gianaroli, happened to be visiting Jacques’ lab and invited me to Bologna with an offer of available material and ample assistance. The confluence of micromanipulation techniques developed mostly at Cohen’s Lab (i.e., the use of acid Tyrode to do assisted hatching and thereby obtain a better day-3 biopsy), the fluorescently labeled probes of Ulli Weier, and the willingness of Luca Gianaroli to contribute material and continued support resulted in what we now call PGS v1, that is, cleavage-stage biopsy and fluorescence in situ hybridization testing of embryos (231, 239). This collaboration resulted in ~100 papers. Talk about getting to be the first to use a technique in something new and basking in your laurels.

The party abruptly ended in 2007 when a series of papers coming from northern Europe showed that in their hands PGS

**FIGURE 11**

# of embryos produced	% of patients with normal blastocysts					
	egg donors	<35 years	35-37 years	38-40 years	41-42 years	>42 years
<b>1-3</b>	75%	67%	57%	44%	29%	19%
<b>4-6</b>	98%	92%	87%	76%	56%	44%
<b>7-10</b>	100%	98%	96%	91%	73%	50%
<b>&gt;10</b>	100%	100%	99%	95%	89%	81%

Frequency of chromosome abnormalities in human blastocysts analyzed by means of NGS.  $n > 100,000$  embryos. Cooper Genomics data, with mosaics not reported (<40% classified as normal and  $\geq 40\%$  as abnormal).

Forty years of IVF. *Fertil Steril* 2018.

v1 did not work or was even detrimental (240). We were astonished by their results because our experience, >10 years already, was to the contrary. Granted, if 50% of embryos were abnormal, we were not getting 100% improvement in implantation, but we were nevertheless getting a significant improvement. We scrutinized and combed the Mastenbroek et al. paper (240) for a clue, and deep in the paper there is one line stating that in 20% of the embryos they biopsied they did not obtain cells but replaced those embryos anyway. Compared with the control group those embryos (an involuntary third arm) had one-half the control group implantation rate. It was their biopsy (aside from other details). We dismissed that work for several more years without recognizing that the technique was too difficult for most labs to perform correctly. It was not until Richard Scott and his team (241) published a clinical randomized trial (CRT) comparing the implantation rate of cleavage-stage with blastocyst biopsies and untested embryos, which showed that the first was detrimental, but not the second. The field largely abandoned cleavage-stage biopsy, but by then PGS (v1) was almost dead. I remember that at the Rome ESHRE meeting Dr. Geraedts presented the European Society for Human Reproduction and Embryology consortium PGD data with a big smile when showing a sharp decrease in PGS procedures. At the time, as for most of its history, the Consortium was not pro-PGS. The Preimplantation Genetic Diagnosis International Society was created by Verlinsky, Gianaroli, Simpson, and me to promote PGS.

I have not yet discussed polar body (PB) analysis. This method was pioneered by Yury Verlinsky's team, one of the only groups practicing it during PGS v1 times, together with Italian (Gianaroli and others) and German (Karsten Held and others) groups, owing to legal prohibition of embryo biopsy in these two countries. However, PB analysis involves double-testing of first and second PBs, having proved that many aneuploidies are not resolved as such until the second meiotic division. More important, 30% of abnormalities are postmeiotic and 10% paternal in origin, therefore undetectable by means of PB analysis. The biopsy of PBs is almost as difficult, or more so, than cleavage-stage biopsy. Finally,

the switch to 24-chromosome analysis meant double amount and cost to analyze PBs than single samples (either single blastomeres or single TE biopsies), and PB analysis has been mostly discontinued.

PGS v2 was made possible thanks to previous improvements in culture media which allowed blastocyst culture and biopsy, vitrification which provided plenty of time for genetic analysis, but mainly the push to analyze all 24 chromosomes with new molecular techniques (aCGH, single-nucleotide polymorphism [SNP] arrays, quantitative polymerase chain reaction [qPCR], and NGS). This second wave of technology took quite long to arrive after the first one, and not all surfers had the stamina to keep afloat. ReproGenetics was the first commercial PGD lab, but we almost went under in between waves. Thanks to the hiring of new star Dagan Wells, we got new blood, skills, and a proper British accent, and we hit it with CGH and thereafter aCGH (242).

Array CGH was the workhorse of PGS v2 in many labs until the advent of NGS. It consists of  $\sim 3,000$  large fragments of human DNA arrayed into a glass slide and spaced at about 1-Mb intervals across each chromosome, allowing accurate copy number analysis as well as counting of regions as small as 6 Mb. The DNA of the cell(s) to be tested is amplified, labeled in green, and cohybridized with the array with the use of control DNA labeled in red in a 1:1 ratio. Analysis is performed by scanning the array and measuring the intensity of both hybridization signals (green and red) relative to each probe (called the logR ratio). If the segment of the chromosome analyzed is euploid, then there would be an equal amount of red and green and the software reads the result as yellow and a 1:1 ratio. Because the control is euploid, this would classify that probe as also euploid. If it shifts to the red (a 1:2 ratio) it means that there is more control than test DNA, indicating the presence of a monosomy; whereas if it shifts to green, there is more test DNA, a 3:2 ratio, and thus a trisomy. A whole chromosome could be trisomic or monosomic, or just a fragment of a chromosome (segmental abnormalities, translocations). Although aCGH was developed years before its use in PGS, it was the Bluegenome



(Graham Snudden, Nick Haan, and Tony Gordon) software and reagents that made it accessible to most laboratories. The aCGH method requires whole-genome amplification (WGA) of the biopsied cells. However, different WGA methods produce different amplification biases, amplifying some areas better than others. There has been a lingering debate (usually between Drs. Treff and Scott vs. the rest of the PGS teams) about whether these amplification biases are critical or not. The biases are normalized by the fact that control DNA is also amplified at the same time. The evidence suggests that the error rates for aCGH are similar to those of qPCR and SNP arrays. Two CRTs have been performed with aCGH showing significant improvement in pregnancy outcomes (232, 235).

Another technique that was initially used in PGS v2 but has now been mostly relegated to PGD of monogenic diseases (by means of karyomapping), are SNP arrays. At the time analyzing >200,000 SNPs simultaneously, it provided quantitative and/or qualitative chromosome copy number (243). About 2.5% of nucleotides are polymorphic in the population, and these biallelic SNPs, in which one of two possible bases are present (A or B), can be genotyped with the use of SNP arrays. By analysis of the intensity ratio of A and B alleles at heterozygous loci, it is possible to detect duplications and deletions of whole chromosomes or small regions. In euploid regions, SNPs would appear as three bands representing AA, AB, and BB combinations of loci at a ratio of either 0, 0.5, or 1. In trisomic regions, the ratio at heterozygous loci appears as two bands representing those that are either AAB or ABB. In monosomic regions, there will be a heterozygous band and only A or B. One peculiarity of this technique, though not very useful, is that if the parents are also analyzed, one can determine the origin of the abnormality. I say it is not very useful because >90% of defects are maternal in origin. However, the karyomapping approach developed by Alan Handyside is very useful for haplotyping and detection of monogenic diseases, as well as fingerprinting embryos in case of a mix-up.

The laboratory of Nathan Treff and Richard Scott was very prolific in developing PGS v2 technology, from the application of SNP arrays (also developed by Natera's Johnson and Rabinowitz team) to the development of qPCR, targeted NGS, and the performing of several key clinical randomized tests (233, 234, 241, 243). In contrast to aCGH and SNP array, which were intended to be comprehensive and covering all regions of each chromosome, qPCR was designed as a fast but low-resolution method, testing only four sites per chromosome. With qPCR there is a preamplification step, followed by a multiplex PCR reaction performed on the sample directly, without WGA, which amplifies about four sequences on each arm of each chromosome. Rapid quantification by means of real-time qPCR is then used for comparison of copy number across the genome. This allows for results in ~4 hours, and it has been extensively validated and several CRTs performed (233, 234), but the trade-off is its low resolution which does not allow for the detection of segmental abnormalities. With mounting evidence that transfer of vitrified embryos in a nonstimulated cycle improves pregnancy outcome over replacement on the same stimulated cycle, this technique has lost its advantage.

The current criterion standard for PGS is now blastocyst biopsy, vitrification, analysis of the biopsy with the use of NGS, and transfer in a thaw cycle (244). This allows ample time for analysis, better endometrial environment for implantation, and centralization of laboratory services with increasing sequencing capacity, which drives lower prices. The most common approach used for NGS was developed by Wells et al. (244). It consists of amplifying the DNA of the biopsy by means of WGA, and then the biopsy DNA is chopped into small fragments and linker oligonucleotides are ligated to the fragments, which can be used also as "barcodes." The fragments are massively sequenced in parallel by the successive addition and removal of nucleotides. The intensity is measured either by counting protons released in each reaction or by measuring fluorescence from the fluorescently labeled nucleotides incorporated. Improving imaging analysis and chemistries is allowing increasing resolution and capacity at decreasing machine prices. Each fragment sequence is then compared with a reference DNA and assigned a locus. Once a certain read depth of fragments is reached, the analysis is performed. By counting fragments in a region and comparing them with the rest of the genome, one can detect if there is a change in copy number in that region. One CRT has been performed so far with this technique (236). An alternative to this NGS approach is to not use WGA and instead applying targeted NGS (developed by the Treff laboratory).

With the use of barcodes, the simultaneous analysis of up to 64 embryos in the same run is possible, thus reducing cost, although at the expense of resolution. However, even at that level, the resolution obtained is superior to all other PGS techniques and cheaper. Accordingly, prices for the genetics part of PGS have been decreasing. Puzzlingly (I am being sarcastic), embryo biopsy prices in the U.S. have not changed, still at ~\$1,500–\$2,000 for a procedure that lasts 1–2 minutes per embryo. This will change with noninvasive PGS, which I will discuss shortly.

In addition to pricing advantages, NGS allows for higher resolution and thus the detection of mosaicism at the 20%–80% ratio of normal-to-abnormal cells in a typical 5-cell blastocyst biopsy (245). Although other PGS v2 techniques, such as aCGH, can detect mosaicism, they do it at much narrower ranges (246). We have shown that euploid/aneuploid mosaic embryos detected by NGS are relatively common, ~20% of blastocysts, and have an intermediate potential between euploid and aneuploid embryos, implanting less and miscarrying more, but resulting in some live births (245), so far all chromosomally normal (246). It also seems that the more abnormal cells or chromosomes involved in the mosaicism, the poorer the prognosis of those mosaic embryos to develop to term (245). As such, Grifo, Wells, Greco, and I proposed that these embryos should be considered as a separate third category when selecting embryos for transfer, prioritizing the transfer of euploid ones, but using mosaic embryos for transfer if other euploid ones are not available or no other cycles of IVF are possible. This reduces further the risk of false positive and negative results, because now euploid and aneuploid embryos are better characterized, at the expense of creating a gray zone of mosaic embryos. NGS thus improves

embryo selection over other techniques, as recently demonstrated by obtaining better pregnancy outcomes when comparing NGS and aCGH techniques (247). Some had argued that a mosaic result is a technical artefact, but the fact that they have a different pregnancy outcome indicates a biologic cause.

Overall, there are at least five CRTs (232–236) showing an improvement in pregnancy outcomes, per transfer, when PGS is performed. Some of these studies were small and underpowered, but a recent large multicentric one also showed positive results in patients 35 years of age and older, where aneuploidy rates are higher (235). This is the same result observed in the Society for Assisted Reproductive Technology (SART) report of 2014 for all U.S. centers reporting. There will be some debate about why PGS does not show improvement in results in the young group when on average 30%–40% of blastocysts in that group are also aneuploid. The reason could be that the STAR trial (235) and the SART data are “real-world” experience, involving not only centers with lots of experience in blastocyst biopsy (actually, many of those did not want to participate in the STAR trial, because their patients were already convinced) but also centers with little experience. This suggests that blastocyst biopsy might not be as benign as we think (241). After all, it has not been standardized and is operator dependent. As an alternative to blastocyst biopsy, an emerging body of research is showing that the embryo sheds DNA into the media and blastocoe fluid and that DNA in that environment is now quantifiable and analyzable with the use of NGS. Although concordance with TE biopsy is still not optimal, in a couple of years we should see noninvasive sampling of embryos as a valid alternative to TE biopsy, at least for PGS. On the other hand, deeper and cheaper sequencing will merge PGS, PGD, and carrier screening into the same procedure. That could be PGS v3.

## REPAIRING THE DAMAGED EMBRYO: CRISPR-CAS9 TECHNOLOGY

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Nuria Montserrat, Ph.D., and  
Juan Carlos Izpisua Belmonte, Ph.D.

### THE HISTORY OF GENE-EDITING TECHNOLOGY

DNA damage can occur after exposure to ionizing radiation or chemotherapy, during the DNA replication process, or after experimental manipulation through the action of endonucleases. Fortunately, the DNA repair machinery helps to repair this damage, preventing DNA mutations that can cause disease, and this is the basis of the gene-editing concept. There are two key repair pathways for DNA double-strand breaks (DSBs): nonhomologous end joining (NHEJ) and homologous recombination (HR) (248).

HR was used in the 1980s to insert and repair genes in mammalian cells. Drs. Oliver Smithies, Mario Capecchi, and Martin Evans, who together pioneered HR-mediated gene editing in mouse embryos, later shared the Nobel Prize in Physiology in 2007 for this work. In the 1990s, Dr. Maria Jasin and colleagues further improved gene targeting in mammalian cells with the use of HR when they used the yeast homing endonuclease I-SceI. These next-generation endonucleases include zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), which combined the DNA-binding specificity of zinc fingers or TALE transcription factors from plants with the DNA-cutting activity of the FokI endonuclease (248).

One of the major breakthroughs achieved in the field of gene editing happened in the late 1980s, when a strange topology at the 3' end of the alkaline phosphatase gene in *Escherichia coli* was discovered. This was the first known description of a clustered regularly interspaced short palindromic repeats (CRISPR) array. In 2005, the molecular mechanism of the transcription of CRISPR arrays into RNA was demonstrated, which is then cleaved and loaded into CRISPR-associated (Cas) proteins (Cas9) (248).

For many years, researchers had been searching for a tool to easily induce mutations in a targeted fashion. Although some progress had been made with the use of engineered meganucleases, ZFNs, and TALENs, these systems had numerous limitations. They were either expensive or labor intensive, or both, because the targeting mechanisms were based on protein–nucleic acid interactions, thereby requiring a custom-designed protein for each genetic locus of interest. The promise of RNA-guided nuclease activity afforded by CRISPR-based approaches led numerous groups to immediately recognize that this technology could potentially be used to induce targeted DSBs in eukaryotes, which previously could be accomplished only with much difficulty. DSBs produced by either previously available technologies or CRISPR-based systems are repaired by low-fidelity DNA repair pathways, leading to the production of indels, a class of mutations characterized by the random insertion or deletion of nucleotides at the site of the DSB.

As described previously, there are four basic nuclease technologies: engineered meganucleases, ZFNs, TALENs, and CRISPR-Cas9 nucleases. The differences between them are described in detail in Table 6.

### THE RELEVANCE OF GENOME-EDITING TECHNOLOGIES FOR POTENTIAL THERAPEUTICS

Gene-editing technologies are powerful tools for basic research, but the final goal is to translate these technologies into therapeutic applications. The potential use of gene editing in the clinic emerged from the idea that the best way to treat monogenic diseases would be to develop a method for correcting the disease-associated mutation, but it would be necessary to make more dramatic changes to the genome to cure diseases with multifactorial origins, which are more common. The use of genome editing to cure monogenic disease is conceptually simple, but the true power of genome-

TABLE 6

The variety of gene-editing tools: a detailed description of the differences between engineered meganucleases, ZFNs, TALENs, and CRISPR-Cas9.

Characteristic	Meganucleases	ZFNs	TALENs	CRISPR-Cas9
Recognition site	14–40 bp. Protein-DNA	9–18 bp per ZFN monomer, 18–36 bp per ZFN pair. Protein-DNA	14–20 bp per TALEN monomer, 28–40 bp per TALEN pair. Protein-DNA	22 bp (20 bp guide sequence + 2 bp PAM sequence for <i>Streptococcus pyogenes</i> Cas9); up to 44 bp for double-nicking. RNA-DNA
Specificity	Small number of positional mismatches tolerated	Small number of positional mismatches tolerated	Small number of positional mismatches tolerated	Positional and multiple consecutive mismatches tolerated
Targeting constraints	Targeting novel sequences often results in low efficiency	Difficult to target non-G-rich sequences	5' targeted base must be a T for each TALEN	Targeted sequence must precede a PAM
Efficiency	Low	High	High	Very high
Design feasibility and delivery	Time consuming and labor intensive	Difficult construction, difficult to assemble, and low delivery	Difficult construction, difficult to assemble, and low delivery	Very rapid construction and easy delivery
Immunogenicity	Unknown	Likely low, because ZFNs are based on human protein scaffold	Unknown	Unknown
Ease of multiplexing	Low	Low	Low	High
Methylation sensitivity	Sensitive	Sensitive	Sensitive	No
Safety	Unknown	Safe in clinical trial	Unknown	Safe in clinical trial
Cost	€€€€€	€€€€€	€€€	€

Note: Cas9 = CRISPR-associated protein 9; CRISPR = clustered regularly interspaced short palindromic repeats; PAM = Protospacer Adjacent Motif; TALEN = transcription activator–like effector nuclease; ZFN = zinc finger nuclease.

Forty years of IVF. Fertil Steril 2018.

editing tools is that they provide a mechanism for making more sophisticated genomic changes, which can be used to cure more common diseases or to modify their course. Indeed, there are currently several companies (CRISPR Therapeutics, Editas, Sangamo Therapeutics, Caribou Biosciences, Editas Company, Precision Biosciences, and Intellia Therapeutics) developing gene editing–based approaches to treat diseases, such as sickle cell anemia,  $\beta$ -thalassemia, cystic fibrosis, Duchenne muscular dystrophy, hemophilia, alpha1-antitrypsin deficiency, lysosomal storage disorders, Huntington disease, human immunodeficiency virus (HIV) 1 resistance/infection/immunization, hepatitis B virus, chimeric antigen receptor T-cell, and others.

## FORTHCOMING POTENTIAL USES OF GENE-EDITING TECHNOLOGY IN THE REPRODUCTIVE FIELD

There are many potential uses for the CRISPR-Cas9 technology in the reproductive field, including those that target pluripotent stem cells, gametes, or embryos (249). The first hypothetical choice for preventing a genetic disorder in progeny is to use gene editing to correct the mutation in pluripotent stem cells (induced pluripotent stem cells or somatic cell nuclear transfer–human embryonic stem cells) that have been obtained from a patient with a specific disease. The pluripotent stem cells are ideal for researching technologies to edit genes because they can be grown faster and more easily. After

gene correction, these pluripotent stem cells can be differentiated in vitro toward germ cells (oocytes or sperm) for future potential uses with the use of assisted reproductive technology (250).

In the gametes, it seems that spermatogonial stem cells (SSCs) are the best male cell type for treating male infertility (especially if the patient has problems generating mature sperm).

In contrast to male gametes, oocytes are much easier to genetically manipulate, although, at present, it is difficult to use gene-editing applications on them owing to the low number collected from the patient as well as the poor efficiency of the gene-editing process. To date, it has been demonstrated that gene-editing technologies (TALEN and CRISPR-Cas9) can be used to eliminate mitochondrial (mt) DNA molecules in oocytes or embryos in mouse models (251). Juan Carlos Izpisua Belmonte and colleagues have used mitochondria-targeting restriction enzymes and TALENs in mammalian oocytes for the first time. To determine whether the enzymes could be used to edit human mtDNA, they fused mouse oocytes with fibroblast cells from patients with one of two mitochondrial disorders (Leber hereditary optic neuropathy or neurogenic muscle weakness, ataxia, and retinitis pigmentosa). Mutant mtDNA was still detectable, although at lower levels, after TALEN mRNA injection. Mutated mtDNA usually causes disease only if >60%–75% of a cell's mitochondria have the error, so the reduction that they observed was more than enough for the phenotype to disappear (251).

The correction of the mutations in the germ line could permit the patients to produce mutation-free gametes, and consequently produce healthy embryos and progeny. However, there are few scenarios in which the application of CRISPR-Cas9 technology could be of benefit for people with risk of having children with monogenic diseases, for example, in families with autosomal recessive diseases, in couples where one partner is affected with an autosomal dominant disease, and in patients who are homozygous for an autosomal dominant disease (Huntington disease, polycystic kidney disease, achondroplasia, Marfan syndrome, myotonic dystrophy type 1, and others) (249), in a rare form of chromosomal aberrations (Robertsonian translocations), and in genetic syndromes (45,X [Turner syndrome], 47,XXY [Klinefelter syndrome], and Y chromosome deletions [oligozoospermia and azoospermia]). Finally, another situation for correction would be the mutations in human embryos that we will discuss later.

## STATE-OF-THE-ART OF THE USE OF GENE EDITING IN EMBRYOS

From 2015 to date, a few studies were published about CRISPR-Cas9 editing in human embryos to verify its specificity, efficiency, and fidelity as well as to understand human development. Basically, the editing system is directly micro-injected into the cytoplasm or pronuclei of zygotes or metaphase II (MII) oocytes, and later some type of screening is needed to identify embryos with a correct edited genome and any off-target modifications. In general, the efficiency of genomic editing in embryos is low. The main problem is the generation of mosaic embryos as a result of inefficient nuclease cutting and/or inaccurate DNA repair before the embryo undergoes cleavage. Still, several studies in different animal models from rats to pigs, even up to monkeys, have demonstrated the achievability of gene editing in animals (252–255). The efficiency of genomic modifications in mammalian zygotes ranges from 0.5% to 40.9% of injected zygotes with the use of TALENs or Cas9 technologies. Low rates of mosaicism have also been achieved in nonhuman primate embryos (255).

In 2015, the technique of CRISPR-Cas9 editing was also performed in human zygotes to verify its efficiency, specificity, and fidelity (256). A Chinese group injected 86 donated tripronuclear (3PN) zygotes with CRISPR-Cas9; 82.6% of the embryos survived the injection, and 51.9% of the genome-edited zygotes were successfully spliced, but only 5.6% of the total contained the correct genetic material inserted through homologous recombination. The gene-edited zygotes were mosaic, with results similar to findings in other model systems. Furthermore, a large number of “off-target” mutations were identified, which probably had been introduced by the CRISPR-Cas9 complex acting in other parts of the genome, intrinsic abnormalities of the 3PN zygotes, or a combination of both.

Later, another Chinese group injected 213 3PN zygotes, which resulted in some of the embryos taking on a mutation that modifies an immune-cell gene called CCR5 (257). This mutation is resistant to HIV infection owing to the CCR5 pro-

tein alteration which prevents the virus from entering into T cells. Genetic analysis showed that four of the 26 embryos targeted were successfully modified, with similar results to Liang et al. However, some embryos contained an unmodified CCR5 $\Delta$ 32 mutation and others had acquired different mutations. In conclusion, the main advance of that paper was the precise introduction of a specific genetic modification in human zygotes by means of CRISPR-Cas9. Very recently, the first report that demonstrates in diploid (2N) human embryos the correction of the hemoglobin subunit beta (HBB) gene and one in the gene encoding the enzyme glucose-6-phosphate dehydrogenase (G6PDH) (258) has been published. These common genetic defects cause different types of anemia. As we mentioned before, previous work has already shown that CRISPR-Cas9 technology can be used to perform specific changes in triploid human zygotes, so these results from the Tang group were not unexpected. The limitations presented in this work were the limited number of ten embryos used compared with previous reports and, similarly to other reports, the generation of mosaic embryos.

Recently, a major milestone in the field of gene editing has been published. Mitalipov et al. addressed several steps to improve the precision and the safety of the CRISPR-Cas9 technique. As we mentioned above, to date the CRISPR-Cas9 technology used in embryos has frequently generated mosaics by repairing the mutation in some cells but also introducing a high rate of unwanted off-target mutations or extra mutations in the targeted gene (nonhomologous repair) (Fig. 12A). However, Mitalipov's team considerably improved on previous efforts by injecting the CRISPR-Cas9 components together with the patient sperm directly into normal MII oocytes (259). Until now, the Cas9 complex had been injected directly into the zygote (Fig. 12A, top). Remarkably, they now found high efficiency of homologous repair, no evidence of off-target genetic changes, and only a single mosaic generated in an experiment involving 58 human embryos (259) (Fig. 12A).

From a developmental point of view, Kathy Niakan and colleagues for the first time optimized the protocol to knock out the OCT4 gene with the sole purpose of understanding the basic principle of human embryonic development, rather than correcting a specific mutation in human embryos as in previous reports (Fig. 12B). Understanding the basic and fundamental aspects of early human embryos will have a direct impact in clinical applications for reproductive medicine (260).

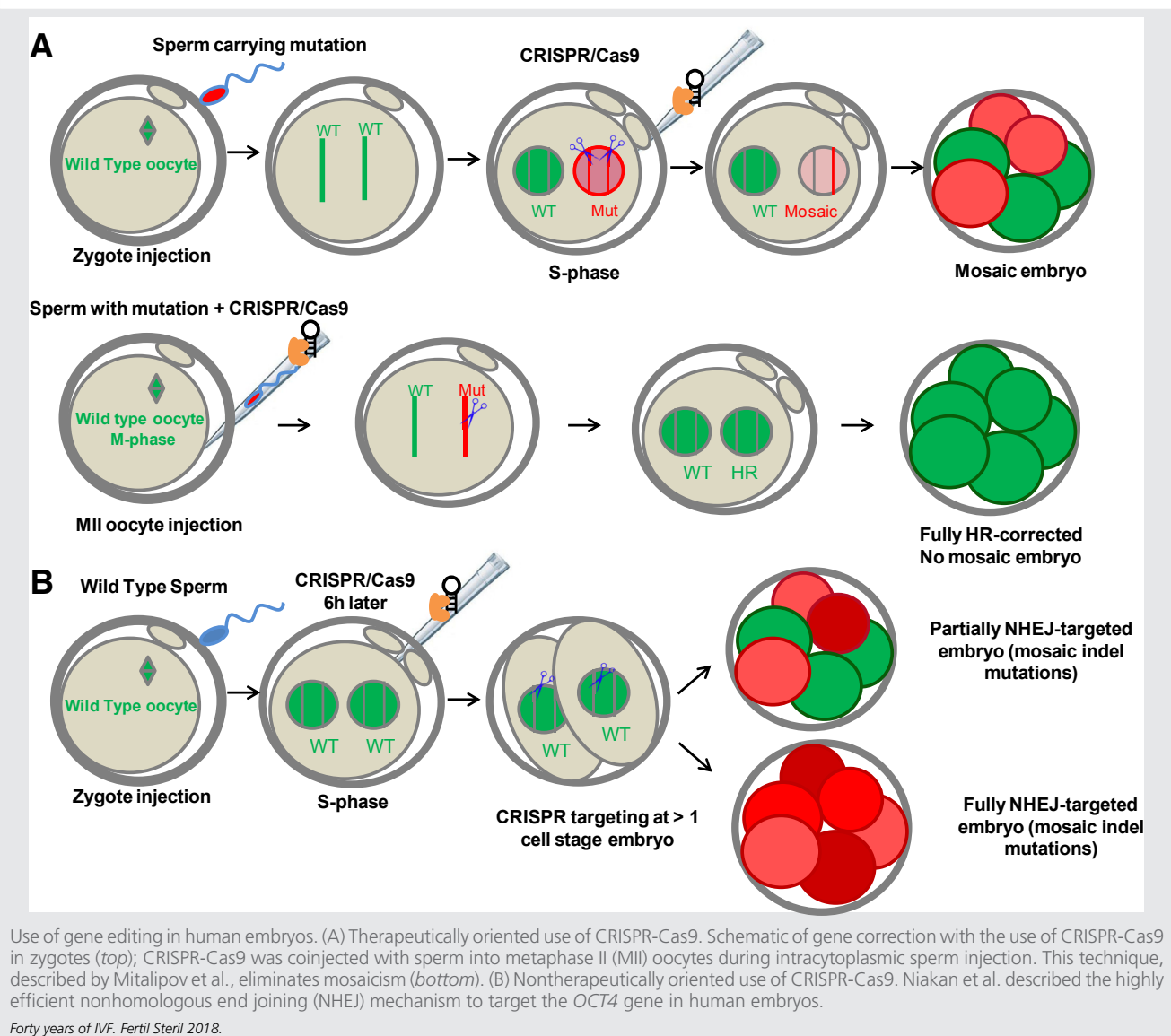
Obviously, more research in animal models is needed to improve the safety and efficiency of this method for germline correction, especially in human embryos, through genome editing.

## GENE EDITING FOR TREATMENT OF GERMLINE GENETIC DISEASES: THE CASE OF MITOCHONDRIAL DISEASES

Mitochondrial diseases are caused by a mutation in a proportion of the mtDNA molecules present exclusively in the oocyte. Mutations in the mtDNA are transmitted exclusively via the oocyte, which can carry between 10,000 to 100,000 mtDNA copies, with the most severe forms causing death soon after birth. Although there is at this time no cure for



FIGURE 12



mitochondrial diseases, scientific advances in this field have provided new ways to prevent disease transmission. One mitochondrial disease prevention method is preimplantation genetic diagnosis (PGD), a technique in which cells taken from in vitro fertilized (IVF) embryos before uterine implantation are screened to select healthy embryos, but this is not a valuable test for all patients. In recent years, a novel method of mtDNA replacement therapy has emerged as a promising approach for preventing mitochondrial disease transmission, including four main techniques for mtDNA replacement: germinal vesicular transfer, MII spindle-chromosome complex transfer, pronuclear transfer, and polar body transfer.

Recent studies in Juan Carlos Izpisua Belmonte's laboratory demonstrated that it is possible to use gene-editing tools to specifically target mtDNA. Mito-TALENs can be used to cut

mutated mtDNA, and have already been used to selectively eliminate defective mtDNA in both unfertilized mouse eggs and murine zygotes. Furthermore, these genetically modified mice gave birth to two successive generations of healthy mice. When mRNA encoding mito-TALENs was injected into an egg from a heteroplasmic mouse that carried two mtDNA haplotypes (NZB and BALB), mtDNA heteroplasmy shift was achieved and the edited embryos grew into normal mice. Importantly, the authors did not observe any off-target effects (251).

Up to this point, we have recounted the massive potential offered by this new CRISPR-Cas9 technology tool, in both research and future clinical applications, particularly in the field of germline genetic modification, such as treating mitochondrial diseases. This is enough motivation to be interested

in the curative potential of CRISPR-Cas9 technologies for carriers of both heteroplasmic and homoplasmic mtDNA mutations. Although gene-editing may not affect all mutant mtDNA, it may modify enough to bring the individual below the disease threshold, conferring therapeutic benefits. Although there is considerable hesitation among the scientific community to use this technique in the germline, as mentioned before, studies using nonviable human embryos have already been performed (256–259), showing that the technique works with low efficiency to generate on-target gene modification, but that off-target mutations are also generated and the resulting embryos are mosaic, with the exception of one recent study (259). CRISPR-Cas9 has already been successfully used to produce mitochondrial sequence-specific cleavage, as proof of the concept that this technique can target specific mitochondrial genes (251). In the same study, researchers engineered a new version of the enzyme Cas9, mitoCas9, whose localization is restricted to the mitochondrial matrix. This is an important step, because it reduces the risk of off-target mutations in the embryo and prospective children.

CRISPR-Cas9 editing of embryonic mtDNA may represent a more socially acceptable alternative to “three-parent IVF.” Instead of combining the genetics of three individuals, this technique may enable a couple to conceive without requiring donor genetic material. Another advantage of trying to treat mitochondrial disease before implantation is the small number of cells; it may be easier to ensure a reduced mutation load if the embryo is in the 8- or 16-cell stage, rather than waiting to provide a therapeutic intervention after birth, when there are exponentially more cells.

## CONCLUSION

In recent years, the development of new technologies based on sequence-specific nucleases has enhanced the feasibility of constructing donor vectors, genome editing with the use of oligonucleotides, multiplexed genome editing, greatly improved efficiency in targeting specific genomic loci, direct genome editing by means of the electroporation of embryos, and, the most important achievement, avoiding mosaicism in human embryos. This technologic breakthrough makes the possibility of editing the human germline much more feasible than in the past. From our point of view, basic studies must be conducted in nonhuman animal models to better understand the target disease before these technologies can successfully be used to edit DNA. Because nonhuman studies have limitations in predicting outcomes in humans, subsequent research in humans will be necessary. Similarly, before CRISPR-Cas9 technology can be translated to the clinic, outstanding problems must be resolved, primarily mosaicism and off-target effects, although apparently it seems to have been solved in the Mitalipov group’s study. Nevertheless, once technical issues are resolved, editing of the human germline to prevent the birth of a child with a genetic defect should be considered only when already established methods, which do not entail manipulation of the genome (such as PGD), are not available.

Considering how far the field has advanced in just the 5 years since the introduction of CRISPR-Cas9 systems in early 2013, and with the approval of the first two in vivo clinical trials dealing with cancer immunotherapy on T cells (USA) and lung cancer (China), we can undoubtedly expect remarkable progress in the next 5 years in the basic research and reproductive fields.

## HUMAN EMBRYONIC STEM CELLS: THE DISCOVERY OF PLURIPOTENCY

Alan Trounson, Ph.D.

The ability of a cultured cell to develop into any cell of the organism, known as pluripotency, was discovered in embryonal carcinoma cells (ECCs) by Kleinsmith and Pierce (261). Pluripotent stem cells derived from the preimplantation mouse embryo were developed and used to establish transgenic offspring and explore functional genomics. Concurrently, developments in human IVF were evolving that provided access to human embryos from fertilization to the blastocyst stage in vitro (262) and an interest to explore the origins of embryonic tissue formation and integration. This led to the research of two separate and independent groups to develop pluripotent human embryonic stem cells (ESCs). One group (263) had been studying monkey embryonic stem cells and the other (264) had been examining the development of isolated inner cell mass (ICM) cells of blastocyst-stage human embryos (265, 266).

## PREIMPLANTATION EMBRYOS AND PLURIPOTENCY

Teratocarcinomas are germ cell tumors most commonly found in the testes and contain cells of all three primary germ cell lineages in a chaotic unorganized state as well as, importantly, undifferentiated malignant cells that are pluripotent (267). These pluripotent cells are ECCs and were used for the founding observations on pluripotency and differentiation into various cell lineages of interest. Importantly they also provided critical data on oncogenesis. However, their malignant properties prevented any consideration of direct clinical applications. Teratomas are nonmalignant and generally arise in the ovary, probably as parthenogenetically activated oocytes. These cells attracted further attention as pluripotent stem cells, particularly in the mouse, where the ECCs could easily be derived in some strains from fetal germ cells, maintained in culture, and differentiated when transplanted in vivo. This in turn led to the development of mouse ESCs from preimplantation embryos with research primarily concentrated on their use for functional genomics with the use of gene knockout techniques and chimeric animals produced by blastocyst injection of gene-edited ESCs. Interest in using human ECCs derived from human fetal germ cells was reported around the time of the discoveries of human ESCs by Gheharhart et al. (268). However, although these human ECCs are pluripotent, they are more difficult to maintain

in culture and to differentiate into a wide range of mature cell types than human ESCs.

## DISCOVERING ESCS AND PLURIPOTENCY WITH THE USE OF IVF BLASTOCYSTS DONATED FOR RESEARCH

Given the success of mouse ESCs in determining gene function by gene knockout and chimeric mouse development in studies of functional genomics and the capacity to generate monkey ESCs, it was of interest for the understanding of early human development and early childhood cancers to try to produce human ESCs. Early studies showed that blastocysts would plate out in culture (265), but these cultures did not resemble the mouse ESC colonies formed with the use of mouse embryos. Further studies in 1995–1996 using the mouse ESC derivation methods on mouse fibroblast cell feeder layer cultures showed typical “lumpy” colonies that were different from mouse ESC colonies, but could be passaged on mouse feeder layers. Although mouse ESC researchers doubted that these were ESCs, scientists experienced in pluripotent ECCs culture believed that they were indeed human ESCs. With this in mind, the collaboration between Monash University and National University of Singapore scientists was reinitiated in 1997 and six ESC lines were established from ~30 human embryos. These cells expressed all the pluripotent markers of ECCs and could be passaged indefinitely on mouse feeder cells. By chance, the Monash group received the Wisconsin group’s paper (263) to review for the journal *Science* in 1998 and knew that their report of successful human ESC derivation was correct. At this time the full characterization by teratoma formation and analyses of multiple lineage on the Monash-Singapore cells had not been completed. It was decided to demonstrate directed neural lineage differentiation as further proof of pluripotency before publishing the Monash data (264) confirming the Wisconsin report of discovery of human ESCs.

The human ESCs formed from human blastocysts are not exactly equivalent to mouse ESCs; they appear to more resemble mouse ESCs derived from the advanced gastrula stage, termed mouse epiblast stem cells. Human ESCs can be converted to the more naïve ground state by defined culture conditions that enable the human ESCs to express molecular markers and functional characteristics that are similar to mouse ESCs (269). Whether this naïve ground state of human ESCs is essential for deriving specific tissues for transplantation has not really been decided yet. However, methods that work well for directing differentiation of mouse ESCs are likely to be shared by these naïve human ESCs.

## DERIVING AND DIFFERENTIATING ESCS

Human blastocysts have been grown in vitro from the earliest days of human IVF research (270). Interest in using spare human blastocysts for deriving ESCs followed the routine production of blastocysts for clinical use (271). The demonstration of the properties of immortality in continuous culture in vitro and ability of these cultured cells to form the major lineages of human development and potentially every cell

type of the human body defined their pluripotentiality. Essentially, the original method for deriving ESCs requires the immune-isolation of the ICM of human blastocysts and culture of these ICMs under defined conditions on mouse fibroblast feeder cells. By regular passage of the dissected colonies before they exhibit spontaneous differentiation, the ESCs develop as colonies attached to plastic culture dishes in the presence of mouse feeder cells (Fig. 13). This method is efficient in producing ESC lines. Variations on this basic method are now in use, including the replacement of the animal feeder cells (feeder-free derivation) and use of earlier-stage human embryos and biopsied cells (272). Up to 2009, it was reported that 1,071 human ESC lines were derived by 87 institutions around the world, but >70% of the publications on ESCs at this time involved just two of those cell lines (266). Therefore, predictions that massive numbers of human embryos would be used to derive ESCs has not been realized. Indeed, there are large numbers of cryopreserved human embryos that remain in storage in the U.S. and elsewhere that could be used for producing ESCs or for other compelling research studies (273).

The expression of key transcription factors is characteristic of pluripotent ESCs. Indeed, expression of just four primary transcription factors (OCT4, cMYC, KLF4, and SOX2) is sufficient to convert mature somatic cells into induced pluripotent stem cells (iPSCs). Some of the SOX family of transcription factors are highly expressed in undifferentiated pluripotent stem cells and others are up-regulated in decisions during differentiation into specific lineages (274). Pluripotent stem cells express common specific cell surface antigens, including SSEA-3, SSEA-4, tumor-related antigen (TRA) 1-60, TRA-1-81, and NANOG. They have high telomerase activity which enables their continuous multiplication in vitro. When ESCs begin to differentiate, single-cell RNA sequencing shows that cells make specific lineage decisions that result in dual or multiple populations developing as different cell types. Therefore, it is important to select the correct cluster from the differentiating populations for those with potential to form efficiently the cell type desired for preclinical studies and applications in cell therapies.

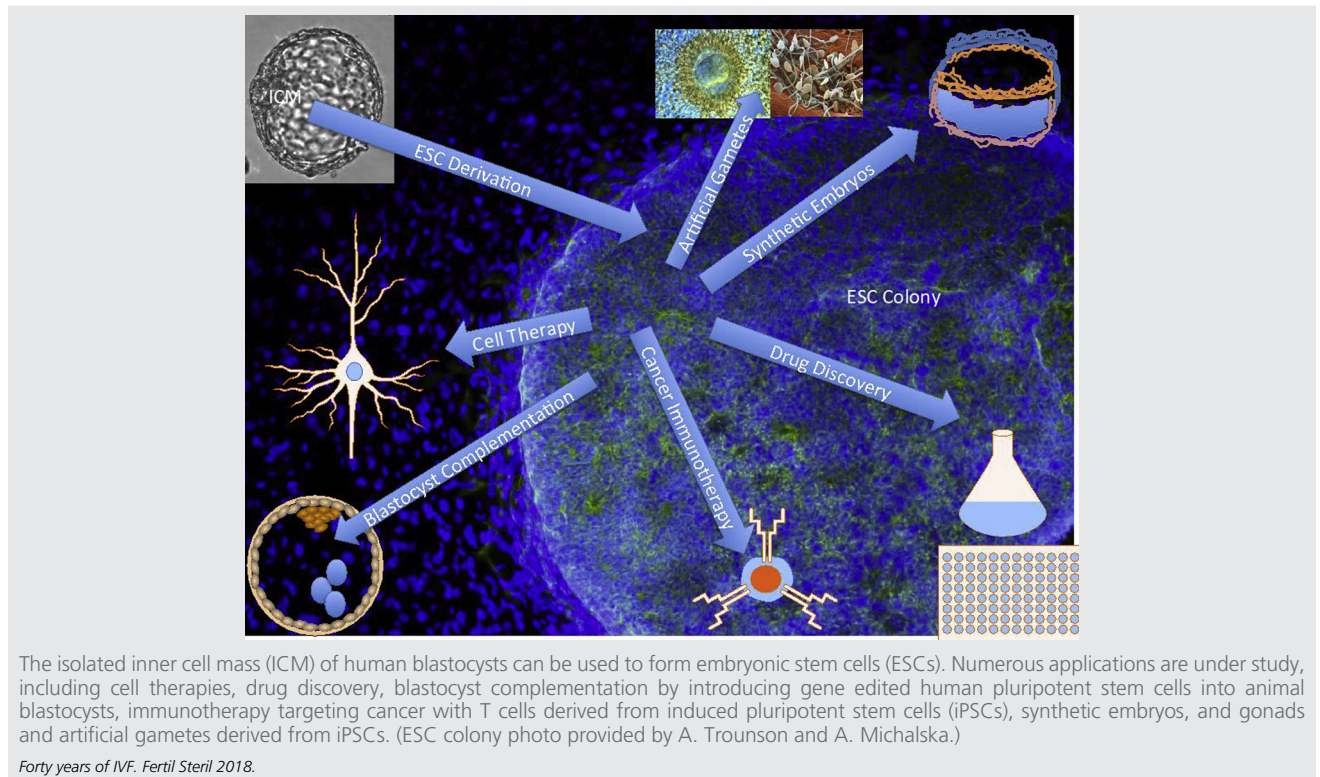
## THE POTENTIAL FOR DISCOVERY WITH THE USE OF ESCS

Human ESCs are now in clinical trials for numerous conditions including spinal cord repair, macular degeneration, type 1 diabetes, myocardial infarct, and Parkinson disease (275). Progress is slowly showing clinical benefits with the use of ESC therapies in many of these applications. The relatively long process has much to do with the emerging cell therapy technologies which have complex regulatory requirements to get to the clinic. Time is needed to judge whether these therapies will eventually become part of clinical practice for regenerative medicine.

Interest in using pluripotent stem cells for immunotherapy has grown with the success of chimeric antigen receptor (CAR) T-cell technology for blood cancer therapy. Work is underway to manufacture T cells and natural killer cells from pluripotent stem cells genetically engineered with CARs that



FIGURE 13



can be multiplied and strongly target and destroy tumor cells. The ability to gene-edit pluripotent stem cells, select for the desired integration, and expand them in vitro provides a very attractive opportunity for cancer therapies.

The discovery of how to derive iPSCs was dependent on data of transcription factor expression in ESCs. These iPSCs are also making their way into similar therapeutic applications as ESCs (275) (Fig 13). Perhaps some of the most important developments may come from the use of pluripotent stem cells, together with human genomics, in drug discovery (276). Pluripotent stem cells may also be used in blastocyst complementation to form chimeric animals that could produce human organs for possible transplantation (277). More recent publications of growing embryos to postimplantation stages has led to consideration and early studies on the development of synthetic embryos with the use of pluripotent stem cells that are not developmentally competent (278) but provide a model for many developmental abnormalities, drug screening, lineage formation and organization, and functional genomics (Fig. 13).

Primitive pluripotent stem cells can be directed into the germ cell lineage. In the mouse, it has been shown that both sperm and oocytes with full developmental competence can be derived from ESCs and iPSCs, although the oocytes require fetal ovarian somatic cells to accomplish the critical conversion to primordial-like germ cells. The development of human germ cells and gametes is also underway, and it is very likely in the near future that human iPSCs could be used to produce

sperm from sterile men and possibly repopulate artificial ovaries for sterile women. Clearly these developments will be desired by many individuals who are sterile because they do not have gametes. The social issues raised for using pluripotent cells for enabling reproduction in transgender individuals will test the support of community ethics. There will also be debate concerning the use of iPSC-derived gametes and artificial gonads for use by postmenopausal women and older men to extend their reproductive life spans. These discussions ought to begin as science moves these new discoveries toward potential application, and key scientific societies should have a strong voice in enabling the community to understand the potential benefits and risks of these possible new medical developments.

## REFLECTIONS ON THE DISCOVERY OF ESCS

The transition from human embryology to pluripotential stem cells was driven by the curiosity of cell biologist members of the research groups described. The availability of human IVF blastocysts enabled the discoveries to occur. My own motivations were to better understand the incredible transformation processes of differentiation that turned primitive embryonic cells into the primary germ lineages for tissue formation and the possible use of resulting cell products in medical therapeutics. I was enthralled with the experiments, and I also thought that we may better understand the origins of childhood cancers and birth defects.



Following the signposts from basic scientific developments in other species provided the necessary information to make the leap to discoveries of human pluripotency that

may possibly change the nature of therapies for regenerative medicine, and even cancer, for evermore. This seems likely.

## PATIENT SELECTION

### THE ENDOMETRIAL FACTOR

Carlos Simon, M.D., Ph.D.

#### WHERE ARE WE TODAY?

Human reproduction is a very inefficient process compared that of the majority of mammalian species. The probability of conception during a given menstrual cycle is ~30% (279) versus 95% in rodents and 96% in rabbits. In humans, only 60% of all conceptions advance beyond 20 weeks of gestation, and 75% of lost pregnancies fail to implant and are not clinically recognized (280).

Between the first live birth from a human embryo generated with the use of in vitro fertilization (IVF) in 1978 and Robert Edwards' Nobel prize award in 2010, reproductive medicine has emerged as one of the most rapidly developing areas of medical science. This achievement has been possible by integrating the knowledge developed in endocrinology, embryology, reproductive science laboratories, and endoscopy, and coupling it with the creation and availability of novel compounds for ovarian stimulation. Additional technologies extending the scope and efficiency of IVF have subsequently been developed and implemented. These include intracytoplasmic sperm injection (ICSI), preimplantation genetic diagnosis, and cryotechnology, which allows the freezing and storage of sperm, oocytes, embryos, and testicular and ovarian tissue. Despite all these technologic advances, when a morphologically normal blastocyst is transferred into a seemingly normal uterus in IVF, cycle reproductive success is very limited (281) and has not significantly improved since the 1990s.

Ovarian function, induction of ovulation, and studies of oocyte and embryo have been by far the preferred areas of investigation by researchers and clinicians. The final success of a crucial function such as embryo implantation relies on two partners, the embryo and the endometrium; we can no longer avert our eyes from one of them, namely, the endometrial factor. The human uterus, and mainly the endometrium, are the anatomic and functional prerequisites for continuing the species. Pregnancy begins with embryonic implantation. Women menstruate in an endless resetting process to synchronize the uterus for the imminent arrival of a blastocyst. Progesterone (P) is the single most important hormone controlling the initiation and course of pregnancy in humans, and P withdrawal is the "trigger" for menstruation in nonpregnant cycles. P induces the acquisition of a 12–48-hour transient functional state by the endometrial epithelium known as the window of receptivity or window of implantation (WOI). This limited period can be found in a personalized

manner from LH+6 to LH+9 in a natural cycle or from P+4 to P+7 in a hormonal replacement therapy cycle.

During this WOI, the endometrial epithelium undergoes a morphologic transition to the receptive phenotype regulated by a specific transcriptional program. As a result, modifications in the plasma membrane occur leading to the disruption of the cytoskeleton and preparing the apical pole for cell-to-cell adhesion known as plasma membrane transformation (282). It allows blastocyst adhesion that is characterized by increased physical interaction between the blastocyst and the uterine epithelium. Shortly thereafter, the WOI closes. The decidualization process is initiated around uterine spiral arterioles, involving the morphologic and biochemical reprogramming of the endometrial stromal compartment. The formation of the decidua is a conceptus-independent progressive process that is driven by P (283), which increases local cyclic adenosine monophosphate production that stimulates synthesis of a complex network of intracellular and secreted proteins. Morphologically, decidualization is characterized by the transformation of elongated fibroblast-like stromal cells into enlarged polygonal/round cells shaped by a complex intracellular cytoskeleton rearrangement. The postimplantation blastocyst is quickly embedded in the stromal tissue previously decidualized by P, which controls trophoblast invasion and subsequent placentation. Finally, the uterine epithelium regrows to cover the implantation site.

The main difference between humans and nonmenstruating species such as rodents and rabbits lies in decidual control of human implantation versus embryo control of rodent implantation. This preponderance of the embryo directing implantation is exemplified by the process known as embryonic diapause or delayed implantation, which is not present in humans. Embryonic diapause occurs in more than 130 species of mammals, ranging from bears and badgers to mice and marsupials. Implantation is withheld as the embryo is suspended in the blastocyst stage for up to 1 year in some cases, and subsequently resumes without deleterious effects for the pregnancy. However, in the human it is the uterus and its endometrial mucosa that are the anatomic and functional prerequisite for continuing the species. They are also an important limiting factor for improving the efficiency of human reproduction.

#### LANDMARK ACHIEVEMENTS TOWARD UNDERSTANDING THE ENDOMETRIAL FACTOR: HOW DID WE GET HERE?

Perhaps the reader knows the most cited paper in obstetrics and gynecology? Published in the inaugural issue of *Fertility and Sterility* in 1950, Noyes et al. described the histologic

evaluation of the human endometrium, providing for the first time an objective classification for endometrial dating (284). This landmark paper, cited 2,606 times, initiated the era of anatomic medicine for the endometrial factor. Fifty years later, the accuracy, reproducibility, and functional relevance of histologic evaluation as a predictor of endometrial receptivity or even fertility was questioned in randomized studies (285). Though Noyes' discovery of the morphologic evidence of endometrial transformation through the menstrual cycle was seminal in the history of our understanding of endometrial functionality, it was not sufficiently specific or precise, owing to the technologies used at that time, for prediction of implantation and is not currently a useful diagnostic test of endometrial reproductive function. Consequently, endometrial histology is not included in today's standard clinical infertility work-up, although it remains relevant for diagnosis of malignancy or endometritis.

The concepts of endometrial receptivity and the existence of a WOI were first suggested by Hertig and Rock in 1956 (286). In their pioneering study lasting 17 years, the authors recovered 84 pre- and postimplantation embryos (known at that time as fertilized human ova) from 210 patients of known fertility (1–14 children) with coital dates around the estimated time of ovulation who underwent hysterectomy during the next 2 weeks. A total of 34 embryos ranging from 2-cell cleavage stage to a 17-day implanting blastocyst (named as villous ovum implanted in early decidua) were found. The take-home message from this magnificent work was that ~30% of the embryos (13/34) were "abnormal" and that embryonic implantation occurs around day 20 in a regular menstrual cycle, demonstrating for the first time the concept of the WOI.

In the 1980s, two critically important technical advances in medical imaging were developed: hysteroscopy and transvaginal ultrasound. The modification of the resectoscope from transurethral prostate resections to hysteroscopic procedures facilitated diagnosis and operation within the uterine cavity under direct visualization. Since then, significant improvements have evolved with rigid complicated instruments evolving into outpatient hysteroscopy as the best diagnostic and therapeutic technique for uterine pathology, though not for predicting endometrial receptivity. In 1986, the introduction of transvaginal ultrasound for aspirating oocytes (287) popularized this technique for the direct visualization of the endometrium and endometrial cavity before, during, and after controlled ovarian stimulation as well as later on for placing the embryo in the correct position in the endometrial cavity. Owing to its resolution, low cost, lack of exposure to ionizing radiation, and technical improvement for three-dimensional imaging, it is today the only diagnostic examination required for evaluation of the endometrial factor. Nevertheless, it is not sufficient for predicting endometrial molecular normalcy and implantation potential.

In the 1990s, with the use of the ovum donation model, clinical WOI, which refers to the time frame in which the embryo must be transferred to the uterus, was demonstrated (288). Endometrial receptivity was tested by replacing 2- to 12-cell embryos (days 2–4 of embryo development) between days 16 and 24 of hormonally and histologically defined

cycles. Of 37 embryo transfer procedures in cycle days 17–19, 15 conceptions occurred (40.5%), whereas of 11 patients transferred on days 20–24, none conceived. Similarly, no pregnancies were achieved with four transfers on cycle day 16.

Further work by Wilcox et al. in 1999 (289) popularized the concept that the human embryo implants 8–10 days after ovulation in natural conceptions. However, ovulation was identified on the basis of changes in urinary excretion of E<sub>2</sub> and P metabolites, measured by means of radioimmunoassay. Now, almost 20 years later, the above method to determine ovulation has not been adopted. The time frame difference between that early urinary metabolite test and today's peak LH blood test used to predict ovulation is unknown. Actual estimates in assisted reproductive technology cycles indicate that the WOI is centered at 5 days after endogenous or exogenous P administration; in a natural cycle it occurs 6–7 days after ovulation (centered at day 20 of the menstrual cycle, ranging from days 19 to 22). Nevertheless, the clinical community has since embraced a permissive time frame in all patients with the same success of implantation for 3 days regardless of individual variations or hormonal treatment received (i.e., natural cycles, controlled ovarian stimulation, hormonal replacement cycles). These are the historical reasons why embryo transfer is usually performed when the embryo is ready, assuming the mother's endometrium is synchronized with the embryo, although it has been demonstrated that this synchrony cannot be assumed.

Also in the 1990s, Barker presented his theory suggesting that "the womb may be more important than the home" to emphasize the concept that the maternal endometrium may have a profound effect on adult physiology by conditioning the embryo, the fetus, and its adult life. Barker postulated that the metabolic effects of depriving a fetus of an adequate supply of nutrients would result in irrevocable "programming," predisposing it to an increased risk for disease in adulthood (290). This groundbreaking concept has been confirmed in a variety of nonhuman animal models and by large clinical studies of complex diseases such as obesity, type 2 diabetes, hypertension, stroke, and atherosclerosis. More recently, the concept has expanded to include the transcriptomic reprogramming of the preimplantation embryo by the maternal intrauterine environment. Even transgenerational effects have been demonstrated with the observation that the intrauterine environment affects the fertility of the offspring by modulating fetal gametogenesis in utero, thereby expanding even further the Barker hypothesis that "the womb of your grandmother may be more important than your home." Because epigenetic changes contribute to the pathogenesis of several complex diseases, it is of crucial importance to identify and modify intrauterine changes that may mediate programming of the developing embryo that will impact adulthood.

The transition from anatomic to molecular medicine in the diagnosis of the endometrial function arrived only two decades ago. Initially, it was unsuccessfully investigated at the single molecular and biochemical levels. All possible molecules and receptors with expression concordant with the WOI time frame were scrutinized without consistent clinical

translational results. However, the search for a transcriptomic signature of endometrial receptivity was a major turning point in the understanding of this function. Pioneering work from the group of Peter Rogers (291), Linda Giudice (292), and Carlos Simon (293) demonstrates the feasibility of the molecular classification of the endometrium with the use of transcriptomic profiling throughout the menstrual cycle, as well as during the window of receptivity/WOI. On this basis, considering that precision medicine is focused on targeted individualized treatment, in 2011 we reported the transcriptomic signature of human endometrial receptivity from 238 genes, leading to the creation of endometrial receptivity analysis (294). This molecular analysis is now performed with the use of next-generation sequencing coupled with a computational predictor and algorithm able to identify the personalized WOI of a given patient regardless of the histologic appearance of their endometrium. We now have the tools to improve synchrony between the embryo and the endometrium: When both are ready, implantation is more successful.

Finally, following thorough work in various nonhuman animal models, uterine transplantation is now a reality since the Brannström group published the first successful live birth after uterine transplantation (295). This latest milestone in reproductive medicine leads to novel therapeutic solutions for the treatment of absolute uterine factor infertility (Fig. 14).

## WHAT WILL THE NEXT 40 YEARS BRING?

Prophets are typically wrong, and I will be no exception, but several unmet needs remain in endometrial biology research that are getting closer to clinical translation. The technologic innovations are coming from three disruptive fields: genetics, stem cell research/bioengineering, and nanotechnology. My forecast is that improvements in the endometrial factor will be application driven with the use of state-of-the-art techniques, rather than technology driven.

## KISMI: keep it simple and minimally invasive?

The search for biomarkers in biologic fluids has emerged as an efficient alternative to classic invasive diagnostic methods. Human endometrial glands actively secrete endometrial fluid (EF), whose main functions are immunologic defense and nursing-reprogramming preimplantation embryos throughout the apposition, adhesion, and early invasion processes. EF can also be aspirated in the same cycle that embryo transfer is performed without affecting pregnancy rates. The secretomic study of EF initiated by Nick Macklon's group may open up a new field for the noninvasive diagnosis of endometrial function.

## Looking for the complete picture: the endometrial microbiome

The recent advent of cost-effective and scalable next-generation sequencing, together with the knowledge of bacterial genomes, has facilitated the discovery of unique microbiomes in anatomic niches previously considered to be sterile (e.g., the uterus). The endometrial cavity has been clas-

sically considered to be a sterile site, but recent reports challenging this dogma support the existence of an endometrial microbiome composed of different microorganisms (specifically *Lactobacillus* spp., *Mycoplasma hominis*, *Gardnerella vaginalis*, and *Enterobacter* spp.) that differs from that of the vagina (296). The study of the endometrial microbiome in IVF patients has revealed that bacterial communities may have an impact in reproductive success; specifically, the presence of dysbiotic or pathogenic bacteria populations has been proposed as an emerging cause of repeated implantation failure or recurrent miscarriage. This fascinating area could lead to "probiotic" treatment to correct an abnormal endometrial microbiome and improve reproductive outcome.

## Treat with cells what cannot be cured with drugs

Regenerative medicine is a new scientific field aiming to replace or regenerate human cells, tissues, or organs to restore normal function. Endometrial cell therapy intending to achieve de novo endometrial regeneration in patients with Asherman syndrome and atrophic endometrium by means of autologous cell therapy with CD133+ bone marrow-derived stem cells is attracting the attention of the clinical community (297). The European Medicine Agency has issued a positive opinion to consider CD133+ cells as the first Orphan Drug Designed therapy for the treatment of Asherman syndrome, categorizing these cells as an Advanced Therapy Medicinal Product, and supervised phase I/II and III trials are underway.

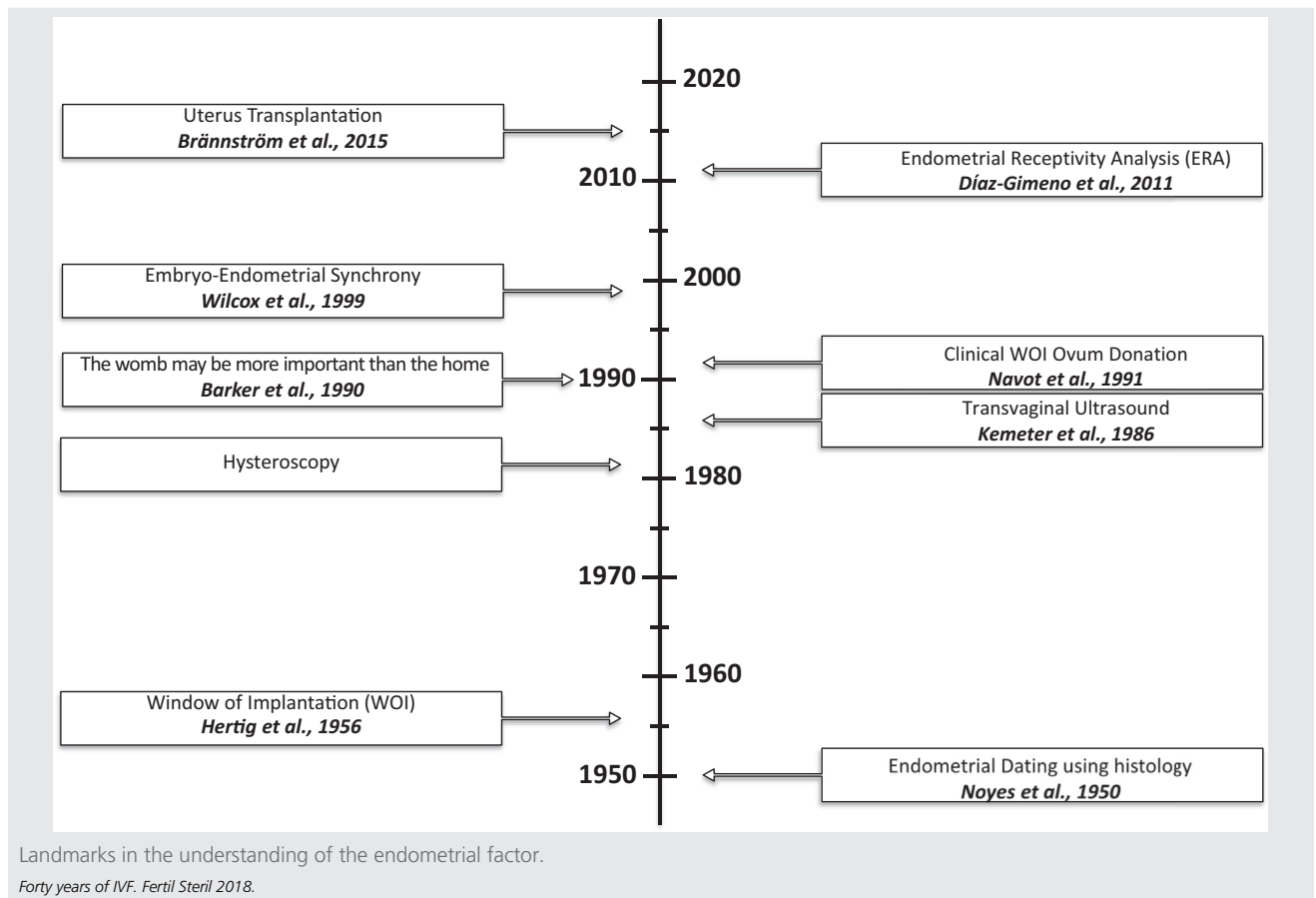
The transplantation of the uterus is inherently limited by familiar problems, including the lack of donor organs and the need for long-term immunosuppression after transplantation. Since 2008, several milestones in uterine bioengineering have been achieved related to the growth of cells on specific three-dimensional structures with the use of different supports or scaffolds: decellularization preserving a reusable/functional extracellular uterine matrix while maintaining its vascular network, and partial recellularization with cells from putative recipients. Thus, the creation of the bioengineered artificial uterus with the use of autologous cells from putative recipients is a natural progression of this field.

## The uterine factor behind gestational pathologies

The maternal decidua controls conception and the course of pregnancy in humans. Failed decidualization or resistance to decidualization is associated with a spectrum of reproductive defects, including failed implantation and clinical miscarriage, but also late gestational complications, such as preeclampsia (298). New emerging data reinforce the concept that a suboptimal uterine environment lies upstream of not only inefficient conception, but also failed trophoblast differentiation and invasion driving placentation, paving the way to better diagnosis, prevention, and treatment of late gestational diseases.

Thus, we are at a turning point in the history of the forgotten maternal endometrium to improve the success and safety of assisted reproductive technologies for the benefit of our patients. We have the opportunity to build on

FIGURE 14



the understanding of the morphologic changes described by Noyes 67 years ago. Going forward we should add the “soil” concept to our clinical practice, combining anatomic medicine with the modern understanding of the genetic and molecular underpinnings of endometrial function to provide accurate diagnostic and therapeutic modalities maximizing the endometrial factor and its critical function in embryonic implantation. Furthermore, understanding the endometrium, the uterine environment, and its contribution to both the successful outcome of a pregnancy and the long-term health of future generations is crucial to maximizing the ultimate success of our treatment for infertile patients and their families.

**Acknowledgments:** The author gratefully acknowledges Cecilia Valdes and Alan Thornhill for their critical review of this manuscript.

## TREATMENT EVOLUTION OF HYDROSALPINX IN INFERTILE WOMEN

Togas Tulandi, M.D., M.H.C.M.

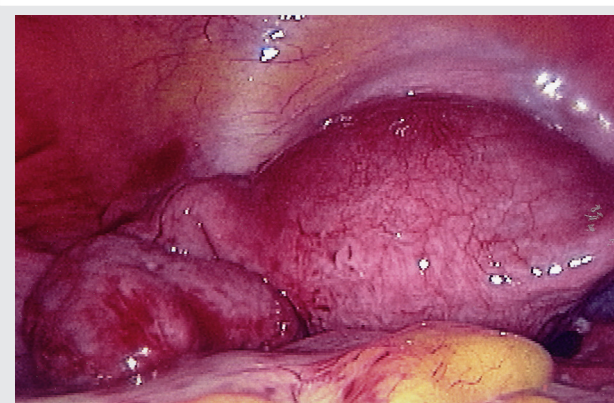
Since my residency years, I have been fascinated with the function and disorders affecting the fallopian tube and their association with infertility. During fellowship, I became passionate about reconstructive surgery to restore fertility. We used the principles of microsurgery, including gentle tissue handling, the use of fine instruments, and fine and nonre-active suture materials. The purpose is to restore the tubal function, enhancing the chance to conceive.

One of the newest energies then was the carbon dioxide (CO<sub>2</sub>) laser. It was thought that the use of CO<sub>2</sub> laser was precise, associated with minimal tissue damage, and rapid tissue healing with no adhesion formation, characteristics that would be beneficial for surgery enhancing fertility. Accordingly, we performed a study comparing surgical treatment of hydrosalpinx with the use of laser versus electrosurgery (Fig. 15). We found that the pregnancy rates after terminal salpingostomy with the two surgical modalities were similar (299). Similar results were found with postoperative adhesion formation and salpingo-ovariectomy in infertile women.

Reproductive outcome of reconstructive surgery for hydrosalpinx is generally unsatisfactory. Depending on the severity of the hydrosalpinx, the rate of pregnancy can be very low if the tube is rigid and thick without mucosal folds, and up to 50% when the tubal damage is minimal. In a randomized study, the rates of spontaneous pregnancy and



FIGURE 15



Dilated fallopian tube consistent with hydrosalpinx. No spillage of methylene blue dye on chromopertubation.

Forty years of IVF. *Fertil Steril* 2018.

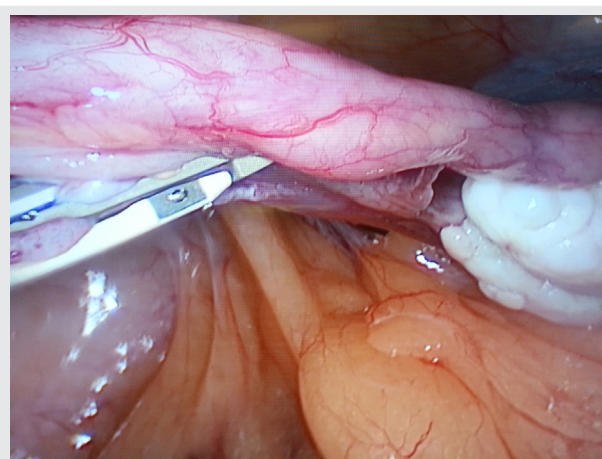
ectopic pregnancy 24 months after salpingostomy by means of laparotomy were 43.7% and 5.0%, respectively, and after laparoscopic salpingostomy were 41.6% and 3.9%, respectively (300, 301).

To evaluate the degree of tubal damage, we used the classification of distal tubal disease of the American Fertility Society (now the American Society for Reproductive Medicine). It is based on the condition of mucosal folds, tubal thickness, and the extent and nature of periadnexal adhesions. Another method to assess the degree of tubal damage is by performing a transvaginal fallopscopy or salpingoscopy at the time of laparoscopy. As such, the endosalpinx can be examined by means of fallopscopy or salpingoscopy and the external tube as well as the distal endosalpinx by means of laparoscopy. It was thought that evaluation of the internal fallopian tube led to a better evaluation of the hydrosalpinx. However, those procedures did not gain popularity and were soon abandoned. The main reason was an improved live birth rate with in vitro fertilization (IVF), making tubal surgery less popular. Furthermore, the recurrence rate of hydrosalpinx after surgery is high.

Indeed, compared with tuboplasty, IVF has become a better treatment option for women with hydrosalpinx. In addition, it eliminates the need for surgery and general anesthesia, and conception can occur much faster than after surgery. As a result, tuboplasty for hydrosalpinx is recommended only for young women with good and preserved tube and no other infertility factors.

However, the presence of hydrosalpinx in women undergoing IVF treatment is associated with reduced pregnancy rates (302). This could be due to entry of the hydrosalpinx fluid into the uterine cavity flushing out the embryo, toxic effects of the hydrosalpinx fluid to the gamete or embryo, or alteration in endometrial receptivity. A few randomized studies showed that hydrosalpinx removal (salpingectomy) increased the efficacy of IVF outcomes (302–304). The benefits seem to be higher when the hydrosalpinx is bilateral and visible on ultrasound. In a meta-analysis

FIGURE 16



To mitigate the interference of ovarian blood supply, salpingectomy should be performed very close to the tube.

Forty years of IVF. *Fertil Steril* 2018.

involving 646 women, pre-IVF treatment with salpingectomy was associated with a higher ongoing pregnancy rate than in those not pretreated (31% vs. 17.6%, odds ratio [OR]: 2.20, 95% confidence interval [CI]: 1.26–3.82) (305).

Yet salpingectomy could also affect the ovarian blood flow and reduces ovarian response during subsequent IVF cycles (306). As a result, salpingectomy before IVF treatment should be performed close to the tube to avoid compromising the blood supply to the ovary (307) (Fig. 16). Thermal energy should be used sparingly. Occasionally, hydrosalpinx is buried in dense adhesions limiting access to the distal tube. Here, one can occlude the proximal part of the tube either by means of electrocautery or preferably with the use of clips, similarly to sterilization. The efficacy seems to be similar to that of salpingectomy (308). However, the resulting iatrogenic tubal occlusion (proximal and distal occlusion) could be followed by further distension of the hydrosalpinx. A meta-analysis shows that the on-going IVF pregnancy rates of proximal tubal occlusion (relative risk [RR]: 3.22, 95% CI: 1.27–8.14) and salpingectomy (RR: 2.24, 95% CI: 1.27–3.95) were superior to no surgical intervention (309).

A less invasive technique is to place a microinsert into the proximal tube by hysteroscope. One such device is Essure (Conceptus, Scottsdale, Arizona), which is approved by the Food and Drug Administration for tubal sterilization. The device and the resulting fibrosis of the tubal lumen occlude the tube. In a two-center randomized study, the ongoing IVF pregnancy rate per patient after hysteroscopic proximal occlusion (26.2%, 95% CI: 7.1%–49.1%; RR: 0.47, 95% CI: 0.27–0.83) was lower than after laparoscopic salpingectomy (55.8%) (310).

A systematic review of 115 women pretreated with Essure placement before IVF showed a 38.6% pregnancy rate (95% CI: 30.9%–46.8%) and 27.9% live birth rate (95% CI: 21.1%–35.8%) (311). However, in the past years, there

have been an increasing number of cases of abdominal pain possibly related to the device and requiring its removal (312). This could be due to nickel allergy or to an enlargement of the hydrosalpinx. Subsequently, the system was withdrawn from the market in some European countries and in Canada. The use of Essure before IVF treatment is off-label.

A minimally invasive procedure before IVF that does not require surgery and could be performed as an office procedure is hydrosalpinx sclerotherapy under ultrasound guidance. The most commonly used sclerosing agent is 98% alcohol, which is injected into the dilated tube at about one-half of the volume of the aspirated hydrosalpinx fluid. In a meta-analysis, we recently reported no difference in the clinical pregnancy rates between hydrosalpinx sclerotherapy and salpingectomy (OR: 0.79, 95% CI: 0.54–1.17) (313).

The treatment of hydrosalpinx has certainly evolved from reconstructive surgery to its removal before IVF treatment. The development in sclerotherapy may make the procedure much less invasive with similar IVF outcome. Whether sclerotherapy can replace other minimally invasive surgeries to enhance the IVF outcome remains to be seen in randomized trials with sufficient statistical power.

## ENVIRONMENT AND INFERTILITY: ITS ROLE IN ASSISTED REPRODUCTIVE TECHNOLOGIES

Linda C. Giudice, M.D., Ph.D.

In 1962, Rachel Carson, a writer, marine biologist, and ecologist, published her landmark book *Silent Spring* (314). This scholarly work put the spotlight on the causal relationship between man-made pesticides and wildlife abnormalities and lethality. Furthermore, it underscored environmental influences, broadly, on health and disease and the need for public awareness and public policy to prevent harm. In the 1990s, Theodora Colborn, Ph.D., a research scientist and environmentalist, coined the term “endocrine disrupters” and presented data supporting the conclusion that exposures in utero, postnatally, and across the lifespan to environmental “endocrine” toxicants disrupt developmental, behavioral, sexual, and reproductive processes (315). Endocrine-disrupting chemicals (EDCs) are now defined as chemicals or mixtures that interfere with any aspect of hormone action at any time of development and/or during the life course (316) and comprise the most widely studied class of environmental toxicants relevant to human health and reproduction.

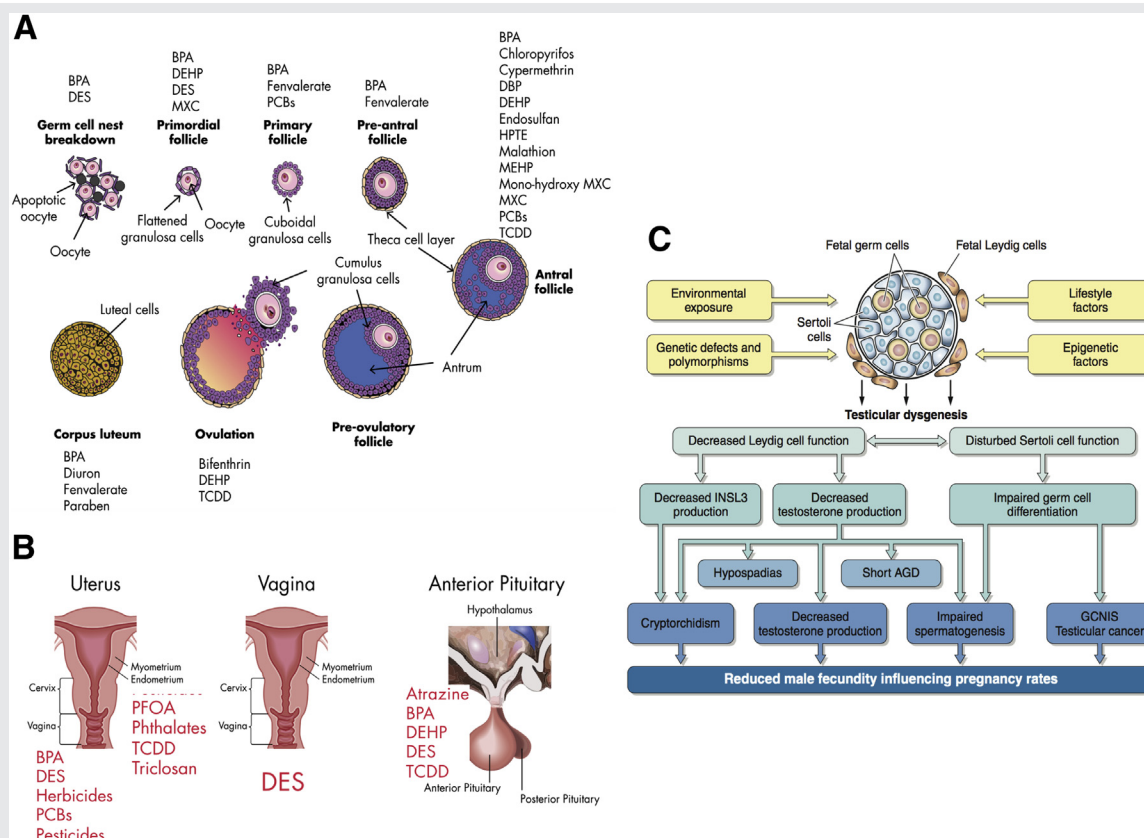
These seminal scientific works triggered intense investigations by mainly laboratory-based scientists and epidemiologists on effects and associations of various environmental toxicants (EDCs, air pollution, heavy metals) on the health of humans and other animals. These studies paralleled efforts by other scientists and physicians who were trying to determine underlying causes of infertility in men and women and who were also advancing therapies for human infertility, including assisted reproductive technologies (ART). Of note, initially, different constituents were thinking about and

working on different parts of the reproductive and infertility paradigm and seeing relevance and research through different lenses. Considerations about the role of “the environment” contributing to reproductive compromise gradually extended to studies on outcomes of clinical therapies for human infertility. The nexus of these approaches over time was almost inevitable given the importance of steroid hormones in reproduction, EDCs in the environment, and processes involved in gametogenesis, embryogenesis, implantation, and placentation. However, the importance of the environment in the ART laboratory was recognized early on, as laboratory air quality and culture media were noted to have profound effects on gamete function, embryo development, and clinical IVF outcomes. The present monograph reviews some of the history of reproductive environmental health research in humans and findings that correlate with or could predispose to infertility in men and women, some of the mechanisms underlying reproductive “disruption,” challenges in conducting such research, and relatively recent information about environmental chemicals and air pollution on IVF clinical outcomes. Highlighted throughout the results of scientific inquiry are roles of professional medical and research societies, the World Health Organization (WHO), the United Nations Environmental Program (UNEP), public health policy, concerned citizens, and patient groups in advocating for scientific discovery and raising awareness and disseminating evidence-based information about environmental impacts on human fertility and during fertility treatment—all to protect human reproductive health now and in the future.

## MAGNITUDE OF THE PROBLEM

Our water, air, soil, and food are increasingly polluted with chemicals and heavy metals. Notable is the explosive increase in chemical manufacturing and processing after World War II, which has contributed to the ~90,000 chemicals currently in use globally ([www.statista.com/statistics/272157/chemical-production-forecast-worldwide](http://www.statista.com/statistics/272157/chemical-production-forecast-worldwide)). In the U.S., ~9.5 trillion pounds of industrial chemicals are manufactured and imported (~30,000 pounds/person), annually. In most countries, including the U.S., there are few if any regulations around tracking chemical production, use, waste, and recycling; moreover, assessments of toxicity to humans, including reproductive health, are minimal. If there is suspicion of possible harm after a chemical enters the marketplace, individuals, affected groups, government agencies, and environmentally aware citizens variably begin ad hoc postexposure observational studies and in vitro and in vivo toxicity testing. This is in marked contrast to pharmaceuticals which have extensive in vitro and in vivo toxicity preclinical studies, followed by randomized clinical trials (RCTs) for safety and efficacy, and if proven to be safe and efficacious, enter the marketplace with postexposure observational studies thereafter. The limited safety testing of environmental chemicals prompted the Director of the U.S. National Institute of Environmental Health Sciences to state that environmental chemicals “act like uncontrolled medicines” (317). This is indeed concerning for reproductive health and human health more broadly, as well as for wildlife and the ecosystem.

FIGURE 17



Processes affected by endocrine-disrupting chemicals (EDCs). (A) Effects of EDCs on the ovary, showing normal developmental stages disrupted by EDCs that are listed in red above or below the likely site of action; from (316) with permission. (B) effects of EDCs on uterus, vagina, and anterior pituitary gland, with EDCs listed in red that adversely affect development and function of these structures; from (316) with permission. (C) hypothesis of testicular dysgenesis syndrome (TDS) involving poor spermatogenesis, hypospadias, cryptorchidism, short anogenital distance (AGD), and testicular cancer, together or individually constituting risk factors for reduced fecundity; from (321) with permission.

Forty years of IVF. *Fertil Steril* 2018.

## ENVIRONMENTAL TOXICANTS AND REPRODUCTIVE PROCESSES: WHAT IS THE EVIDENCE?

During the past two decades, there has been an explosion of research reports and scholarly reviews on EDCs and other environmental toxicants on animal and human development and reproduction. The data support causal and associative effects of EDCs (Fig. 17) on gonadal, the hypothalamic-pituitary-gonadal (HPG) axis, and reproductive tract development and function relevant to fertility potential. Associated disorders include aneuploidy, premature ovarian insufficiency (POI), polycystic ovarian syndrome, endometriosis, fibroids, miscarriage, endocrine cancers, lactation, and altered pubertal timing (316–319) ([www.who.int/ceh/publications/endocrine/en/](http://www.who.int/ceh/publications/endocrine/en/)). Figure 17 shows “hotspots” of ovarian follicle disruption associated with prenatal and postnatal exposures to EDCs (Fig. 17A) and perturbations of development and function of the female reproductive tract and HPG axis (Fig. 17B). Recently a systematic review and meta-analysis of 97 studies evaluated POI and environmental toxicants and found that bi-

sphenol A, phthalates, pesticides, and cigarette smoking most commonly had a negative impact on ovarian function (increased follicular depletion, earlier age of menopause), regardless of when exposures occurred (320). EDCs can disrupt each step in steroidogenesis in the ovary (316) and testis (321). Moreover, Leydig and Sertoli cell function disrupted in the developing and adult testis results in decreased INSL3 and testosterone production and impaired germ cell differentiation (Fig. 17C). Strong data implicate environmental and genetic effects resulting in the “testicular dysgenesis syndrome,” resulting in reduced male fecundity and influencing pregnancy rates (321). Much of the nonhuman animal data provide information about mechanisms of EDC actions, including affecting steroid hormone-signaling pathways, altered gene expression, and epigenetic alterations affecting gene expression (316).

## ENVIRONMENTAL TOXICANTS AND ART: WHAT IS THE EVIDENCE?

In 1998, a Modern Trends article in *Fertility and Sterility* reviewed then-current data regarding effects of environmental



toxicants (EDCs, heavy metals, solvents, other industrial chemicals, pesticides, and cigarette smoke) on female reproduction in laboratory animals, wildlife, and humans (322). Although the authors found that most of these agents had effects on reproductive outcomes in animals, effects on women's reproductive health were inconclusive except for cigarette smoking. The latter altered success rates in ART cycles in female smokers. Eight reviewed studies gave a common odds ratio (OR) for conception of 0.6 (95% confidence interval [CI]: 0.5–0.7) among smokers, and diminished ovarian reserve was found in smokers versus nonsmokers (OR: 2.8, 95% CI: 1.2–7.9) as well as elevated basal or stimulated FSH levels in smokers versus age-matched nonsmokers (322). We now know that second-hand smoke in women and smoking in men reduce gamete quality and IVF outcomes (323).

In 2005, Foster et al. (324) reviewed literature from 1979–2004 linking infertility and IVF success with occupational exposures to hazardous substances. They concluded that “there was a paucity of data linking exposure of humans to hazardous substances and IVF success” and extrapolated the plausibility of adverse events in humans from animal and cell culture studies. Only effects of cigarette smoking on IVF outcomes cited in Sharara et al. (322) were identified.

Although the majority of environmental health studies over the past 20 years have focused on human health and reproductive health and factors that can affect infertility risk (Fig. 17), more recently effects and correlations of EDCs and air pollution on parameters that influence IVF outcomes (e.g., ovarian reserve) and IVF outcomes per se (oocyte quality, sperm parameters, aneuploidy, follicular and embryo dynamics, peak serum E<sub>2</sub> levels, fertilization rates, endometrial thickness, implantation rates, pregnancy rates, and miscarriage rates) have been evaluated. A recent systematic review was undertaken of well-designed prospective cohort studies published from 2000 to 2016, wherein EDC exposure assessment was based on biomarkers of exposure, documented associations of exposures to environmental EDCs, and the reproductive potential of women undergoing IVF (323). It included data from the Environment and Reproductive Health study ([www.hsph.harvard.edu/earth/](http://www.hsph.harvard.edu/earth/)), the Longitudinal Investigation of Fertility and the Environment ([www.nichd.nih.gov](http://www.nichd.nih.gov)), the Study of Metals and Assisted Reproductive Technologies ([www.albany.edu/sph/bloom](http://www.albany.edu/sph/bloom)), and others and demonstrated that exposures to EDCs are negatively associated with several IVF end points (Table 7).

Recently, higher consumption of high-pesticide-residue fruits and vegetables was found to be associated with lower probability of pregnancy and live birth after treatment with ART, underscoring that typical human pesticide exposure from dietary sources may compromise IVF outcomes (325). A recent systematic review of nonhuman animal studies and human epidemiologic studies from 2000–2016, including data from the U.S. Nurses' Health Study II, on exposures to environmental air pollutants (air quality, O<sub>3</sub>, NO<sub>2</sub>, SO<sub>2</sub>, particulate matter <2.5 μm [PM<sub>2.5</sub>], PM<sub>10</sub>, diesel exhaust, and proximity to major roads) and fertility underscored challenges in conducting such research (326). Human reports included retrospective birth cohort studies measuring average monthly exposure during the 2–4 months before attempting concep-

TABLE 7

Negative IVF outcomes associated with endocrine-disrupting chemicals in women.

Outcome

Decreased serum E<sub>2</sub> levels: BPA  
Decreased serum AMH levels: PCBs  
Low antral follicle count: PGA, parabens, phthalates  
Poor oocyte quality: BPA, triclosan, phthalates, PCBs  
Low fertilization rates: PFCs, PCBs  
Low implantation rates: BPA, phthalates, PCBs  
Poor embryo quality: triclosan, PCBs, BPA  
Low clinical pregnancy and live birth rates: parabens, phthalates

Note: AMH = antimüllerian hormone; BPA = bisphenol A; PCB = polychlorinated biphenyl; PGA = poly(glycolic acid).

Forty years of IVF. *Fertil Steril* 2018.

tion, prospective cohort studies, and retrospective cohort studies examining associations between numbers of oocytes retrieved, fertilization rates, embryo morphology, and pregnancy outcomes, with exposures assessed during specified time periods from stimulation to live birth. Overall, the data support decreased fertility for couples living in close proximity to major roads, and mixed data were found on effects of specific air pollutants and PM exposures with live birth rates. One study found decreased odds of live birth with NO<sub>2</sub> throughout stimulation to pregnancy outcome, but for O<sub>3</sub> only exposure during the latter period correlated with decreased live birth. Another study revealed that high exposures to PM<sub>10</sub> were not correlated with adverse IVF outcomes, although they correlated with increased odds of clinical early pregnancy loss (326). Clearly, more studies are needed to determine effects of air pollution in reproductive and ART success.

ASSESSING THE EVIDENCE:  
HOW GOOD ARE THE DATA?

Historically, expert literature reviews formed the basis of evaluating health care research and guided decision making for recommendations. After challenges in the 1970s and with demonstrated superiority of systematic reviews and patient outcomes (327), in 2000 the Grading of Recommendations, Assessment, Development, and Evaluation (GRADE) system was initiated ([www.gradeworkinggroup.org](http://www.gradeworkinggroup.org)). GRADE, a formal, systematic approach that includes rigorous methodologies to evaluate specific questions, grades the quality of scientific evidence and strength of recommendations and is widely used in clinical and public health research. In contrast, literature on effects of environmental toxicants on human health and reproduction have relied on expert literature reviews, mainly because the methodologies for grading quality of evidence and strength of recommendations are difficult to shoehorn into clinical and public health reporting. Recent systematic reviews (above) are comprehensive and conformed to the Preferred Reporting Items for Systematic Reviews and Meta-analysis guidelines ([prisma-statement.org](http://prisma-statement.org)), although strength of the evidence and recommendations are challenging to deduce. To overcome these limitations and bridge the gap between clinical and environmental health sciences, international experts were convened in 2010 by scientists Tracey Woodruff and Patrice Sutton at the University of California, San Francisco (UCSF) to develop the



Navigation Guide (327). This systematic review guidance has four parts: specify the study question; systematically select evidence from the literature; rate quality and strength of the evidence in nonhuman and human systems (sufficient, limited, or inadequate, or evidence of lack of toxicity with a final overall strength of evidence integrated from the human and nonhuman evidence as known to be toxic, probably toxic, possibly toxic, or not classifiable); and grade strength of the recommendation (Strong or Discretionary recommendation) (327). This systematic approach requires dedicated personnel and resources and has had led to more scientifically robust evidence evaluations for human pregnancy outcomes, reproductive effects and neurodevelopmental effects ([prhe.ucsf.edu/navigation-guide](http://prhe.ucsf.edu/navigation-guide)), although effects of select chemicals on fertility and IVF outcomes await further study.

## GETTING ALL THE STAKEHOLDERS TOGETHER AND MOVING FORWARD

In general, patients, scientists, policy makers, funders, and health care providers rarely have an opportunity to strategize together about impacts on all stakeholders. To overcome this in the emerging field of reproductive environmental health and based on the accumulating literature and increasing concerns about environmental effects on reproductive health and fertility, the Vallombrosia Workshop was convened in 2005 in Menlo Park, California. The workshop, “Understanding Environmental Contaminants and Human Fertility: Science and Strategy,” was organized by Stanford University and the Collaborative on Health and the Environment (CHE) and had 40 participants. Its goal was to build a network of scientists, policy makers, and affected communities to assess the state of the science about contributions of environmental contaminants, specifically synthetic compounds and heavy metals, to human infertility and associated health conditions, identify gaps regarding impacts of environmental contaminants on human infertility, and support and advocate for enhanced research in the field. This was the first time in the U.S. that researchers in reproductive epidemiology, biology, toxicology, and clinical medicine convened with representatives of relevant professional societies—infertility support, women’s health, and reproductive advocacy organizations—government officials, and funders to review the state of environmental health science relevant to infertility. The outcomes of this workshop included the Vallombrosia Consensus Statement on Environmental Contaminants and Human Fertility Compromise (328) and a lay monograph, “Challenged Conceptions: Environmental Chemicals and Fertility” (<https://www.healthandenvironment.org/environmental-health/health-diseases-and-disabilities/reproductive-health-research-and-resources>).

The content and format of the Vallombrosia Workshop were subsequently expanded to a multidisciplinary, summit, the “UCSF-CHE Summit on Environmental Challenges to Reproductive Health and Fertility,” in San Francisco in 2007. More than 400 participants came from around the globe. Its goals were to increase awareness and expertise among medical and public health professionals, basic and clinical scientists, policy makers, advocates, funders, and

reproductive and community health leaders about these issues and expand the network to support enhanced reproductive health research agendas, educational programs, and public health policy actions. A report on the summit, “Shaping Our Legacy,” and the scientific proceedings that covered the state of the science in male and female reproductive tract and fertility compromise with a focus on humans and published as an online supplement in *Fertility and Sterility* in February 2008, are available, respectively, at [prhe.ucsf.edu/sites/prhe.ucsf.edu/files/shapingourlegacy.pdf](http://prhe.ucsf.edu/sites/prhe.ucsf.edu/files/shapingourlegacy.pdf) and [www.fertstert.org/issue/S0015-0282\(08\)X0190-6](http://www.fertstert.org/issue/S0015-0282(08)X0190-6).

Momentum has been carried and expanded by other groups with a focus on reproduction, pregnancy, fertility outcomes, developmental origins of health and disease, and children’s environmental health. Stakeholders include some academic medical centers, the Endocrine Society, American Society for Reproductive Medicine, American College of Obstetricians and Gynaecologists, European Society for Human Reproduction and Embryology, Society for Canadian Obstetricians and Gynecologists, Royal College of Obstetricians and Gynaecologists, International Federation of Obstetricians and Gynaecologists, International Federation of Fertility Societies, American Pediatric Association, the Targeting Environmental Neuro-Development Risks Project, WHO/UNEP, EDC Gordon Research Conference, and others. These groups have issued evidence-based scientific statements and conducted special scientific symposia and postgraduate courses, and are working globally with other stakeholders to establish patient information access, incorporate environmental health into reproductive and developmental science and medicine research and education, and advocate for policy changes regarding protecting the environment and human health. Some medical schools, e.g., UCSF, are including curriculum about environmental reproductive health to train future physicians. Remarkably, even with all these efforts, most physicians are not aware of data supporting adverse health effects of the environment on reproductive health and fertility and therefore are hesitant to discuss these issues with patients ([www.ncbi.nlm.nih.gov/pmc/articles/PMC4070906/](http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4070906/)). Globally, minimizing environmental threats to human health and reproduction is part of the WHO Sustainable Development Goals 2015-2030 (<https://una-gp.org/the-sustainable-development-goals-2015-2030>), although political, economic, and social challenges remain. It is hoped that stakeholders will continue to collaborate and move the agenda forward, because there is much at stake for reproductive health and fertility for this and future generations.

## OBESITY AND REPRODUCTION

Robert J. Norman, M.D., C.R.E.I.

### Early studies on weight and reproduction

The original description of what is now known as the polycystic ovary syndrome (PCOS) noted the correlation of obesity and anovulation with infertility. Much later, classic studies confirmed these findings in very much larger groups of

women, few of whom had PCOS. Mitchell and Rogers (329) noted that obesity was four times higher in women with menstrual disturbances than in women who had regular cycles. Hartz et al. (330) questioned over 26,000 women and noted that grossly obese women had a three times higher rate of menstrual disturbance than those in the normal weight range. They also noted that teenage obesity was more closely related to menstrual irregularities later in life. In 1958, Lake et al. (331) studied 5,800 children and noted very strong correlations between childhood obesity and risks of menstrual abnormalities in adult life. All these studies were subject to confirmation by many other investigators, and the concept that obesity and being overweight impacts menstrual dysfunction became well established.

The Nurses' Health Study showed a very strong correlation between body mass index (BMI) and infertility, which was partially but not completely explained by abnormal menstrual periods (332). Many of the participants who desired pregnancy and were obese were shown to be anovulatory and required fertility treatment with an emerging group of fertility drugs, including clomiphene citrate and gonadotropins (333). In turn, the increasing success and availability of in vitro fertilization (IVF) and assisted reproduction (ART) procedures meant that more overweight and obese women were entering treatment programs.

### Trends in weight in women of reproductive age

In past centuries, infertility and anovulation were probably linked to being underweight, with a hypothalamic origin leading to delayed puberty and subsequent abnormal periods. In the past few decades, particularly in the West, the problem of obesity has become extremely consequential (334) and now has a great impact on fertility management in general and assisted reproduction in particular. This increase is not confined to developing countries; it is a worldwide problem with many other health effects, including diabetes mellitus and hypertension, that overlap with reproduction.

### Metabolic consequences

Overweight and obese individuals have a much higher prevalence of insulin resistance, glucose intolerance, hypertension, and hyperlipidemia contributing to the metabolic syndrome. This is particularly pronounced in PCOS. This may contribute to the lack of ovulation or the failure to become pregnant in a woman who has regular periods. It has been shown that the prevalence of diabetes mellitus is at least four times higher in patients with PCOS, even those with relatively mild weight problems (335). The effects may vary depending on the distribution of fat—central or peripheral—as shown by studies from the Netherlands on the use of donor sperm in women of the same average BMI but different waist and hip circumferences. This may be related to well-recognized insulin and glucose homeostasis differences in these women.

The discovery of leptin in rodents raised the prospect that high leptin levels in obese women might lead to anovulation, but the role of leptin in infertility in humans is not as clear as it is in other species. However, there are many other adipocy-

tokines that may play an important role in modulating the reproductive axis.

### Treatment consequences of obesity in women

Fertility drug distribution is hampered in overweight people, who require higher doses of oral agents or gonadotropins. Different oral ovulation induction drugs may be required, depending on BMI, and the beneficial effects of one agent may be preferred over another. With procedures such as egg recovery in overweight women there also is a higher likelihood of anesthetic problems, difficulty in egg recovery, and postoperative complications such as thromboembolic problems. Once a pregnancy commences, a higher BMI may be correlated with a greater rate of miscarriage, regardless of whether a euploid embryo has been replaced. Gestational diabetes, pregnancy-induced hypertension, congenital abnormalities, difficulties during delivery, and puerperal complications also may compromise outcomes. Many large studies have examined the effect of obesity on pregnancy outcomes as well as the long-term consequences for the offspring. The problems of large babies and exposure to an abnormal intrauterine environment are thought to be potentially consequential for the children of obese mothers (336).

### Obesity and male fertility

An emerging literature has linked poor endocrine and semen analysis parameters to obesity. An obese male, regardless of the weight of his partner, may be more prone to producing lower fertilization, poor-quality embryos, and a lower pregnancy rate in an in vitro fertilization (IVF) program (337). As the partners of many overweight men are overweight women, the fertility effects are compounded. Recent data from rodents suggest that there are epigenetic transmission mechanisms wherein male obesity may influence offspring for several generations. There are no data as yet for humans, but there is sufficient concern to recommend that male partners should be advised about the impact of obesity on infertility and poor outcomes.

### Implications for IVF procedures

As many more men and women are seeking fertility treatment through IVF, it has become apparent that pregnancy rates are probably lower when BMI is higher. Several meta-analyses have indicated that especially high BMIs are associated with significantly lower rates of ongoing pregnancies and greater fetal losses (338). At least two mechanisms appear to influence these outcomes. First, the quality of the egg is affected by exposure to metabolic derangements in the mother, including high levels of insulin, glucose, and lipids. Studies have shown a much higher lipid level in oocytes from female rodents given a high-fat diet and the presence of endoplasmic reticulum stress leading to derangements of mitochondrial function, increased apoptosis, and lower fertilization (339). Some of these effects can be eliminated through change of diet, exercise, or effective drugs that alter endoplasmic reticulum stress.

Second, in a less well-understood mechanism, endometrial dysfunction appears to manifest even when a normal embryo that developed well in the laboratory is transferred

to an obese recipient. This is also reflected by lower pregnancy rates in donor egg recipients who have a high BMI, although with less effect if only the donor is overweight. And, as noted earlier, there are substantially increased risks with the procedure of IVF and the subsequent pregnancy.

### Lifestyle management

Initial studies from Adelaide and London (340, 341) suggested that weight loss of about 5% before fertility interventions can lead to far better reproductive outcomes, regardless of whether the women undergoes treatment. These relatively small, uncontrolled studies need larger, randomized, controlled trials to confirm their findings. In women with polycystic ovary syndrome several studies have shown the benefit of lifestyle intervention, but there is less evidence from randomized controlled trials for patients with general infertility who are overweight women undergoing medical fertility treatment. Because many patients drop out from lifestyle studies and others demand more-rapid medical intervention, none of these studies can be shown to be definitive at present. Traditional weight-loss drugs have also generally been shown to be ineffective.

As noted previously, the presence of an overweight male partner may complicate studies. Our group has taken the approach of offering lifestyle programs to couples rather than only to the woman. This intervention allows both the male and female partners to obtain metabolic benefits, and it is much easier to arrange within a household.

### The role of weight loss and insulin-reduction drugs

Because many patients present relatively late in reproductive life and do not readily accept long-term lifestyle programs, there has been some demand for weight-loss agents, bariatric surgery, and insulin-lowering drugs such as metformin. Drugs such as orlistat and appetite suppressants do not have convincing evidence in treatments, but metformin may reduce the risk of ovarian hyperstimulation syndrome and increase pregnancy rates on IVF programs.

Bariatric surgery has generally been considered to produce better fertility results, but the patient normally must wait for at least 12 months after surgery before trying to become pregnant. Surgery is probably the only truly effective option for extremely overweight and obese women to regain some metabolic normality; procedures such as gastric stapling or bypass can produce substantial weight loss. However, the surgery has significant risks in very overweight patients, and it may be expensive. Also, excessive weight loss attempts that occur very rapidly around pregnancy can have bad outcomes.

### Ethics issues in fertility treatment of obese individuals

Some health authorities, funding bodies, and clinics have instituted protocols or rules that prohibit funded fertility programs unless the female patient reduces her weight to a certain BMI, such as 32 kg/m<sup>2</sup>. Other official professional bodies have produced guidelines that indicate treatment beyond a certain BMI is inappropriate, and that it may open practitioners to legal risk if they operate outside these parameters. The epidemiological and medical logic for this approach

**TABLE 8**

#### Key points on the effect of obesity on fertility.

##### Obesity in women and men

- Obesity is related to many systematic diseases and abnormalities in hormones and reproductive function.
- Obesity can be measured clinically by body mass index (BMI), waist circumference, and waist-hip ratio. A waste-hip ratio >0.8 defines an increased risk of cardiovascular disease and a reduced cumulative pregnancy rate.

##### Obesity with PCOS

- Among women with PCOS, 30% to 50% are overweight or obese. Obesity, particularly abdominal obesity, amplifies hyperinsulinemia and the metabolic syndrome.

##### Obesity with reproduction

- Obesity is related to menstrual abnormalities; amenorrhea, oligomenorrhea, and menorrhagia are fourfold higher in obese women.
- Obesity contributes to anovulatory and ovulatory infertility via an imbalance among estrogen, androgen, and sex hormone-binding globulin.
- Hyperandrogenism and hyperleptinemia are related to anovulatory and ovulatory infertility in obese women.
- Obesity may affect sexual activity, sperm quality, and DNA fragmentation.

##### Obesity with assisted reproduction

- The adverse effects of obesity include oocyte, sperm, and endometrial components.
- Obesity may reduce the likelihood that a woman will be accepted for assisted reproductive techniques (ART) treatment, particularly when outside funding is involved rather than private.
- Obesity is associated with an impaired response to ovarian stimulation and a lower chance of a live birth after in vitro fertilization and intracytoplasmic sperm induction.

##### Obesity with obstetric care

- Being overweight or obese increases obstetric risks in a BMI-dependent manner, including the risks of diabetes mellitus, hypertension, and preeclampsia. The risk of perinatal death and congenital abnormalities is doubled with an obese mother.

##### Obesity management

###### Lifestyle intervention

- Weight loss improves menstrual regularity, ovulation, and fertility, and it should be promoted as an initial treatment option for obese women with infertility. A weight loss of only 3% to 5% is needed to restore ovulation and natural pregnancy.
- Evidence is presently lacking for the full effect of lifestyle modification on IVF outcomes.
- Lifestyle modifications are the best way to achieve and sustain weight loss. These include sensible dieting, regular exercise, cognitive behavior therapy, and a supportive group environment.

###### Pharmacologic and surgical intervention

- Pharmacologic intervention with ovulation-inducing drugs can improve pregnancy and live-birth rates among obese women with PCOS.
- Pharmacologic intervention with weight-loss inducing drugs are not generally helpful for improving IVF results.
- Surgical intervention should be reserved for when other weight-reduction measures have failed. Although surgery can be effective, it must be accompanied by dietary modifications and behavioral changes.

Forty years of IVF. Fertil Steril 2018.

is strong, but others have argued that when there is a reasonable although reduced chance of pregnancy, personal autonomy and patient choice should override any guidelines from professional and official bodies. Thus, decisions are often made on the basis of available funding and personal considerations rather than true medical evidence.

### Obesity, reproduction, and the future for fertility treatment

Forty years ago, the issue of obesity or other lifestyle-related conditions was irrelevant compared with the technical challenges and failures that beset IVF. Sorting out ovarian stimulation, egg recovery, embryo growth, and implantation were issues of far greater import. We are now looking for much smaller improvements, given our great leaps forward over the past four decades. We also are facing a much higher proportion of patients with lifestyle-related conditions, including obesity. We now understand the importance of preconception advice and care for anyone who is planning a pregnancy, whether it be natural or medically induced (342, 343).

All fertility programs now have enough evidence to provide preconception advice on weight for both partners (Table 8), and these programs should consider providing sufficient resources to allow their patients to access weight treatment interventions. Obesity is known to be associated with diabetes mellitus, hypertension, cardiovascular disease, and cancer, but its shadow on the early and established stages of reproductive life is equally damaging—to the individual, to his or her relationship with a partner, and to any pregnancy that may result. We have an obligation to provide information, intervention, and support for our patients affected by this condition.

## CONTROLLED OVARIAN STIMULATION AND MONITORING

### OVARIAN MECHANISMS UNDERLYING EVOLVING ASSISTED REPRODUCTIVE TECHNOLOGIES

Aaron J. Hsueh, Ph.D., and  
Yingpu Sun, M.D., Ph.D.

#### History of in vitro fertilization and evolving assisted reproduction technology

The original in vitro fertilization (IVF) procedure developed by Steptoe and Edwards was based on obtaining mature oocytes during natural cycles for patients with fallopian tube defects. Successful application of IVF was followed by the use of gonadotropins to stimulate multiple preovulatory follicles for treating infertile patients without tubal defects. This was followed by the development of diverse, related assisted reproduction technologies (ART). Because the evolution of ART relies on advances in ovarian physiology, this review addresses the underlying ovarian mechanisms. Oocyte vitrification and/or donation have become accepted techniques, and ovarian tissue freezing is gaining acceptance. And, although the mitochondria content of oocytes has been negatively correlated with implantation potential, there is no evidence that oocyte selection through noninvasive measurement of the mitochondria DNA load increases live-birth rate (344). We will discuss ovary-associated ART starting from preovulatory follicles back to primordial follicles.

#### Does controlled ovarian stimulation yield suboptimal oocytes?

In the human menstrual cycle, ~20 early antral follicles are found at the early follicular stage, but only one progresses to the preovulatory stage, capable of releasing one mature oocyte for fertilization. The follicle-stimulating hormone (FSH) threshold model for preovulatory follicle selection in primates (345) proposes that regression of the corpus luteum at the end

of the cycle releases the negative feedback suppression of FSH secretion, which leads to a rise in serum FSH above threshold levels. One (or occasionally more) of the maturing preantral follicles containing sufficient granulosa cells expressing FSH receptors is stimulated by the increased FSH and acquires aromatase enzyme and luteinizing hormone (LH) receptors. The acquisition of aromatase results in a rise in circulating estrogens capable of suppressing FSH secretion; this in turn prevents the maturation of less mature follicles. The FSH-stimulated induction of LH receptors, together with the enhanced secretion of local estrogens and diverse growth factors—such as insulin-like growth factor I (IGF-I) and vascular endothelial growth factor (VEGF)—increases the LH responsiveness of granulosa cells in the “selected” follicle and permits it to mature in the presence of FSH concentrations that are insufficient to stimulate other less mature follicles.

Although recruitment of a large number of preovulatory follicles after controlled ovarian stimulation (COS) could lead to the retrieval of subpar oocytes, several studies have indicated that, for younger patients, the pregnancy rates per cycle after COS are higher than in natural cycles, and the cumulative live-birth rates are comparable between COS and natural cycles. In addition to gonadotropins, paracrine factors—such as growth differentiation factor 9 (GDF9) and bone morphogenetic proteins 15 and 6 (BMP15, BMP6)—secreted by oocytes also promote follicle growth (346); follicular fluid levels of these factors are associated with increased IVF success.

However, in older patients follicles containing oocytes of high quality could secrete more paracrine factors and promote folliculogenesis for recruitment during natural cycles whereas COS could lead to retrieval of suboptimal oocytes. Indeed, COS has led to lower implantation rates as compared with the natural cycle in older patients (35–42 years of age).

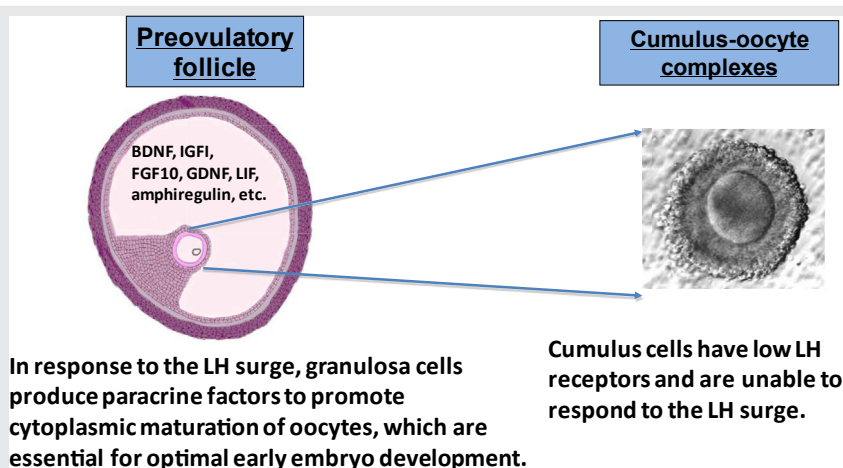
*Answer:* A qualified no.

#### Can in vitro maturation of oocytes be optimized?

Because in vitro maturation (IVM) is addressed more fully elsewhere in this supplement, we will only describe the ovarian mechanisms. Mainly a technique for avoiding



FIGURE 18



Preovulatory luteinizing (LH) surge stimulates granulosa cells to secrete local growth factors essential for oocyte development into early embryos.

Forty years of IVF. *Fertil Steril* 2018.

ovarian hyperstimulation, IVM involves aspirating germinal vesicle-stage oocytes from early antral follicles in unprimed or minimally stimulated cycles, especially from PCOS patients. These oocytes, complexed with cumulus cells, spontaneously resume meiosis to metaphase II before fertilization.

Rodent studies have indicated that granulosa cells in pre-antral and antral follicles secrete C-type natriuretic factor (CNP) to suppress germinal vesicle breakdown of oocytes. Furthermore, CNP levels decrease after granulosa cells are exposed to the midcycle LH surge (347), leading to meiosis resumption and nuclear maturation. Thus, the isolation of cumulus-oocyte complexes or denuded oocytes from antral follicles during IVM allows spontaneous germinal vesicle breakdown. The cytoplasmic maturation of oocytes essential for early embryonic development is inadequate for IVM oocytes. Indeed, animal studies have indicated that multiple granulosa cell-derived paracrine factors are stimulated by the LH surge to act on oocytes or cumulus cells in promoting early embryonic development. These include brain-derived neurotrophic factor (BDNF) (348), IGF-I, fibroblast growth factor 10 (FGF10), glial cell-derived neurotrophic factor (GDNF), leukemic inhibiting factor (LIF), and amphiregulin (Fig. 18).

Exposure of preovulatory follicles to the LH surge also is important for gene transcription and post-transcriptional modification. Rodent and human studies have indicated that cumulus cells express low levels of LH receptors as compared with mural granulosa cells, rendering the supplementation of LH/human chorionic gonadotropin (hCG) in IVM media inadequate. Although some IVM studies have used oocytes from patients after both FSH and hCG priming, it is uncertain whether the granulosa cells from treated follicles acquired adequate levels of LH receptors to respond to LH/hCG.

Because the beneficial effects of many of these paracrine factors have been extended to human early embryo cultures (349), inclusion of these factors during culture could promote

IVM success. Because most IVM involves the practice of minimizing or even avoiding exogenous gonadotropin administration to collect cumulus-enclosed immature oocytes from small antral follicles of PCOS patients, IVM of these oocytes should be optimized by adding ovarian paracrine factors, including CNP (to delay nuclear maturation) and above-mentioned paracrine factors (to promote cytoplasmic maturation).

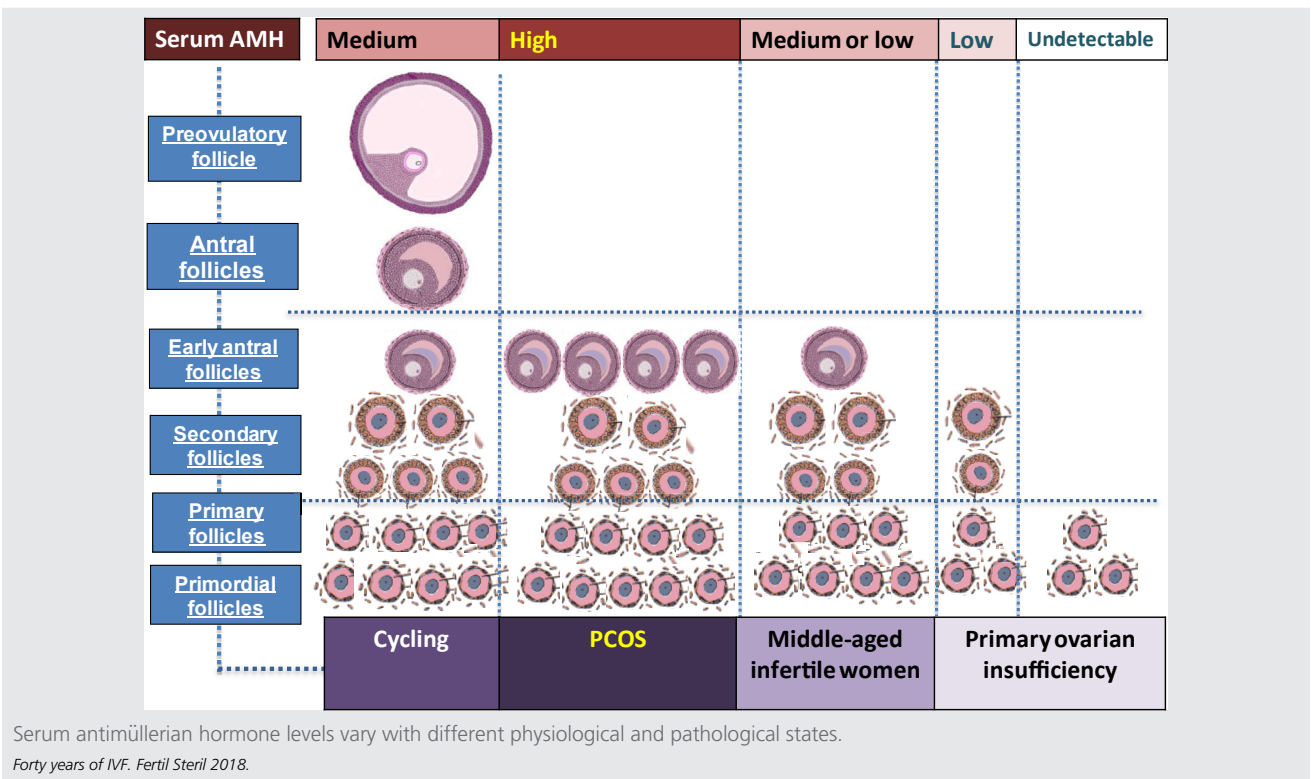
Answer: Yes.

### Can ovarian damage lead to preantral follicle activation?

Classic studies using wedge resection and laparoscopic laser drilling have demonstrated the efficacy of ovarian damage procedures for promoting early antral follicle growth in polycystic ovaries. Our group has demonstrated the important role of Hippo signaling disruption in promoting preantral follicle growth in patients with primary ovarian insufficiency (POI) who underwent the in vitro activation (IVA) procedure (350). Our POI patients at 4 to 6 years after menses cessation were treated with IVA therapy consisting of fragmentation of ovarian cortical strips into cubes (Hippo signaling disruption) followed by incubation with drugs to stimulate protein kinase B (Akt) to promote follicle growth. After grafting ovarian cubes to a “pouch” constructed in the fallopian tube, about half of our POI patients with residual follicles showed follicle growth, and oocytes were retrieved in some patients for IVF and embryo transfer, leading to successful pregnancies and delivery. Because ovarian fragmentation without Akt drug exposure also promoted follicle growth (350), further improvement of ovarian disruption procedures using in situ disruption of ovarian Hippo signaling via mechanical or chemical approaches could promote preantral follicle growth in POI patients or patients with diminishing ovarian reserve.

Answer: Yes.

FIGURE 19



DO SERUM ANTIMÜLLERIAN HORMONE LEVELS REFLECT FOLLICLE RESERVE?

This supplement deals with the topic of antimüllerian hormone (AMH) elsewhere; our discussion is limited to ovarian physiology. At any given time, there are multiple follicles at different developmental stages inside the ovary. Antimüllerian hormone is a paracrine hormone, and AMH secretion increases in primary and secondary follicles, reaching high levels in the early antral stage but decreasing in preovulatory follicles. Follicles 5–8 mm in diameter contribute ~60% of the circulating AMH; the granulosa cells of preovulatory follicles >10 mm in diameter fail to produce AMH in normal women.

Because AMH is not an endocrine hormone, the serum levels of AMH mainly reflect “leakage” from the ovary, and absolute serum levels of AMH do not necessarily reflect the number of ovarian follicles. Although detection of serum AMH indicates the presence of secondary or early antral follicles, one cannot rule out the presence of non-AMH-secreting primordial follicles (Fig. 19). Indeed, patients with undetectable AMH levels have responded to IVA treatment due to presence of residual primordial follicles (351). Thus, detection of serum AMH ensures the presence of secondary follicles, but serum AMH levels do not accurately reflect ovarian reserve.

Answer: Only partially.

IS IT POSSIBLE TO DIAGNOSE THE PRESENCE OF PREANTRAL FOLLICLES IN VIVO?

Because the prevailing transvaginal ultrasound approach does not allow imaging of preantral follicles, our group recently conjugated a small molecular near infrared-II fluorophore to FSH for imaging FSH receptors in granulosa cells of preantral follicles in live mice (352). Because FSH receptors are expressed in primary and larger follicles and the fluorophore has low cell toxicity and rapid clearance, future clinical studies could reveal whether this approach is useful for detecting preantral follicles in patients with low ovarian reserve.

Answer: Yes.

Is DHEA supplementation useful for patients with diminished ovarian reserve?

Dehydroepiandrosterone (DHEA) supplementation has been reported to improve ovarian function, increase pregnancy chances, reduce aneuploidy, and lower miscarriage rates in patients with diminished ovarian reserve (DOR) (353). However, a meta-analysis that included ~200 IVF cycles indicated there is insufficient evidence for the efficiency of DHEA supplementation in women with DOR or poor responders (354). Furthermore, no improvement in ovarian response markers, ovarian response to gonadotropin stimulation, or IVF outcomes was found in poor responders receiving DHEA pretreatment (355).

The requirement of androgens for folliculogenesis is based on studies in mice with granulosa cell-specific deletion of androgen receptors, which showed more preantral and atretic follicles together with fewer antral follicles and corpus lutea. However, gain-of-function mutations in the LH receptor gene led to male-limited precocious puberty with elevated androgen secretion, but the mothers of these patients carrying the same mutation showed normal fertility; this suggested that elevation of androgen biosynthesis by a constitutively activated LH receptor in thecal cells has minimal impact on follicle development. Because there is minimal evidence for androgen deficiency in DOR patients, the exact effects of exogenous androgens on follicle growth are difficult to investigate *in vivo*.

In hypophysectomized, estrogen-primed rats, exogenous androgens induced atresia in preantral follicles that was associated with apoptosis in granulosa cells. Furthermore, DHEA is a “prehormone” that is converted to androgens and estrogens by  $3\beta$ -hydroxysteroid dehydrogenase and aromatases, respectively. In addition to their endocrine actions on the hypothalamic-pituitary axis, both estrogens and androgens have potent paracrine actions in the ovary. Therefore, it is difficult to decipher the mechanisms underlying putative “positive” effects of exogenous DHEA on follicle development in infertile women. Oral administration of DHEA is further complicated by varying absorption rates. Due to expected variations in DHEA absorption, metabolism, and sites of actions in DOR patients, it is unlikely that DHEA supplementation could yield consistent outcomes.

*Answer: No.*

### IS IT POSSIBLE TO OBTAIN MATURE OOCYTES AFTER IN VITRO GROWTH OF HUMAN PRIMORDIAL FOLLICLES?

Using a two-step *in vitro* growth (IVG) approach, a living mouse pup was derived from an oocyte in follicles cultured from the primordial stage. The ovaries of newborn mice were first placed in organ culture for 8 days before isolating oocyte-granulosa cell complexes for 2 weeks. The mature oocytes obtained could be fertilized and the resultant embryos transferred, leading to a live birth. In humans, cortical tissue explants were cultured to allow primordial follicle growth into preantral follicles followed by dissection of isolated follicles for culture in microwells for potential IVM (356). Using another two-step follicle culture strategy, the human follicles developed from the preantral to antral stage, and they produced meiotically competent metaphase II oocytes after IVM. Similarly, secondary human follicles developed to small antral follicles and remained hormonally active in an alginate-encapsulation culture system for several weeks. Because the growth rate of the cultured follicles appears to be accelerated compared with estimates *in vivo*, it has raised the prospects of future clinical utility. However, the prolonged growth duration and large size of human preovulatory follicles represent major challenges.

*Answer: Maybe.*

### IS IT POSSIBLE TO USE OVARIAN GERM STEM CELLS OR THEIR MITOCHONDRIA FOR INFERTILITY TREATMENT?

Earlier studies showed the presence of a finite, nonreplenishable pool of follicles after birth. However, oocyte formation by mitotically active germ cells purified from human ovaries has been reported (357). A comprehensive review concluded that the “evidence strongly denies that new follicles are formed continuously after birth, but the survival of a population of stem cells of uncertain potency cannot be summarily dismissed,” and asked, “Could [ovarian germ stem cells] OSC be a sub-population of germ cells that failed to make the grade?” (358).

In addition, mitochondria from putative OSC were transferred into oocytes of low responders to “augment” oocyte functions (359) with limited improvement in clinical outcomes (360). However, the oocyte mitochondria DNA copy numbers per cell and accumulation of mitochondria DNA mutations were not found to be different between older patients with low ovarian reserve and younger ones (361). Furthermore, if OSCs are homologous with primordial germ cells or oogonia, they are expected to have only a few thousand copies of mitochondrial DNA compared with the 160,000 copies found in mature oocytes. It is also unclear how mitochondria transfer restores oocyte competence if the aneuploidy is preexisting at metaphase II in oocytes from aging patients. It is thus unlikely that mitochondria transfer could boost the embryonic developmental potential of low-quality oocytes obtained from subfertile patients.

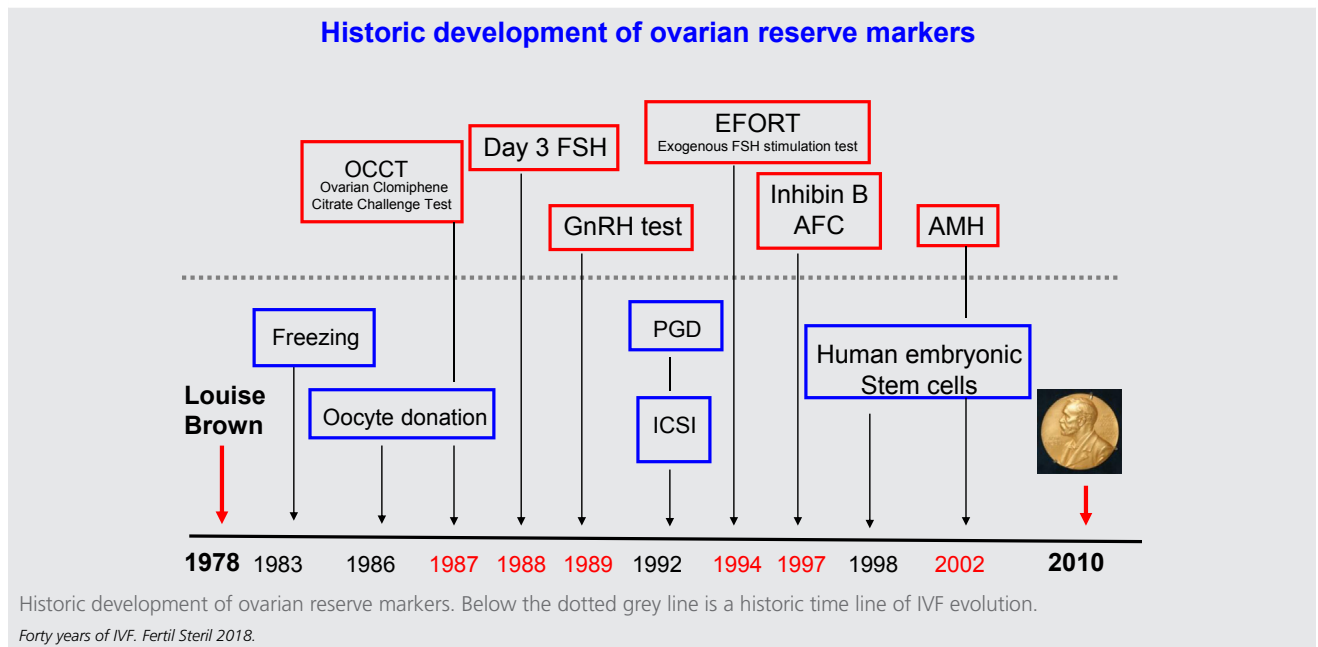
*Answer: No.*

### MARKERS OF OVARIAN FUNCTION FROM FOLLICLE-STIMULATING HORMONE TO ANTIMÜLLERIAN HORMONE

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The birth of Louise Brown in 1978 was achieved in a natural cycle; after this breakthrough, it was clear that to achieve an acceptable pregnancy rate, more than one embryo should be transferred into the uterus and ovulation induction should be used. In 1980, Lopata and colleagues from Monash University in Australia described the first *in vitro* fertilization (IVF) birth in Australia employing clomiphene citrate (CC) followed later by CC with human menopausal gonadotropin (hMG), which improved pregnancy rates to 6.3% and 18%, respectively. Jones, heading the Norfolk group, achieved the first IVF baby in the United States in 1982 by employing a low-dose hMG regimen. Also in 1982 as well, we used a high-dose hMG protocol for ovulation induction. With this relatively aggressive treatment, two opposing phenomena were noted in young patients: an unexpected reduced or absent

FIGURE 20



ovarian response in 8% of patients and mild ovarian hyperstimulation syndrome (OHSS) in 1.5% (362). In view of similar observations throughout the first decade of IVF, it became imperative to develop ovarian reserve tests (ORT) that would effectively screen patients and help to identify those with a potentially decreased ovarian response to gonadotropin stimulation and those who were at the greatest risk of developing ovarian overstimulation (363).

Two types of ORT were developed over time: static and dynamic. The dynamic tests are evaluated elsewhere (364), and are beyond the scope of this review. The static tests assess specific parameters at a single point in time, and dynamic tests assess ovarian response to exogenous stimulation. Figure 20 depicts the time line of these tests. The first static ORT was the CC challenge test, introduced by Navot and colleagues in 1987. After that were the day-3 follicle-stimulating hormone (FSH) test developed by Muasher and colleagues in 1988, the gonadotropin-releasing hormone (GnRH) agonist test by Padilla and colleagues in 1990, the exogenous FSH ovarian reserve test (EFORT) by Fanchin and colleagues in 1994, inhibin B measurement by Seifer and colleagues in 1997, antral follicle count (AFC) by Tomas and colleagues in 1997, and the antimüllerian hormone (AMH) test by Van Rooij in 2002. This review summarizes the development history of the three markers used most frequently in the clinical practice of IVF: basal FSH, AFC, and AMH levels.

### Early follicular follicle-stimulating hormone levels

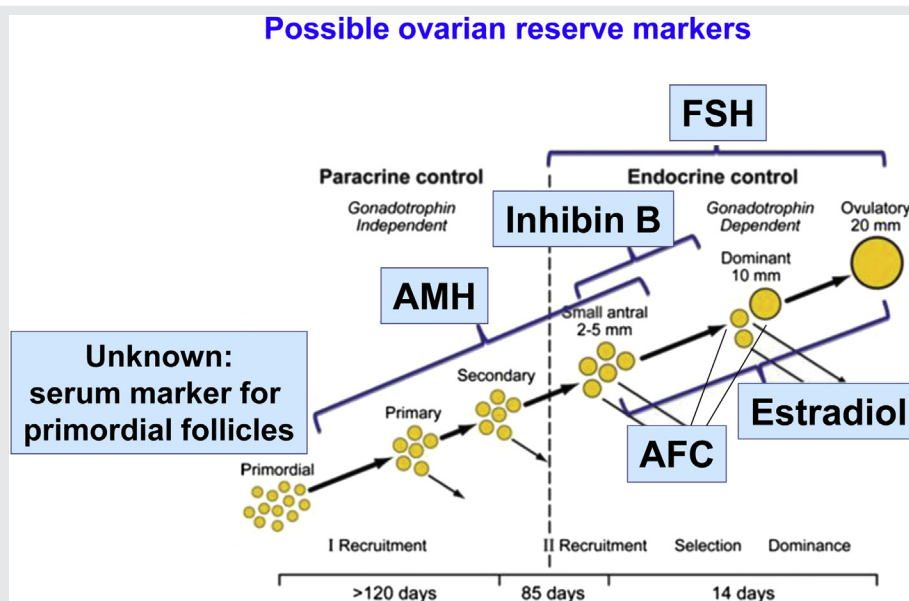
The decline in the follicular pool leads to a decrease in inhibin B production, which in turn leads to elevated early follicular phase FSH levels and a shortened follicular phase. Accord-

ingly, early follicular phase serum FSH levels are inversely correlated with the number of follicles in the ovary as determined histologically. The use of early follicular basal FSH as a marker for ovarian reserve and a tool in assessing ovarian response to controlled ovarian stimulation (COS) was first suggested by the Norfolk group in the late 1980s and early 1990s. This group demonstrated that higher day-3 FSH levels (but not day-3 LH levels) positively correlated with the age of patients and that the estradiol ( $E_2$ ) response to stimulation and the number of oocytes correlated with low levels of FSH and high levels of LH, as did conception rate per transfer (365). The same group showed that high baseline FSH levels ( $>25$  mIU/mL) were associated with a low ongoing pregnancy rate (3.6%) compared with patients with moderate levels (15–24.9 mIU/mL) (9.3%) and patients with low FSH levels ( $<15$  mIU/mL) (17%). On the basis of these results it was suggested that basal FSH levels are predictive of pregnancy outcome and stimulation characteristics, and may be used to counsel patients. This group later found that basal FSH testing was limited by intercycle variability, which weakened its reliability. These variations in basal FSH values were found to be wide, did not predict changes in ovarian response to gonadotropin stimulation, and could not be used to select an optimal cycle in which to stimulate an individual patient. In addition, patients with large intercycle variations responded poorly to gonadotropin stimulation independent of their basal FSH concentration.

Because reduction in ovarian reserve and the ensuing FSH increase in the first days of the follicular phase stimulate  $E_2$  production, it was hypothesized that combining  $E_2$  and FSH would enhance the sensitivity and specificity of the markers. In a study conducted at Weill Cornell Medicine in New York



FIGURE 21



Potential markers of ovarian reserve and their ovarian site of evaluation.

Forty years of IVF. *Fertil Steril* 2018.

from 1989 through March 1992 it was demonstrated that the simultaneous evaluation of FSH and  $E_2$  levels appeared to predict pregnancy outcome better than either of these hormone levels used alone (366).

Contrary to the results obtained from the initial retrospective analyses, the predictive value of basal FSH was shown by numerous additional retrospective and prospective studies to be considerably weaker (367). From this large body of data it may be concluded that basal FSH may serve as a predictor of decreased ovarian response, and that this probability and failure to conceive increase substantially only in women with very high threshold levels. This fact, combined with a ~5% false-positive rate, precludes the use of basal FSH as a diagnostic test to exclude patients from treatment; rather, it only functions as a screening test for further investigation. An abnormally high basal FSH value has a high predictive value for decreased ovarian response but a normal value has a low negative predictive value for poor response. A single abnormal result in women younger than 40 years of age may not predict poor response or failure to conceive. In addition, basal FSH has no value in predicting OHSS. The marked shortcomings of basal FSH level as a predictive marker have been a driving force behind the search for and development of new markers of ovarian reserve, which began in the second and third decades of IVF history.

### Antral follicle count

The evolution of assisted reproduction technology and ultrasound (US) imaging are tightly connected as the latter provides a reliable platform to visualize reproductive organs

noninvasively. Hackeloer (368) was the first to describe the progressive growth of the maturing follicle. In a pioneering report as early as 1982, the Norfolk group described the routine daily use of a real-time abdominal probe for evaluating follicular growth. Soon, US monitoring rather than serum  $E_2$  level was emphasized as the more definitive clinical tool for assessing follicular development (369). The same abdominal US served as a guide for transvesical percutaneous needle aspiration of oocytes, replacing the original laparoscopic technique of egg retrieval (370). After the development of transvaginal high-resolution US probes, they were readily adopted for follicular monitoring and oocyte pickup. Consequently, by 1990 the laparoscopic approach for oocyte recovery had been largely replaced by US-guided techniques.

Consideration of ovarian morphometrics first began for women with Stein-Leventhal syndrome. Enlarged ovaries with multiple small peripheral follicles of 5–8 mm were considered the classic US picture of the polycystic ovary. Later, as US measurements of the ovaries became quick, accurate, and cost effective, ovarian volume became the first significant predictor of ovarian reserve and response to superovulation (371).

The ovary contains three distinct populations of developing follicles: primordial follicles, early growing follicles, and antral follicles (Fig. 21). A small proportion of early growing follicles develops into antral follicles larger than 2 mm. These are highly responsive to FSH and can be readily visualized using transvaginal US.

Early in the evolution of AFC as a marker of ovarian reserve, it was shown to have the closest association with the chronologic age of normal women with proven fertility

and could detect age-related decreases in follicle counts (372). The model of AFC declining with age in a biphasic pattern corroborated the decline of primordial follicles observed in autopsy studies. Following this initial report, Tomas et al. (373) and Chang et al. (374) introduced AFC by vaginal US, employing a measurement of 2–5 mm. They showed it to be a promising tool for evaluation of ovarian responsiveness to gonadotropin stimulation in IVF. Subsequently, most reports on AFC employed a 2–10 mm range without consistent decline in its predictive performance. Early comparative prospective studies demonstrated the superiority of AFC over ovarian volume and endocrine biomarkers to predict poor ovarian response as well as hyper-response in IVF programs (373).

When the intercycle variation of AFC was examined, cycle-to-cycle measurements revealed only moderate agreement in any range of counts. The difference in AFC between cycles was explained by the variable size of the growing follicle cohort among separate cycles. In addition, it was demonstrated that AFC tended to overestimate the number of FSH-responsive follicles and collected eggs because it invariably also measured atretic follicles of the same size (375).

Greater intercycle variability in AFC was detected in overweight and obese women, limiting its predictive value in this growing subpopulation (367). In two meta-analyses and a systematic review of AFC, the estimated receiver operating characteristic (ROC) curves showed AFC to perform well (area under the curve [AUC] 0.76; specificity 73% to 97%) in the prediction of poor ovarian response (<3–4 oocytes or cycle cancelation), and to be more accurate than basal FSH and ovarian volume. Yet the prediction of failure to conceive was poor in meta-analyses for AFC, basal FSH, and ovarian volume. Furthermore, AFC was shown to be predictive of ovarian response to gonadotropins and oocytes retrieved, but it did not predict implantation rate, pregnancy rate, or live-birth rate.

In 2010, a group of experts led by Broekmans (375) published simple, practical recommendations for standardizing the assessment of AFC. The group focused on establishing the basic clinical and technical requirements for its evaluation. The clinical setting included selection of patients with regular menstrual cycles and with no coexisting pathologic condition that could technically affect the counting of follicles; counting of follicles between days 2 and 4 of a spontaneous menstrual cycle or an oral contraceptive cycle to avoid the effect of intracycle variation; and inclusion of all antral follicles of 2–10 mm in diameter.

Presently, AFC is an easy-to-perform, noninvasive approach that immediately provides essential predictive information on ovarian responsiveness. With acceptable intercycle and interoperator reliability, AFC has become an established biomarker of ovarian reserve. It is a readily available bedside tool and is the first choice in patient assessment before IVF, as well as an excellent tool in predicting ovarian hyper-response. Very low AFC is not predictive of failure to conceive.

## Antimüllerian hormone

Two transforming growth factor- $\beta$  superfamily members, inhibin-B and AMH, are secreted exclusively from ovarian

granulosa cells in response to FSH stimulation: AMH by primary and secondary preantral (but not primordial) and small antral follicles (up to 5–6 mm), and inhibin-B primarily by more differentiated preantral and small and medium sized antral follicles up to 12–14 mm (see Fig. 21).

Women with decreased ovarian response have fewer preantral and small antral follicles, and thus reduced inhibin-B secretion. In 1997 Seifer and colleagues showed that women with low early follicular serum inhibin-B concentrations had poor ovarian response and were less likely to conceive after IVF treatment (364). However, inhibin-B levels exhibit high intercycle variability; many studies have shown that it is not a reliable marker of ovarian response or of pregnancy success through IVF.

A well-known stimulator of müllerian duct regression in the male fetus, AMH was first found in follicular fluid by Seifer et al. (376) in 1993. It inhibits the transition from primordial to primary follicles and decreases FSH-induced follicle selection and aromatase activity. In 2002, van Rooij et al. (377) first suggested AMH could serve as a marker of ovarian reserve. Its serum levels positively correlate with histologically determined primordial follicle number and negatively correlate with chronologic age (378). Additionally, AMH plays an important role in the pathogenesis of PCOS. Because AMH is secreted from the earliest differentiated follicles, its measurement is closest to the primordial follicle phase, thus providing the most direct assessment of ovarian reserve (see Fig. 21). Furthermore, AMH secretion is FSH-independent, remains relatively consistent within and between menstrual cycles, and can be measured throughout the menstrual cycle. Some external factors—such as oral contraception, GnRH analogues, smoking, or obesity—may temporarily decrease serum AMH levels (378, 379).

Early studies found that AMH levels were associated with ovarian response, embryo quality, and pregnancy outcomes in IVF. Later meta-analyses of individual patient data, of 5,705 and 4,786 women from 28 studies and 32 databases, respectively, demonstrated that AMH was an excellent predictor of poor ovarian response as well as excessive response (380, 381). AMH or AFC clearly add to patient age in predicting poor response (AUC 0.78 and 0.76, respectively). Predicting excessive response using a model with AFC and AMH, with or without patient age, had an AUC of 0.85. Although both tests appear to have clinical value, four prospective multicenter trials consistently showed AMH to be better at predicting oocyte number as well as poor response and over-response as compared with AFC (382). The AMH cutoffs used for determining sensitivity and specificity are between 0.1 and 1.66 ng/ml for poor response, and 3.4 and 5.0 ng/ml for hyperstimulation. According to meta-analyses, AMH cannot accurately predict pregnancy (378). The consistency of the results supports the notion that AMH represents oocyte quantity but not quality, and pregnancy rates depend on many other factors.

The findings from these studies opened the way to explore the potential of individualized FSH dose regimens based on ORT. In a recent multicenter randomized controlled trial, Nyboe et al. (383) showed that optimizing ovarian response in IVF by individualized dosing according to pretreatment

patient characteristics (serum AMH and body weight) improved safety and resulted in similar efficacy compared with conventional ovarian stimulation.

The main limitation of AMH is its assay methods (378, 382). Initially AMH was assayed by two different enzyme-linked immunosorbent assays (ELISA) kits and later by second-generation ELISAs. Due to dissimilarity in the antibodies and assay sensitivities, in addition to interlaboratory variations, a considerable difference was found between the different assays, particularly for low AMH values, complicating interpretation of the AMH values and their clinical implications. The highly sensitive, fully automated AMH assays that have been available since 2014 have replaced the older ELISA assays, thereby both providing faster results and improving interobserver reliability (382). However, no reliable converting factor has been identified, and no international assay standardizations have been agreed upon. Therefore, the cut points developed and reported for one commercial AMH assay are not generalizable to others.

At present, AMH is considered the earliest, most sensitive ORT. Objectivity and the convenience of testing at any time throughout the menstrual cycle make AMH concentration the gold standard ovarian reserve biomarker and the most reliable predictor of ovarian response in IVF. Development of an international standard of AMH level is necessary for future clinical use.

## Conclusion

Ovarian reserve tests have three main goals: counseling IVF patients based on ovarian response prediction and the probability of live birth; employing predicted ovarian response to optimize ovarian stimulation and minimize safety risks; and assessing current and future fertility potential to allow women to decide when and how to proceed with family planning, fertility treatment, or fertility preservation. Both AFC and AMH clearly have an added value together with female age and basal FSH for predicting poor (380) and excessive (381) ovarian response in IVF. Antimüllerian hormone, recognized as the best biomarker of ovarian reserve, has the advantage of being cycle-stage independent.

Because ORTs reflect oocyte quantity but not quality, no ORT can accurately predict the probability of pregnancy. Young women with decreased ovarian response have a small number of high-quality oocytes but reasonable pregnancy rates. Thus, it is important to emphasize that an ORT should not be the sole criterion used to deny patients access to fertility treatments (384). Evidence of decreased ovarian response in all age groups does not necessarily correlate with an inability to conceive. Whether ORT can predict the specific stages of ovarian aging and timing of menopause is still a matter of debate.

We may be approaching a time when the value of these markers should be reevaluated. The shift from long GnRH-agonist to short GnRH-antagonist protocols combined with GnRH-agonist trigger and a freeze-all policy have diminished the importance of using markers to predict ovarian response and prevent OHSS.

## THE DEVELOPMENT AND EVOLUTION OF GONADOTROPINS IN ASSISTED REPRODUCTION TECHNOLOGY

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### DISCOVERY OF GONADOTROPINS

The link between the gonads and the pituitary was established in the 1920s through animal experiments that involved removal of the hypophysis leading to gonadal atrophy, which in turn could be reversed with pituitary implants (385). The link was further demonstrated by implantation of anterior pituitary glands in immature animals, which resulted in precocious sexual maturation (385). In 1929, Zondek proposed the existence of two different products secreted by the pituitary gland, which he named prolan A and prolan B, both of which stimulated the gonads (386, 387). He proposed that follicular growth was stimulated by prolan A and that “folliculin” secretion was stimulated by both prolan A and prolan B (386, 387). Furthermore, prolan B was proposed to induce ovulation, corpus luteum formation, and lutein and folliculin secretion. These two hormones were described as gonadotropins (reflecting their effect on the gonads), and were subsequently renamed according to their specific actions: prolan A was called follicle-stimulating hormone (FSH), and prolan B was called luteinizing hormone (LH).

### HUMAN CHORIONIC GONADOTROPIN: DISCOVERY AND PLACENTAL/URINARY EXTRACTION

In 1927, Ascheim and Zondek demonstrated that the blood and urine of pregnant women contained a gonad-stimulating substance, which was believed to have originated in the pituitary (385). This discovery led to the Ascheim-Zondek pregnancy test, in which a woman's urine was injected into an immature female mouse; if the woman was pregnant, the ovaries would enlarge (two to three times normal size) with red dots visible (due to hemorrhage into the follicles), or luteinization would occur and corpora lutea would be visible despite the mouse's immaturity (385). Studying in vitro cultures of the chorionic villi, Seegar Jones and colleagues subsequently concluded that the placenta rather than the pituitary was responsible for the secretion of the hormone (386). Consequently, the material was named human chorionic gonadotropin (hCG).

In 1931, Organon released an hCG extract for use in humans (originally named Pregnon, and renamed Pregnyl in 1932) (386). However, reproducibility with this product was limited, partly due to the use of animal units to measure its bioactivity (385). One “rat” unit was defined as the

amount of the hCG preparation required to produce vaginal opening and estrus when injected into immature female rats (385). The international standard for hCG, the first for a gonadotropin, was introduced in 1939 by the League of Nations (385), wherein 1 IU of hCG was defined as the activity contained in 0.1 mg of the reference hCG preparation (386). Subsequently, in 1940 purified urinary preparations of hCG became available, with a bioactivity up to 8,500 IU/mL, extracted from urine collected during the first half of pregnancy in women (385). During infertility treatment hCG is used to induce final follicular maturation and ovulation as well as to support the luteal phase; in addition, hCG can be used to treat male hypogonadism because it stimulates the Leydig cells to produce testosterone.

## HUMAN CLINICAL USE OF ANIMAL GONADOTROPIN EXTRACTS

The studies demonstrating the physiologic action of the gonadotropins suggested that gonadotropin extracts may be of use for the treatment of infertile patients with gonadotropin insufficiency (385). The first extract, which became available in 1930, was derived from swine pituitaries. Later, preparations from hog and sheep pituitaries and from the serum of pregnant mares—pregnant mare serum gonadotropin (PMSG)—became available. These extracts were effective in women for increasing urinary estrogen secretion, resulting in enlarged cystic ovaries (386). This led in 1941 to the introduction of the “two-step protocol” by Mazer and Ravetz that involved ovarian stimulation using animal gonadotropins (PMSG, or hog or sheep pituitary gonadotropin) to stimulate follicular growth and development, followed by the induction of ovulation using hCG (385, 386, 388).

However, it was observed that the ovarian response was maintained for only a short period of time, gradually weakening before disappearing altogether (385). In 1942, monographs by Ostergaard, and Zondek and Sulman claimed that this decreased responsiveness was due to the production of “anti-hormones” (antibodies) to animal gonadotropins (385). In 1948, Leatham and Rakoff asserted that antihormone production rendered nonprimate gonadotropins of limited clinical use (385). The development of antihormones was confirmed by Maddock in 1956, who detected them between days 44 and 76 after prolonged treatment with animal FSH preparations (386).

## HUMAN PITUITARY GONADOTROPIN

Because nonprimate gonadotropins were of limited clinical use, Gemzell originally extracted gonadotropins from cadaveric human pituitaries in 1958; in the same year, reports of successful follicle stimulation after administration of an hCG preparation to induce ovulation induction were released (386). In 1963, Bettendorf demonstrated that ovarian stimulation with human pituitary gonadotropin (hPG) was possible in hypophysectomized individuals, and between 1958 and 1988 hPG preparations were successfully used for ovulation induction (386). However, the production of hPG required human pituitaries from

cadavers, and their limited supply could not cover the growing demand for gonadotropin preparations (386). In addition, more than 20 years after its introduction, fatal cases of iatrogenic Creutzfeldt-Jakob disease were linked to the use of hPG and human pituitary growth hormone in the United Kingdom, France, and Australia (386), where all the affected individuals had received products produced by government agencies; hPG was subsequently withdrawn from the market (386).

## HUMAN MENOPAUSAL GONADOTROPIN

In 1954, Borth et al. (389) discovered that postmenopausal urine contained increased FSH and LH that could be extracted, although the extracts needed to be lyophilized for storage to prevent oxidation. Injecting this product into female hypophysectomized infantile rats resulted in a linear increase in ovarian and uterine weights. Injection into male hypophysectomized infantile rats induced stimulation of Leydig cells to produce testosterone, resulting in a linear increase in ventral prostate weight (385, 386). Subsequently the therapeutic use of human menopausal gonadotropin (hMG) in humans was suggested in 1957 (385, 386).

The first hMG preparation (Pergonal 25) was registered for clinical use by Serono in Italy in 1950, but no clinical results were reported in the medical literature. Later preparations benefited from improvements in purification technology, and the proportions of LH and FSH were standardized (Pergonal 75 contained 75 IU FSH and 75 IU LH, measured using *in vivo* assays). However, these products still contained other unwanted urinary proteins (390). Moreover, a large volume of source material was required in the production process (about 3.5 L of urine were required to produce one ampule of Pergonal 75 (Serono internal data).

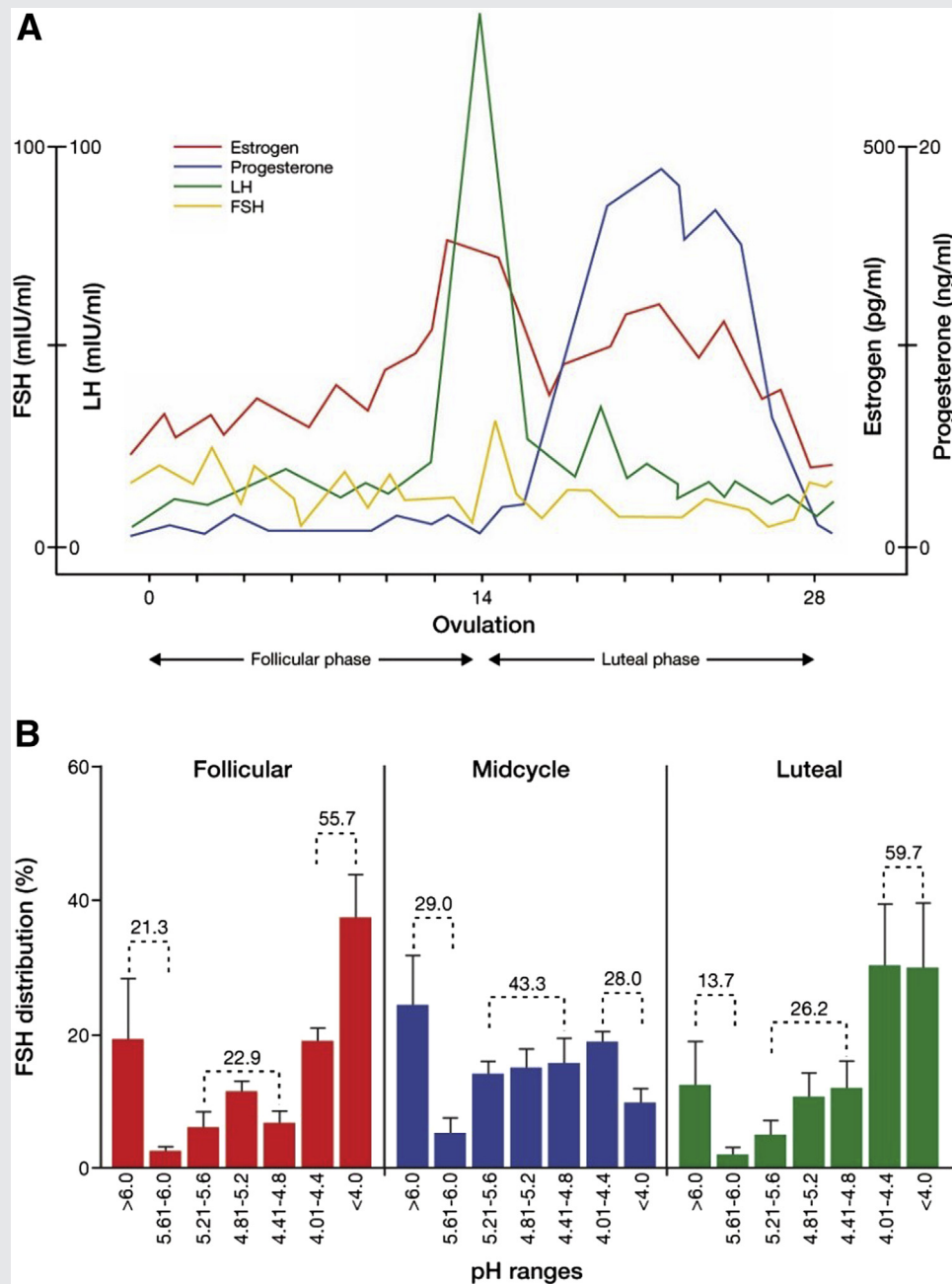
At the time the first hMG preparations were registered, “animal” units (mouse or rat) were used to define their bioactivity (386). One “rat” unit was the amount of the preparation required to induce estrus in 28-day-old prepubertal female rats, and this unit varied according to the strain of rat used. Mouse units were defined in a similar manner (386). However, as with hCG, the lack of a defined international unit meant that clinical trials could not be started.

In the 1950s, batches of menopausal urine kaolin extract (hMG 20, hMG 20a, and hMG 24) were provided by Organon Newhouse for use as reference products to standardize preparations and assay methods (386). The availability of a reference product also enabled the study of the day-to-day variation of gonadotropins and steroid secretion during a normal menstrual cycle with the aim of learning to imitate the pattern for use in future clinical trials (Fig. 22) (385, 386).

By 1959, most of the reference product had been used up, and further batches from the same source could not be provided (386). Therefore, in 1959, Pietro Donini from Serono donated 50 g of Pergonal 23 to be used as the reference product (386). Each ampule of this reference preparation contained 5 mg of hMG, equivalent to 40 IU of FSH and 40 IU of LH



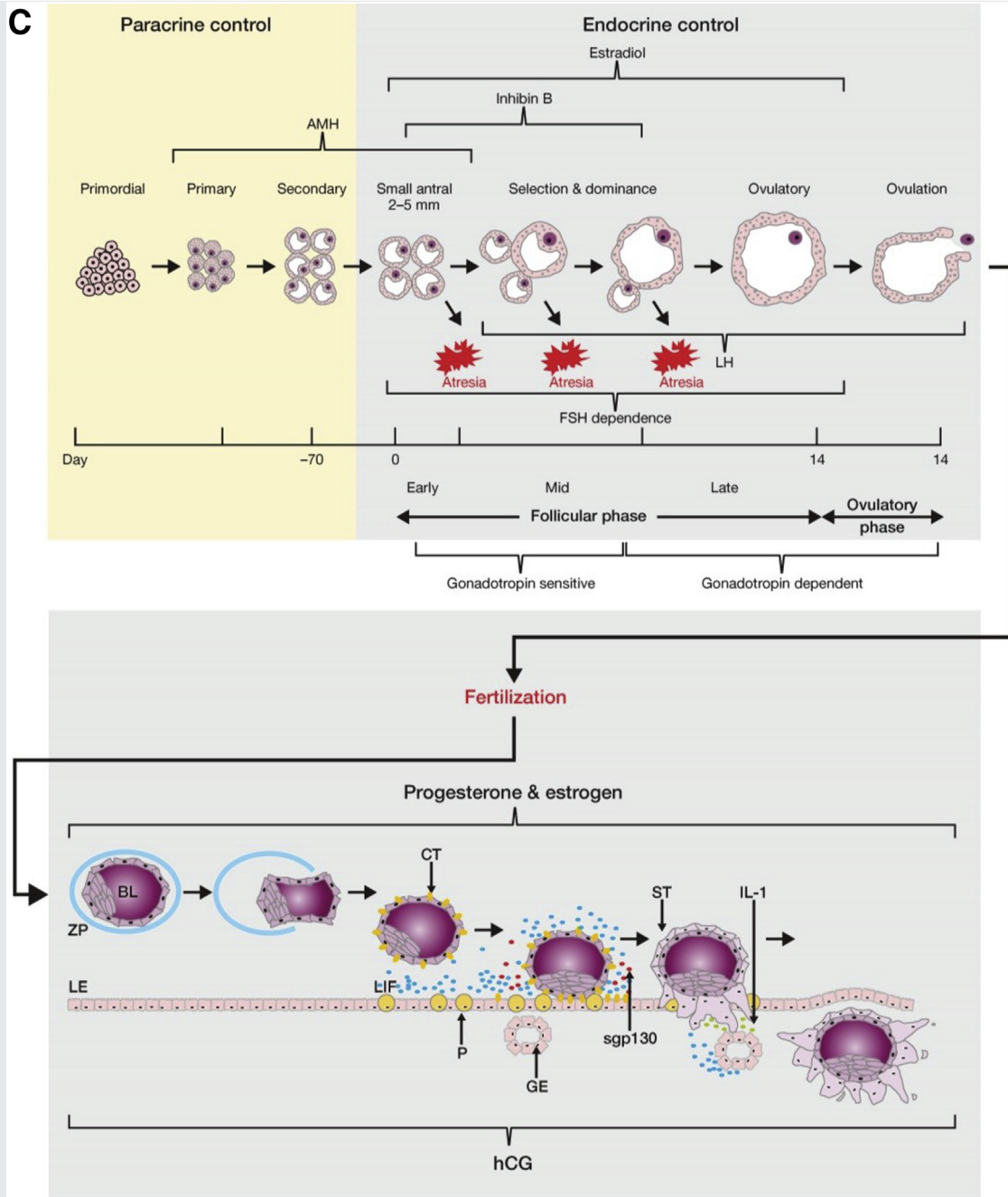
FIGURE 22



Schematic figures showing the (A) levels of pituitary and ovarian hormones during the menstrual cycle (394), (B) follicle-stimulating hormone (FSH) isoform secretion during the menstrual cycle (404), (C) ovulation and oocyte fertilization up to implantation, and (D) hormone pattern during follicular phase, ovulation, luteal phase, and pregnancy after stimulation with human menopausal gonadotropin (hMG) of a primary amenorrheic patient with hypopituitary hypogonadism which led to the first pregnancy using hMG for ovulation stimulation. (\*The starting dose was 240 mg of IRP-hMG [corresponding to 150 IU], and this was increased to 360 mg [225 IU], then to 480 mg IRP-hMG [300 IU], before being gradually reduced to 360 mg [225 IU] and 240 mg [150 IU] IRP-hMG. Ovulation was induced with 10,000 IU of hCG followed by 10,000 and 5,000 IU of hCG on consecutive days.) AMH, antimüllerian hormone; BL, blastocyst; CT, cytotrophoblast; FSH, follicle-stimulating hormone; GE, glandular epithelium; h, hours; hCG, human chorionic gonadotropin; IL-1, interleukin-1; IU, international units; LH, luteinizing hormone; LIF, leukocyte inhibitory factor; P, pinopode; sgf130, soluble gp 130; ST, syncytiotrophoblast; Temp, temperature; ZP, zonapellucida. [1C: Includes data from <https://www.ncbi.nlm.nih.gov/books/NBK29/#A1223>. 1D: Adapted from B. Lunenfeld, Treatment of anovulation by human gonadotrophins, J Int Fedn Gynecol Obstet 1963;1:153.]

Forty years of IVF. Fertil Steril 2018.

FIGURE 22 Continued

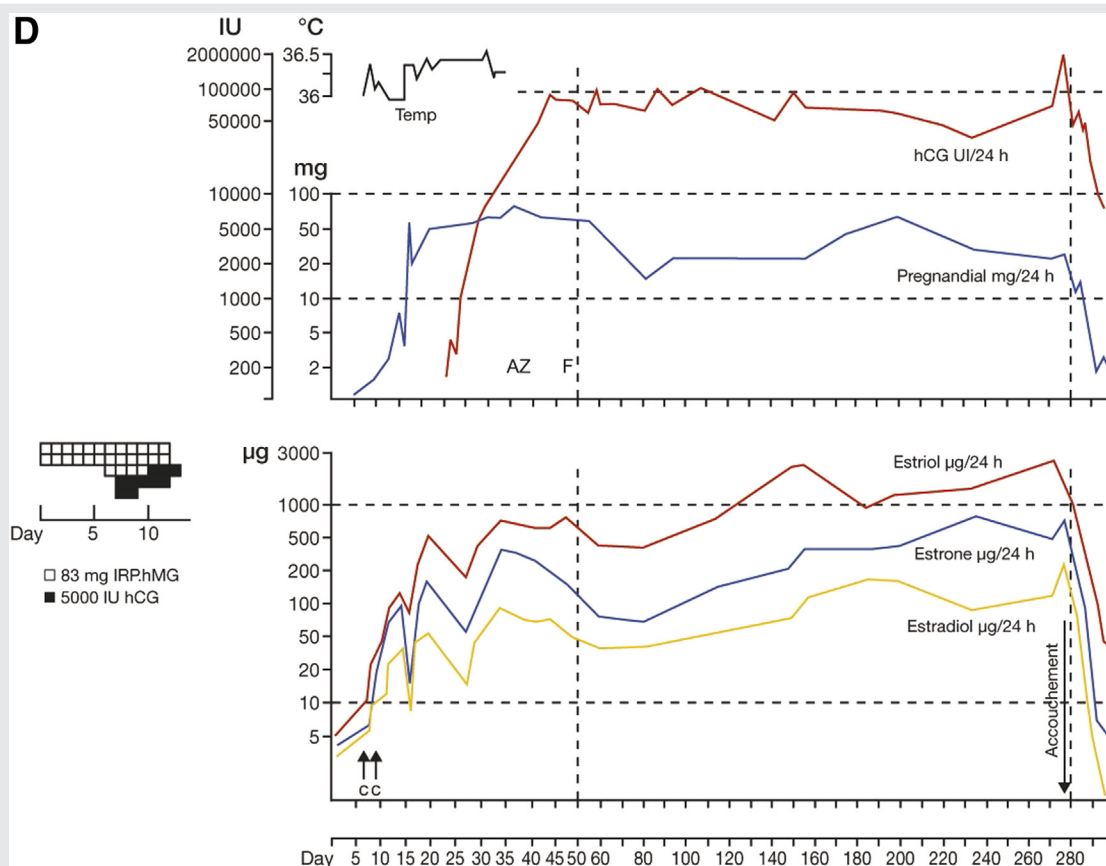


Forty years of IVF. *Fertil Steril* 2018.

(386). This material became the International Reference Preparation (IRP), and in 1964 became the international standard for FSH and LH for the quantitation of hMG (386). This enabled determination of the relative gonadotropin content of a preparation, allowing more precise and repeatable dosing.

Clinical trials using hMG preparations were initiated, with the preparations being accepted by both the scientific community and regulatory agencies despite their low purity, as no other alternatives were available (385). In 1960, use of hMG in anovulatory, hypopituitary, hypogonadotropic, primary amenorrheic women was demonstrated to induce

FIGURE 22 Continued

Forty years of IVF. *Fertil Steril* 2018.

the expected, desirable changes in the endometrium and the vaginal epithelium as well as to induce steroid secretion (385). Then, in 1962, Lunenfeld et al. (391) observed that amenorrheic hypogonadotropic women required 120–240 mg equivalent of the IRP to stimulate their ovaries to produce estrogens (385). Based on the hMG content of the IRP (120 mg IRP = 6.8 mg hMG), this would be 6.8–13.6 mg of hMG (392). This was followed by the first report of hMG being used for successful ovulation stimulation followed by pregnancy in amenorrheic, hypogonadotropic women, by use of a sequential step-up/step-down regimen (385). The hormone pattern during the follicular phase, ovulation, luteal phase, and pregnancy are shown in Figure 21D.

After successful ovulation induction and pregnancies were demonstrated in hypopituitary hypogonadic amenorrheic women, Pergonal 75 was registered by Serono in Israel in 1963 and in Italy in 1965 (386). A similar preparation, Humegon, was marketed by Organon in the Netherlands in 1963 (385).

In 1973, the first World Health Organization (WHO) guidelines on the diagnosis and management of infertile

couples were developed (385). These recommended an effective daily dose of 150–225 IU hMG for hypogonadotropic patients (WHO Group I), and 75–150 IU for anovulatory normogonadotropic patients (WHO Group II). These guidelines also noted that the FSH/LH ratio varied in the different hMG and hPG preparations, and that available evidence suggested preparations with ratios of 0.1–10.0 were acceptable, provided a sufficient FSH dose was given (385, 386). Currently, for gonadotropins, a variability of 20% to 25% is accepted in the pharmacopeia (i.e., a 75-IU vial of hMG may contain 80% to 125% content of FSH and/or LH) (393).

In 1981, following the pioneering work of Steptoe and Edwards using natural cycles for IVF, Howard and Georgeanna Jones established the hMG/hCG protocols that had been described by Lunenfeld et al. in 1963 as the standard approach for ovulation stimulation and induction for assisted reproduction technologies (ART) (385). These protocols were later revised, with the standard for ovarian stimulation in ART treatment changing from monofollicular development to the production of multiple follicles (385). Frequent cycle cancellations were associated with these protocols as a result

of a premature LH surge and premature follicular luteinization (385). This was overcome by the use of GnRH agonists to down-regulate the pituitary during ovarian stimulation with hMG (385).

Highly purified urinary hMG (HP-hMG) (Menopur, Ferring Pharmaceuticals) received marketing approval in Europe in 2004 and is extracted from menopausal urine via eight purification steps (394). However, this increased purity also results in a loss of LH activity, and hCG is added to reestablish the FSH/LH ratio. As a result of this, HP-hMG contains approximately 30% identified impurities that vary from batch to batch. In addition, LH and hCG are different from molecular, functional, and clinical points of view (395). Unlike LH, hCG is only produced by pregnant women, and when used in nonpregnant women it has a longer half-life, resulting in accumulation in peripheral blood. In addition, hCG has a very different effect on granulosa cells than LH, down-regulating the LH receptor; it is more potent on cAMP production and less potent on extracellular-regulated kinase and protein kinase B (395).

## PURIFICATION OF FSH FROM hMG

Despite the availability of hMG preparations, clinicians wanted to be able to individualize the dosing of FSH and LH according to specific patient characteristics (385). A number of methods were proposed to purify FSH from hMG (386), but none of these methods could realistically be used on a large scale; it was not until developments in immunological techniques enabled the preparation of specific antibodies to FSH or LH that purification on a large scale became feasible (385).

Initially, in 1960, polyclonal anti-hCG antibodies were generated and used to produce an affinity column to remove LH activity from hMG (385). The eluted FSH could then be further purified and lyophilized as with earlier hMG preparations (385). This produced a product (Metrodin, urofollitropin) that contained 150 IU of FSH and 1 IU of LH per mg of protein (385). However, a large proportion of the nongonadotropin urinary proteins remained present (385).

Further advances enabled the replacement of the polyclonal antibodies with highly specific monoclonal antibodies to FSH (385). In these techniques, hMG passed through an affinity column containing the monoclonal antibodies, which would bind selectively to the FSH molecules; all unbound urinary proteins and LH passed through the column and were removed (386). The FSH could be extracted from the column, providing highly purified FSH with very minimal LH or other contaminating proteins (386). The final product (Metrodin HP; highly purified urofollitropin) contained <0.1 IU of LH activity and <5% of unidentified urinary proteins and had about 9,000 IU of FSH per mg of protein (386). The FSH content per mg was 60 times greater with Metrodin-HP compared with Metrodin, and the proportion of nongonadotropin urinary proteins was significantly lower. This improvement meant that the product could be injected subcutaneously rather than intramuscularly as had been the case for all previous gonadotropin preparations.

As the demand for gonadotropins increased, the shortcomings of sourcing menopausal urine became more apparent

(385). In the year 2000, 120,000,000 L of urine from 600,000 donors were needed to satisfy the demand, and increasing safety measures had to be put in place as each woman could not be evaluated individually (385). After Creutzfeldt-Jakob disease had been reported in women who had received hPG treatment, prion proteins were identified in the urine of affected individuals (385). Concern about the potential for similar disease transmission with hMG led to countries publishing resolutions to replace urinary with recombinant gonadotropins, owing to their higher purity and safety (385).

## RECOMBINANT GONADOTROPINS

Recombinant human FSH (recombinant hFSH) was developed to reduce the inherent variability that resulted from the inconsistent starting materials of urinary FSH production, and to make FSH production independent of urine collection, thus ensuring greater availability. Because glycosylation is very important to the function of FSH, the recombinant molecules must be produced using mammalian cell lines capable of this post-translational modification (385). Hence, recombinant hFSH was first expressed in Chinese hamster ovary (CHO) cells, an industry standard for the production of biologics at that time (Fig. 23A) (386).

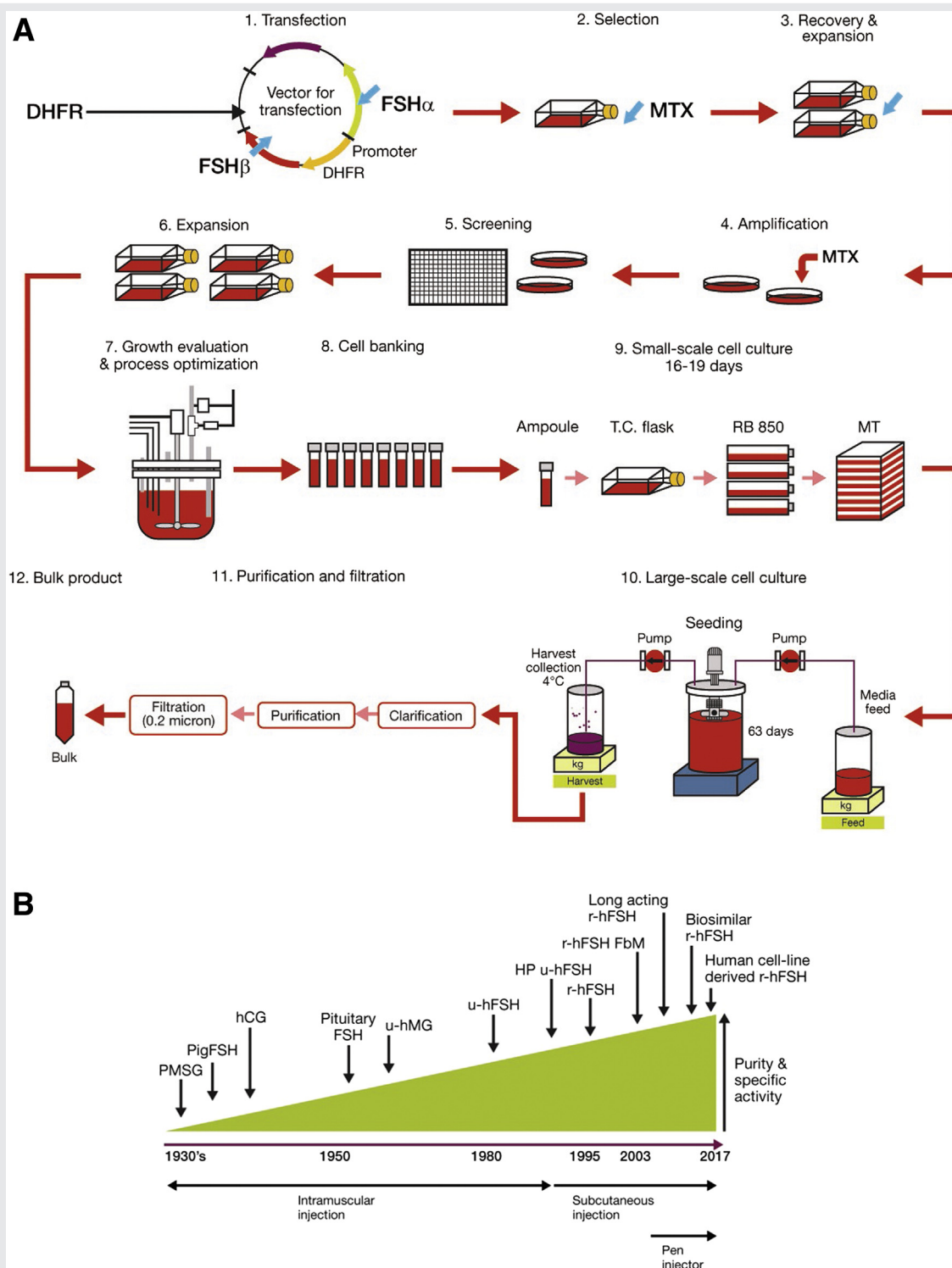
During the development of recombinant hFSH, clones were selected based on activity, consistency of production, and the glycosylation pattern (386). It was important that stable cell lines that expressed the product in relative abundance were used for efficiency and to avoid changes in the product over time (386). The glycosylation pattern of gonadotropins varies throughout the menstrual cycle (see Fig. 22B), and the desired glycosylation profile of the recombinant hFSH also had to be selected (386). After considering the feasibility of producing three FSH preparations with different glycan profiles, matching the glycosylation at the start, middle, and end points of the menstrual cycle, the decision was made to use the midcycle glycosylation pattern, as it was the most bioactive.

The resulting recombinant hFSH is pure (99%) and has a more homogenous glycosylation pattern than highly purified urinary and pituitary FSH or hMG preparations, and the manufacturing process allows high batch-to-batch consistency in both isoform profile and glycan-species distribution (386). This higher purity permits bioactivity to be determined using physicochemical analysis and vials to be filled by protein mass rather than specific activity, which reduces batch-to-batch variation to  $\pm 2\%$ . Furthermore, the greater purity of the formulation reduces the possibility of oxidation, thereby enabling the production of liquid gonadotropin formulations. These liquid formulations could then be used in prefilled pen-injection devices.

To determine the bioactivity of the filled-by-mass product, a conversion factor between international units of biological activity based on the Steelman-Pohley bioassay and micrograms had to be obtained. The Steelman-Pohley bioassay determines the FSH activity in a gonadotropin sample by comparing ovarian weight in immature rats (six to eight per group) treated for 3 days with a daily injection of either the test FSH preparation plus hCG or a standard FSH



FIGURE 23



(A) The development and production of recombinant products in dihydrofolate deficient Chinese hamster ovary cells and (B) time line of gonadotropin development. dhfr, dihydrofolate reductase; FbM, filled-by-mass; FSH, follicle-stimulating hormone; hCG, human chorionic gonadotropin; HP, highly purified; MTX, methotrexate; PMSG, pregnant mare serum gonadotropin; Q.C., quality control; r-hFSH, recombinant human follicle-stimulating hormone; T.C., tissue culture; u-hFSH, urinary follicle-stimulating hormone; u-hMG, urinary human menopausal gonadotropin. [1A: Adapted from B. Lunenfeld, Gonadotropin stimulation: past, present and future, *Reprod Med Biol* 2012;11:11–25; 1B: Adapted from B. Lunenfeld, Historical perspectives in gonadotrophin therapy, *Hum Reprod Update* 2004;10:453–67.]

Forty years of IVF. *Fertil Steril* 2018.

preparation plus hCG (396). Potency ratios are then calculated based on dose–response curves for the test and standard FSH preparations, enabling the biopotency of the test preparation to be determined. This took 2 years for the production, analysis, and clinical validation of different batches of follitropin alfa (385). This work determined that recombinant hFSH has a bioactivity of 13,745 IU/mg of protein, which leads to a conversion factor of 75 IU per 5.5  $\mu$ g of FSH.

The first recombinant hFSH (follitropin alfa) for clinical use was produced by Serono Laboratories (now Merck KGaA, Darmstadt, Germany) in 1988 and was licensed for marketing in the European Union in 1995, and a similar recombinant hFSH (follitropin beta) produced by Organon (now Merck & Co., Kenilworth, NJ) received its license for marketing in 1996 (385). These two products are similar but have biochemical differences resulting from their production (386). Follitropin alfa is produced using two separate plasmids to construct the FSH-producing cell line, one for the  $\alpha$ -subunit and one for the  $\beta$ -subunit (386); follitropin beta is produced using a single plasmid encoding both the  $\alpha$ -subunit and the  $\beta$ -subunit (386). In addition, the downstream purification processes differ for the two products: follitropin beta is purified using ion exchange chromatography, hydrophobic chromatography, and size exclusion chromatography whereas follitropin alfa is purified using these processes plus an additional immunoaffinity step (386). During the early 1990s recombinant preparations were shown to be at least as efficient for ART as urinary preparations when pregnancies from fresh transfers were compared (386).

After the successful production of recombinant hFSH, a similar process was used to produce recombinant human versions of LH (recombinant hLH) and hCG (recombinant hCG) (386). In 1997, Agarwal et al. reported the first birth in a hypopituitary–hypogonadotropic (WHO Group 1) woman treated with recombinant hFSH and recombinant hLH to stimulate follicular growth and recombinant hCG to induce ovulation (386). An estimated 100 million units of gonadotropin was used in 2016, and this amount is estimated to continue to increase by 6% each year until 2025 (data based on IMS MIDAS data of IQVIA).

Two biosimilar versions of recombinant hFSH are now available. Ovaleap (Teva B.V., Haarlem, the Netherlands) received marketing authorization in Europe in 2013, and Bemfola (Afolia, Finox Biotech AG, Balzers, Liechtenstein) received marketing authorization in Europe in 2015. Biosimilars are not exact copies and are not considered generic versions of the reference product. Differences result from the manufacturing processes, which are not identical for each product. Biochemical differences from the originator product (strength, purity, and differences in the composition of isoforms and/or glycosylation profiles) can cause differences in biological activity (including biopotency, receptor binding, postreceptor biochemistry in the cell, pharmacokinetics, and pharmacodynamics). Phase III trials for both Bemfola (Afolia) (397, 398) and Ovaleap (399) consistently observed a tendency toward a lower ongoing pregnancy rate per cycle with the biosimilar preparations compared with GONAL-f, highlighting that more data are needed regarding efficacy.

In December 2016, a new recombinant FSH, follitropin delta (Rekovele, Ferring Pharmaceuticals, Saint Prex, Switzerland) produced using a human retina-derived cell (PER.C6 cell line), received marketing authorization in Europe. The peptide structure is the same as the other recombinant FSH preparations, but the glycan structure is different (including both  $\alpha$ 2,3 sialylation and  $\alpha$ 2,6 sialylation), with higher sialylation than follitropin alfa. This results in differences in pharmacokinetics and pharmacodynamics, which causes higher exposure and lower serum clearance compared with follitropin alfa (400). Because comparison of the bioactivity of follitropin delta with the bioactivity of a reference standard determined by the Steelman–Pohley assay cannot directly predict the proportional pharmacodynamic activity of follitropin delta in women (400), it has to be dosed according to mass ( $\mu$ g) rather than bioactivity, and the starting dose has to be calculated according to patient's antimüllerian hormone level and weight (401). Once treatment has been initiated, dose adjustments are not allowed with follitropin delta, unlike with other follitropin preparations.

## LONG-ACTING GONADOTROPIN PRODUCTS

Follicle-stimulating hormone has a relatively short biological half-life (24 hours), and daily injections are required over a number of days during fertility treatment. There has therefore been interest in producing longer-acting versions of the product that would reduce the treatment burden. One early approach was to extend the half-life of FSH in the bloodstream through hyperglycosylation (GM1-Serono). This compound remained in the bloodstream for 5 days; however, development of this product was discontinued, despite achieving the same results as follitropin alfa in clinical trials (402). A different approach was used for the development of corifollitropin alfa (Elonva, Merck & Co.), which has a modified  $\beta$ -subunit fused with the CTP component of the hCG  $\beta$ -subunit, which reduces its metabolism and enables it to remain in the bloodstream for 7 days. This product received marketing authorization in Europe in 2010, and no evidence has been observed of a difference in live-birth rates for medium doses (150–180  $\mu$ g) compared with daily FSH treatment (403).

## CONCLUSION

The development of gonadotropins has been a process focused on producing purer products that are safer and more efficacious (see Fig. 23B). It is estimated that their use in ovulation induction and ART has led to the birth of more than 15 million children. The currently available products can be injected subcutaneously rather than intramuscularly, and pen injection devices are available, eliminating the need to reconstitute the product before injection and improving ease-of-use compared with syringe and vial. This has led to the current state of the art, where fertility treatment can be personalized using pure gonadotropin preparations (recombinant hFSH, recombinant hLH, and recombinant hCG) at the start of and during ovarian stimulation with the aim of maximizing safety and efficacy. However, work to

develop new preparations is still ongoing. There has been a lot of interest in developing orally active FSH agonists and antagonists as FSH itself cannot be absorbed orally, although to date all the efforts have failed (386).

**Acknowledgments:** The authors thank all the scientists and physicians who have contributed to this endeavor (owing to space restrictions we were unable to cite many of the important manuscripts that informed the development of gonadotropins for use in ART treatment); and Alexander Jones of inScience Communications, who provided medical writing support, funded by Merck KGaA, Darmstadt, Germany.

## PROGRESS IN OVARIAN STIMULATION FOR IVF OVER TIME

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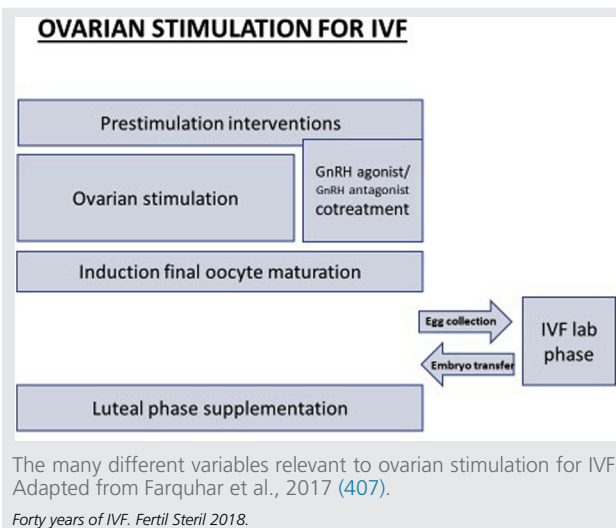
Despite the notion that the first baby Louise Brown was born in 1978 following IVF performed in the natural menstrual cycle without exogenous stimulation, soon thereafter ovarian stimulation became standard of care in clinical IVF. It is generally believed that starting the laboratory phase of IVF with multiple oocytes retrieved following ovarian stimulation effectively compensates for suboptimal fertilization of oocytes and the development of embryos in vitro and is therefore mandatory for a successful IVF program.

### Historical perspective of ovarian stimulation

In the early 1980s, births after IVF in clomiphene citrate-stimulated IVF cycles were reported from Australia, followed by the use of urinary gonadotropins after multiple failed natural cycle IVF attempts in the U.S. (405). Associated with the ongoing development of multiple follicles and the coinciding supraphysiological production of estradiol ( $E_2$ ), a premature late follicular phase rise in luteinizing hormone (LH) and progesterone (P) frequently occurred, resulting in compromised IVF outcomes. For this reason, down regulation of endogenous gonadotropin synthesis resulting from the co-administration of gonadotropin-releasing hormone (GnRH) agonists was introduced in the late 1980s and rapidly became standard of care. The timed induction of final oocyte maturation during the late follicular phase and before the retrieval of oocytes was induced by administering a single bolus dose of human chorionic gonadotropin (hCG).

The duration of stimulation protocols increased significantly because stimulation had to be postponed due to the initial stimulatory (so called “flare”) effect of GnRH agonists on pituitary gonadotropin release. Over the last several years, clinicians slowly shifted from GnRH agonists co-treatment towards the more recently introduced GnRH antagonists. Due to competitive binding to the GnRH receptor, suppressive effects of GnRH antagonists are immediate. Therefore, ovarian stimulation can be initiated early during the normal menstrual cycle, rendering stimulation shorter and more patient friendly. After many years of controversy, the most

**FIGURE 24**



recently updated meta-analysis including all randomized controlled trials to date comparing GnRH agonist versus antagonist co-treatment, revealed similar IVF success rates coinciding with lower overall consumption of gonadotropins and reduced ovarian hyperstimulation (OHSS) rates (406).

Ovarian stimulation protocols may be preceded by interventions such as estrogens, combined steroid contraceptives or long-term down regulation by GnRH agonists for reasons of timing and convenience for the clinic creating the possibility to plan IVF cycles, to “synchronize” the cohort of ovarian follicles or suppress benign steroid dependent diseases which may interfere with IVF outcomes, such as endometriosis (Fig. 24) (407).

The luteal phase is distinctly abnormal following ovarian stimulation. This was already reported by Professor Bob Edwards in the 1970s when he was initially aiming to develop IVF using clomiphene citrate stimulation. Although luteal phase supplementation (predominantly by daily progesterone administration) is widely applied, the true reason underlying abnormal luteal function remains insufficiently understood. Many different features may cause insufficient endogenous support of corpora lutea by pituitary gonadotropins, such as the follicular phase GnRH agonist coadministration, pharmacological follicular phase  $E_2$  concentrations, the bolus dose of hCG to induce final oocyte maturation, follicular puncture and disruption of the follicular integrity, and finally markedly increased early luteal phase P concentration produced by multiple corpora lutea. Different drugs and medication regimens for luteal phase supplementation have been developed over the years (Table 9).

Initially, ovarian stimulation was predominantly performed using extracts obtained from the urine of postmenopausal women (human menopausal gonadotropin [HMG]) with both follicle-stimulating hormone (FSH) and LH bioactivity. In more recent years, next to purified urinary products, recombinant DNA technology allowed for the mass production of human recombinant FSH, a long acting FSH chimera,

**TABLE 9****Summary of key questions related to ovarian stimulation for IVF.**

Issue	More detailed discussion
Aim of ovarian stimulation	Retrieve multiple oocytes for IVF, resulting in: More embryos for transfer and cryopreservation Increased pregnancy rates Cost/access to IVF care Complexity/treatment burden Side effects
Drawbacks ovarian stimulation	Complications (especially ovarian hyperstimulation) Reduced endometrial receptivity and lower embryo implantation rates Uncertain effects on embryo quality
What is the optimal number of oocytes retrieved?	Uncertain, this may vary under different clinical circumstances, according to most, in between 8-15 oocytes. Mild stimulation approaches aim for lower numbers (3-8 oocytes).
Drug classes used for ovarian stimulation	Gonadotropins (follicle-stimulating hormone, luteinizing hormone, human chorionic gonadotropin) Anti-estrogens (clomiphene citrate, tamoxifen) Aromatase inhibitors (letrozole, others) Insulin sensitizers (metformin, inositol) Androgens (testosterone, dihydroepiandrosterone, others) Others (growth hormone, ...)
Can stimulation dosing/ compounds be individualized?	Several patient characteristics predict ovarian response to exogenous stimulation (especially antimüllerian hormone and antral follicle count), with the potential to generate algorithms for individualized dosing. More prospective studies are required to confirm a robust clinical benefit, using different compounds in diverse populations.
Do effective intervention for women with low response exist?	The addition of androgens (and possibly growth hormone) seem to exhibit clinical benefit.
Stimulation pretreatment	Gonadotropin-releasing hormone agonist down regulation Estrogens Combined steroid contraceptives
Monitoring ovarian stimulation	Monitoring of ovarian function by either ultrasound, hormone assays or both. Required intensity of monitoring varies with rigor of stimulation.
Luteal phase supplementation	Progesterone Progesterone and estrogens Human chorionic gonadotropin Micro dose gonadotropin-releasing hormone agonist

Forty years of IVF. Fertil Steril 2018.

recombinant LH, and hCG. Numerous different ovarian stimulation regimens have been developed using exogenous gonadotropins combining different preparations, different starting days of stimulation, different doses in either fixed or flexible regimens dependent on the ovarian response observed. Starting doses of FSH may vary from <75 IU/day up to 600 IU/day!

Oral drugs, such as anti-estrogens, aromatase inhibitors, insulin sensitizers, androgens, and many others, have been increasingly used in the context of ovarian stimulation in recent years, either alone or in combination with exogenous gonadotropin preparations (Fig. 24, Table 9). Reported clinical outcomes vary, but stimulation cost are significantly reduced.

The optimal number of oocytes to be retrieved remains a topic of ongoing debate. Many clinicians and embryologist believe that more oocytes are associated with higher IVF pregnancy rates, also referred to as “the more, the better” approach. A high oocyte yield associated with high pregnancy rates in IVF is still advocated by some (408, 409), but disputed by others (410, 411). The aim should be to titrate the stimulation dose in such a way that the optimal number of follicles develop. Too few follicles (also referred to as low response) usually means poor IVF outcome, whereas too many developing follicles induce a risk for developing the

potentially hazardous OHSS (412). When assessing the optimal number of oocytes, next to pregnancy rates, other important features associated with ovarian stimulation should also be considered such as cost, burden of treatment and risks. For instance, possible detrimental effects of high dose ovarian stimulation on perinatal outcomes have recently been reported (413, 414).

For sure, chances to develop OHSS are directly linked to the number of developing follicles. Since serious forms of OHSS often coincides with pregnancy, approaches have been advocated to segment IVF, separating in time the stimulation phase of IVF from the transfer of embryos. This so called freeze all strategy only transfers frozen-thawed embryos in subsequent natural or artificial cycles. Several well designed and sufficiently powered randomized controlled trials (RCTs) are on their way so the jury is still out to definitively assess whether OHSS risks can indeed be eradicated without compromising IVF risks and success.

### Ovarian stimulation in women with low ovarian response

From the early days of ovarian stimulation for IVF it became apparent that ovulatory women do not respond the same way



to stimulation. A proportion of women exhibit a reduced ovarian response, characterized by lower  $E_2$  levels and a low number of retrieved oocytes, which is associated with significantly reduced pregnancy rates. Although this observation has been reported by many investigators, the exact definition of low ovarian response remained variable among the different studies (415). To address this issue of heterogeneity, a consensus expert meeting proposed the Bologna Criteria for defining poor ovarian response (416), which, although not perfect, represent an important step forward in homogenizing this group of patients.

Several interventions have been developed aiming to improve ovarian response in poor responders undergoing IVF. However, the available evidence, derived mostly from retrospective and few prospective studies which used very variable definitions of low response, indicated very disappointing results in improving the probability of pregnancy (417). In a first meta-analysis comparing the different interventions reported, the addition of growth hormone (GH) to ovarian stimulation protocols, based on its important role in ovarian steroidogenesis and follicular development, seemed quite promising. This was further assessed in a subsequent meta-analysis of six relevant RCTs, involving a total of only 169 patients (415). In this small group of women, the addition of GH was associated with significantly increased live-birth rates (rate difference +17%; 95% confidence interval: +5 to +30). These encouraging results, however, should be viewed with caution since they are based on a very limited number of patients, whereas the safety of GH use for the patient and the offspring and the cost of treatment need to be further evaluated. Finally, in several countries the use of GH is tightly regulated, thus making access to the drug problematic.

Another group of substances that has been hypothesized to be potentially beneficial in improving poor ovarian response is androgens or androgen-modulating agents. This contention is based on several studies showing that androgens play a critical role in early follicular development and granulosa cell proliferation, increasing the number of preantral and antral follicles and enhancing ovarian responsiveness to FSH. Hence, another meta-analysis examined the effect of pretreatment with transdermal testosterone or dihydroepiandrosterone, the addition of aromatase inhibitors or recombinant LH or hCG during ovarian stimulation in poor responders undergoing IVF (418). Two studies involving 163 patients showed that pretreatment with transdermal testosterone for days was associated with a significant increase in live birth rates (risk difference +11%; 95% confidence interval: +0.3 to +22%) in poor responders stimulated for IVF. Moreover, pretreatment with transdermal testosterone, decreased significantly the total dose of gonadotropins and the number of stimulation days required and increased significantly the number of oocytes retrieved. Conversely, no significant differences in clinical pregnancy rates were observed in patients receiving dihydroepiandrosterone, aromatase inhibitors, recombinant LH and hCG.

These positive findings from the meta-analysis concerning transdermal testosterone pretreatment were further tested in a proper RCT in 48 patients fulfilling the Bologna Criteria (419). Transdermal testosterone was given in the preceding

cycle during extended GnRH agonist down regulation and was discontinued when FSH stimulation was initiated. No significant difference was observed in the duration of FSH stimulation, the number of oocytes retrieved, the fertilization rates and live birth rates per randomized patient. Hence, the data from this study, together with another four eligible RCTs, were included in a meta-analysis not yet published involving 362 patients evaluating testosterone pretreatment either with transdermal ( $n=4$ ) or with oral ( $n=1$ ) preparations. These findings confirm that testosterone pretreatment is associated with significantly higher clinical pregnancy and live birth rates, significantly lower total dose of gonadotropins and significantly more retrieved oocytes.

### Current ovarian stimulation challenges in perspective

It seems unjustified to assess the benefits of ovarian stimulation in isolation. For sure pregnancy chances of a single IVF cycle will be higher as compared to natural cycle IVF in case multiple oocytes resulting from ovarian stimulation are involved. However, multiple potential drawbacks of ovarian stimulation cannot be ignored, including; the complexity of regimens and resulting patient compliance issues, exceptionally high cost of drugs also inducing the need for intense ovarian response monitoring requiring frequent hospital visits, ultrasound scans often combined with endocrine assessments in blood, considerable side effects related to the GnRH agonist induced initial flare and subsequent down regulation along with high  $E_2$  concentrations due to ovarian stimulation, and finally and most importantly the risk for developing OHSS, which is usually reported somewhere between 1-8% depending on OHSS criteria used and applied stimulation regimen.

More recently, convincing evidence has been provided that the abnormal endocrine milieu during the follicular phase also negatively impacts on endometrial receptivity and therefore compromised implantation of embryos transferred in an IVF cycle. In an attempt to circumvent these negative effects of ovarian stimulation at the level of the endometrium, the freeze all strategy has also been advocated where only frozen-thawed embryos are transferred in subsequent cycles.

Following GnRH antagonist co-treatment during ovarian stimulation, final oocyte maturation at the end of the follicular phase can also be induced by a bolus dose of a GnRH agonist inducing a relative short endogenous LH (and FSH) surge, just like in the normal menstrual cycle. Luteal phase supplementation with low-dose hCG has been advocated, although such an approach again introduces the risk of developing OHSS (420). The pros and cons of the later approach should be balanced against the freeze all strategy.

Although not without controversy, some data seem to suggest that generating a great number of oocytes, may also give rise to more aneuploidy of embryos generated (421). The concept that profound ovarian stimulation is a prerequisite for a successful IVF program has been challenged by some in recent years. When cumulative outcomes are considered involving fresh and frozen transfers of a given IVF cycle, or even multiple IVF cycles over a given period of time, similar live-birth rates following mild compared to conventional stimulation have been reported (422). Moreover, a

recent systematic review considering all RCTs involving mild ovarian stimulation in both normal responders (15 studies and a total of 3,721 women involved) and low responders (5 RCT, and 1,493 women) revealed comparable pregnancy rates, reduced cost and reduced OHSS rates (423). These ongoing efforts aiming to improve the experience of patients as well as the health economics of IVF will likely contribute to the more widespread acceptance of mild IVF, both by patients and health care providers, and hopefully increasing access to IVF care worldwide.

As mentioned earlier, the individual ovarian response to stimulation varies significantly. Often clinicians are taken by surprise by individual ovarian response differences to stimulation and they aim to circumvent unwanted response by either increasing or decreasing the dose of the drug given. It should be mentioned, however, that the clinical utility of such an approach has never been proven. Much effort has been directed towards the identification of features which might predict ovarian response to standard stimulation, including female age, early follicular phase serum FSH concentrations, the number of ovarian follicles assessed by transvaginal ultrasound (antral follicle count), and more recently antimüllerian hormone levels exclusively produced by preantral and early antral ovarian follicles. Many retrospective studies demonstrated convincingly that both antral follicle count and antimüllerian hormone correlate well with ovarian response to standard stimulation. Such factors predicting ovarian response of a given woman may aid in selecting the most appropriate starting dose for stimulation.

Only recently, few prospective RCTs were undertaken involving individualized dosing for ovarian stimulation based on prediction models involving initial screening characteristics for ovarian response. A recent systematic review summarized outcomes of these 4 RCTs involving 1,982 women in total (424). Based on these studies it can be concluded that the desired oocyte number was more often reached following individualized dosing, less drugs were being used and chances for OHSS may be reduced. An even more recent RCT not included in the systematic review was unable to demonstrate a clinically relevant difference in cumulative live birth rates. Clearly, more studies are needed to refine the concept of individualized dosing further, using different algorithms and different stimulation regimens in diverse populations.

Ovarian stimulation in the context of oncofertility should be considered separately because the potential effects of the disease itself or the treatment required on ovarian function, the need for developing novel and immediate stimulation regimens because often there is no time to postpone chemotherapy, and finally the need for different approaches and compounds in steroid dependent cancer such as breast cancer (425). Moreover, safe and effective ovarian stimulation strategies are being developed for special conditions like oocyte donation or oocyte freezing for own later use.

### Summary of most pressing current challenges

Let us end with a personal account of the most pressing current questions regarding ovarian stimulation for women undergoing IVF today:

Can we agree on the optimal number of oocytes needed for successful IVF, balancing benefits versus risks?

Should GnRH antagonist cotreatment during ovarian stimulation for IVF be preferred for all IVF patients based on simplicity, less use of drugs, lower OHSS rates and similar IVF success?

Does ovarian stimulation impact negatively on embryo quality and endometrial receptivity, and if so how can we circumvent these detrimental effects?

Should hCG for inducing final oocyte maturation be replaced by a late follicular phase GnRH agonist bolus?

What is the optimal stimulation regimen, if any, for low responders?

How can we reach an agreement regarding compounds and dosing for ovarian stimulation?

Can ovarian stimulation be individualized, and if so what would be the best way to do this?

Is there a place for mild stimulation or natural cycle IVF, and if so in which patients or context?

## GONADOTROPIN-RELEASING HORMONE AGONISTS AND ANTAGONISTS IN THE CONTEXT OF COS AND TO TRIGGER OVULATION

David R. Meldrum, M.D. and  
Robert F. Casper, M.D.

At the beginning of the 1980s, researchers were starting to experiment with a new class of drug called gonadotropin-releasing hormone (GnRH) agonists, named due their enhanced ability to stimulate pituitary GnRH receptors. In 1982, in collaboration with the Salk institute, where much of the work on characterizing GnRH had been done, we (D.R.M., R.J.C., J.L., W.V., H.L.J.) were first to describe the use of repeated daily administration of a GnRH agonist in women with endometriosis (426). At the end of 28 days of their Nal-Glu agonist, mean estradiol levels were in the postmenopausal range. We proposed that a medical oophorectomy could be used as a new approach in treatment of endometriosis. Making the deadline for submission to the next Society for Gynecology Investigation meeting was an interesting challenge. The UCLA pharmacy told me to get in a long line to have the drug put into vials. I went to the associate dean for research and after explaining the potential paradigm shift we were proposing, a pharmacist, with me assisting, had the drug in vials that next weekend and I made the Society for Gynecology Investigation abstract deadline.

Researchers and clinicians have used downregulation to describe suppression of the pituitary due to repeated agonist administration. Although we subsequently showed that continued secretion of fragments of the gonadotropin alpha subunit demonstrated continued stimulation of the GnRH receptor and a post-receptor mechanism of suppression (427),

the term downregulation became ingrained in the literature rather than the more appropriate term, desensitization.

During the early to mid-1980s the most difficult thing we and our IVF couples had to deal with was the 20% to 30% incidence of ovulation prior to oocyte retrieval. Patients would come in prepared for laparoscopy only to be sent home because ovulation had occurred. Even at retrieval, we sometimes found oocytes in the culdesac fluid, observed the ovarian capsule spreading open before our eyes, or aspirated an oocyte from a stigma where fluid had released but the oocyte had been retained.

It was therefore inevitable that we and others in the U.S. began to use a newly available agonist, leuprolide acetate, to block ovulation, although its use was off-label. In 1989, we proposed that GnRH suppression should be routinely used, based on our own experience with excellent results that appeared to be superior to cycles without agonist (428). Due to the greater agonist effect and more cyst production when the drug was started in the follicular phase, we advocated routine mid-luteal administration. The outcomes may also be better due to greater synchronization of the follicular cohort in the luteal phase. Pellicer et al. (429) also reported improved stimulation when the agonist was started at least 8 days after ovulation. Even without having a higher success rate, later shown in a meta-analysis to be two-fold, simply the avoidance of premature ovulation by the agonist was sufficient to justify routine use. Still today, after almost 30 years, use of leuprolide acetate in IVF patients remains “off-label”.

A further advantage of agonist suppression prior to ovarian stimulation was that suppression could be varied in duration, offering patients and the IVF center the ability to program cycles. Extending the agonist suppression caused a minimal increase in cycle cancellation, but some women had side effects such as severe headaches and short-term memory loss due to the rapid and profound drop of the estradiol level. In one unfortunate anecdote, a woman was about to give an important talk and could not remember what she had planned to say. Clearly such effects were unacceptable, leading us and others to routinely precede the agonist with a variable duration of oral contraceptive administration to provide that same programming but without prolonged estrogen deprivation. That approach had the additional advantages of blunting any agonist effect and reducing the incidence of cyst formation. As long as a reasonable level of luteinizing hormone (LH) activity was provided during stimulation, use of the oral contraceptive did not appear to affect stimulation response or cycle success.

Since that early use of agonist, due to the introduction of recombinant follicle-stimulating hormone (FSH) and the goal of doing away with use of urinary human menopausal gonadotropins (hMG), one of the main issues was whether stimulation could be equally effective with using only FSH. We had continued using LH due to a study we had carried out showing that bioactive LH/hCG was significantly suppressed on the day of human chorionic gonadotropin (hCG) compared to control subjects on day 3 of menses who had not received agonist (430). The lower levels were in spite of the leuprolide acetate group receiving 225 IU of hMG. In the normal menstrual cycle, bioactive LH is low in the early follicular phase

and rises markedly prior to ovulation. Over the next several years it was confirmed that there was a small but, significant benefit in the live birth rate with use of FSH and LH compared to FSH alone (431).

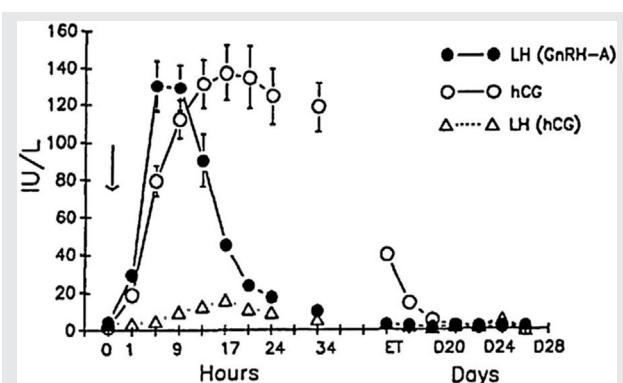
Another early question to investigate was whether the agonist would reliably prevent premature luteinization. We were first to report, using extracted serum specimens and radioimmunoassay (RIA), that in spite of suppression of endogenous gonadotropins with agonist, women with serum progesterone (P) on the day of hCG administration exceeding 0.9 ng/mL had a reduced pregnancy rate (432). The women with elevated serum P had received significantly higher levels of ovarian stimulation. Our finding dovetailed with the current cut-off using enzyme immunoassay. When we changed to that platform, by assaying specimens using both methods, our cut-off of 0.9 ng/mL with RIA was equivalent to the 1.5 ng/mL currently commonly used for those newer, although less accurate, assay techniques. Although originally assumed to be an LH effect, it was later realized that FHS was largely responsible for the progesterone rise, and further, that the balance of FSH and LH activity determined the chance of occurrence of a significant P rise (433). Now, with the advent of vitrification of embryos for deferred transfer, measurement of P has assumed greater importance for those programs doing fresh transfer for some or all of their patients. However, due to the inaccuracies of P measurement using enzyme immunoassay on unextracted serum compared to mass spectrometry, reliable clinical decision making regarding deferred transfer is problematic. Among other reasons, that argues in favor of routine “freeze all”.

It now appears that comparisons of outcomes using all FSH versus all hMG for stimulation were confounded by the greater stimulation of P secretion with either an excess amount of FSH or LH activity. Werner et al. (433) showed that lower P levels were achieved with a balance of FSH and LH activity (for example 150 IU of FSH and 150 IU of hMG), due to complex biochemical mechanisms described in their publication. Comparisons of outcomes using varying amounts of FSH and LH for freeze-all cycles will require re-investigating outcomes with all hMG or a balance of hMG and FSH contrasted to the use of FSH alone with deferred transfer. In the absence of such information we have continued to assume that some LH is required for optimal health of the mature oocyte and have therefore used a balance of the two gonadotropin preparations for freeze all cycles.

Unfortunately, agonist suppression increased the incidence of ovarian hyperstimulation syndrome (OHSS), most likely due to synchronization of the follicular cohort. As will be described, that drawback, together with requiring a longer series of injections, set the stage for a wholesale change in stimulation to use of GnRH antagonists. That paradigm shift yielded multiple advantages over use of agonists, and also allowed agonist trigger of ovulation with an associated dramatic reduction in OHSS.

In 1988, I (R.F.C.) joined the University of Toronto and was asked to develop an academic REI division and an IVF program using vaginal ultrasound oocyte retrievals instead of the current laparoscopic retrievals. Consequently, the physicians in the division needed training in vaginal ultrasound.

FIGURE 25



Mean ( $\pm$  standard error of the mean) serum luteinizing hormone (LH) concentrations ( $\bullet$ — $\bullet$ ) in the group of 9 women who were given gonadotropin-releasing hormone analogue (GnRH-a), compared with of human chorionic gonadotropin (hCG) ( $\circ$ — $\circ$ ) and LH ( $\triangle$ — $\triangle$ ) concentrations in the group of 9 women who were given hCG for follicular maturation. The arrow indicates the time of GnRH-a and hCG administration. ET = embryo transfer. Figure reproduced with the permission of Elsevier Inc. (434).

Forty years of IVF. *Fertil Steril* 2018.

A young Israeli fellow, Dr. Yael Gonen, who had already been in Toronto for about a year, was looking for research projects and during the intensive vaginal ultrasound experience we observed and published a correlation between endometrial thickness and the three-line endometrial pattern with pregnancy rates. However, even controlling for endometrial pattern and thickness, pregnancy rates with IVF were quite low back then and it was not clear whether the poor results were related to stimulation protocols and oocyte quality or to suboptimal embryo culture systems. We hypothesized that we might improve oocyte quality by trying to mimic the endogenous gonadotropin surge in both duration (48 hours) and quality (LH and FSH rise) when triggering final oocyte maturation. Based on work as a fellow with Sam Yen at UCSD, we chose a single dose of GnRH agonist as a trigger and did an initial study to compare the results with the standard dose of hCG (434).

Eighteen women undergoing IVF stimulation with 5 days of clomiphene citrate (CC) 100 mg and 75 to 150 IU of human menopausal gonadotropins (hMG) were randomized to receive either a single hCG (5000 IU) or leuprolide acetate (0.5 mg) injection when at least 2 follicles of 1.8 cm diameter were seen on ultrasound. We followed the women with serial blood sampling for LH, FSH, hCG, estradiol, and progesterone at baseline and after triggering. We determined that serum LH and FSH levels were elevated for 34 hours after GnRH agonist administration (Fig. 25) whereas serum hCG levels were still detectable 6 days after hCG injection, without any rise in serum FSH. Luteal phase levels of progesterone and estradiol were lower in the GnRH agonist triggered cycles compared to the hCG cycles, but interestingly the luteal phase length was similar in both groups. There were three live births in the GnRH agonist triggered group in the absence of any luteal support. This study provided evidence that a single bolus of GnRH agonist in an IVF cycle resulted in adequate release

of LH and FSH to complete the final stage of follicular maturation, the retrieval of fertilizable oocytes, normal embryo development and pregnancy (434). In this study, no luteal support was given and the luteal phases were similar to natural cycles. I now believe that this rather remarkable finding resulted from the CC used for follicular stimulation since we showed many years later that CC results in prolonged LH elevation in natural cycles when given for augmentation of ovulation. The LH elevation may have been long enough to rescue the corpora lutea.

Independent of our group, Itzkovitz et al. (435) published a similar study the following year using one or two doses of GnRH agonist to trigger final oocyte maturation in women undergoing IVF. This group also found good oocyte maturity, fertilization and pregnancies with the GnRH agonist trigger and lower serum estradiol and progesterone levels in 8 women with excessive response to gonadotropins. There were no cases of OHSS and the authors suggested that the GnRH agonist trigger may be a useful strategy to prevent OHSS.

We then published a prospective randomized trial in 179 women undergoing IVF triggered with hCG 5,000 IU or a single 0.5 mg dose of leuprolide acetate (436). We observed the same pregnancy rates with both triggers although luteal phase deficiency was now observed in several of the GnRH agonist triggered cycles. Luteal progesterone levels were significantly lower despite low-dose progesterone administration (50 mg two times per day vaginally). We recorded 16 women (18%) in the GnRH agonist group who had clinically short luteal phases. By the time of this study, ovarian stimulation for IVF had progressed so that many patients received the new highly purified FSH without CC and this might explain the luteal phase defects that were not seen in the first trial. This study's results were a harbinger of the luteal phase defects and low pregnancy rates seen with the GnRH agonist trigger once GnRH antagonist was developed for use in IVF cycles.

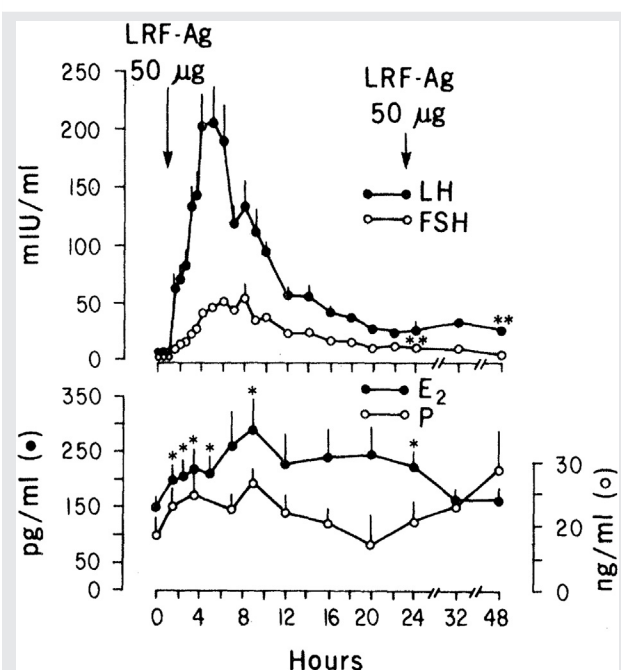
Around the same time as these publications, GnRH agonist was utilized for the long luteal phase stimulation protocol for IVF, which revolutionized the field by eliminating the occurrence of premature LH rises. However, the desensitization of pituitary GnRH receptors in this protocol prevented induction of a gonadotropin surge by a bolus of GnRH agonist.

In 1994, GnRH antagonist was introduced in controlled ovarian stimulation protocols to prevent unwanted LH surges. This resulted in renewed interest in the GnRH agonist trigger as an alternative to hCG and to prevent OHSS in women who had an excessive response to stimulation. Since GnRH agonists have greater affinity for GnRH receptors than GnRH antagonists, the agonist can displace the antagonist from the receptor resulting in LH and FSH release. The evidence that GnRH agonist could successfully trigger final follicle maturation in GnRH antagonist cycles was first published in 2000 in overstimulated IVF cycles to prevent OHSS (437) and later confirmed in normal response cycles.

It quickly became apparent that GnRH agonist triggering together with GnRH antagonist was associated with inadequate luteal phases and lower pregnancy rates with fresh embryo transfer (438). The luteolytic effect of this protocol was beneficial in preventing OHSS as shown by several publications. The cause for the luteal phase defects was not clear



FIGURE 26



Time course of luteinizing hormone (LH), follicle-stimulating hormone (FSH), estradiol (E<sub>2</sub>), and progesterone serum concentrations (mean  $\pm$  standard error of the mean) in response to two injections of gonadotropin-releasing hormone agonist (LRF-Ag) 24 hours apart in the mid-luteal phase of 4 normal cycling women. There was a vigorous and sustained release of LH and FSH after the first GnRH agonist injection, but no response following the second injection. \* $P < .05$ ; \*\* $P < .005$ . Figure reproduced with permission from American Association for the Advancement of Science.

Forty years of IVF. Fertil Steril 2018.

but speculation ranged between pituitary desensitization with central LH deficiency versus a direct effect of GnRH agonist on luteal steroidogenesis. I believe the evidence supports a central effect, although not pituitary desensitization. We showed in 1979 (439) that one or two boluses of GnRH agonist in the mid-luteal phase of natural cycles resulted in luteolysis and a short luteal phase. When two doses of 50 mcg of GnRH agonist were given 24 hours apart, there was a massive release of LH and FSH (double the spontaneous LH peak level) with the first dose and no response at all to the second dose (Fig. 26). This 24-hour time frame is too short for pituitary receptor desensitization and likely represents complete depletion of stored LH and FSH with too little time to regenerate more gonadotropin for release with the second GnRH agonist dose. Alternatively, it could represent disruption of the processing of gonadotropin as demonstrated by Meldrum et al. (427) in his early work on agonist effects on the pituitary. We ruled out a direct inhibitory effect of GnRH agonist since the corpora lutea could be rescued by addition of exogenous hCG, and we also showed the absence of any direct inhibitory effect of GnRH or GnRH agonist on human luteal cell steroidogenesis in vitro.

Today, the main use of the GnRH agonist trigger is to prevent OHSS in women who hyper-respond to gonadotropin stimulation in GnRH antagonist protocols. The mechanism is likely through both luteolysis as discussed above as well

as release of pigment epithelial derived factor from granulosa-luteal cells and consequent inhibition of vascular endothelial growth factor secretion (440). Endometrial receptivity can be preserved by adding low dose hCG for luteal support or by adding high dose estrogen and progesterone to maintain the endometrium. However, the most common strategy is cycle segmentation by freezing all embryos for transfer in a subsequent cycle.

## UNDERSTANDING THE LUTEAL PHASE IN STIMULATED ASSISTED REPRODUCTIVE TECHNOLOGY CYCLES

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The luteal phase is defined as the period between ovulation and either the establishment of a pregnancy or the onset of menses two weeks later. Following ovulation, a corpus luteum is formed which secretes steroids, necessary for secretory transformation of the endometrium (441). If conception and implantation occur, the developing blastocyst secretes human chorionic gonadotrophin (hCG), the role of which is to maintain the corpus luteum and its secretions (441). Human chorionic gonadotrophin produced by the embryo will maintain the secretory activity of the corpus luteum due to its structural similarity with luteinizing hormone (LH) and thereby activating the same receptor.

Under the influence of progesterone, the endometrium undergoes secretory transformation. Furthermore, local vasodilation and relaxation of the uterine muscle is promoted.

Implantation is a dynamic process involving the apposition, adherence and invasion of the endometrium by a blastocyst. Successful implantation is dependent on both embryological and endometrial factors. The endometrium must be receptive to the embryo with synchrony between the embryo stage and the endometrium. Despite the many advances in assisted reproduction implantation continues to be the rate-limiting step.

Throughout the menstrual cycle, the endometrium undergoes cyclical changes that prepare for embryonic implantation. The endometrium is only receptive to the embryo during a very limited time period generally referred to as the window of implantation (WOI) in the luteal phase. In a natural and idealized 28-day cycle, it is thought to occur approximately between day 22 to day 24. Previously, it was assumed that the WOI is more or less constant in time in all women. However, it has been shown that the WOI can differ and displacement of the WOI may result in infertility and repeated implantation failure.

### Cause of the luteal phase defect in stimulated in vitro fertilization cycles

In 1949, Georgeanna Jones, first described luteal phase deficiency as the premature onset of menses resulting from

deficient progesterone production, correctable by exogenous progesterone administration. It is well established that the luteal phase of almost all stimulated in vitro fertilization (IVF) cycles is defective (442). The etiology of luteal phase defect in stimulated IVF cycles has been debated for more than three decades.

Initially, it was incorrectly thought that the removal of large quantities of granulosa cells during the oocyte retrieval might diminish the most important source of progesterone synthesis by the corpora lutea, leading to a defect of the luteal phase. Another proposal suggested that the prolonged pituitary recovery that followed the gonadotropin-releasing hormone (GnRH) agonist co-treatment designed to prevent spontaneous LH rise in stimulated cycles resulted in a luteal phase defect.

The introduction of GnRH antagonists in IVF raised speculation that a rapid recovery of the pituitary function would obviate the need for luteal phase supplementation. However, studies have confirmed that luteolysis is also initiated prematurely in antagonist co-treated IVF cycles, resulting in a significant reduction in the luteal phase length and compromising the chances for pregnancy. Despite the rapid recovery of the pituitary function in GnRH antagonist protocols luteal phase supplementation remains mandatory (442). Today, it is clear that the main cause of the luteal phase defect in stimulated assisted reproductive technology (ART) cycles is related to the supraphysiological levels of steroids secreted by a high number of corpora lutea during the early luteal phase, which directly inhibits LH release via a negative feedback action at the hypothalamic-pituitary axis level (443). Luteinizing hormone support during the luteal phase is entirely responsible for the maintenance and the normal steroidogenic activity of the corpus luteum and withdrawal of LH, causes premature luteolysis.

### **Influence of progesterone on the endometrium in natural and stimulated cycles**

In 1950, Noyes et al. (444) studied the different histological appearances of the endometrium. Under the influence of progesterone, the epithelial glands and vasculature continue to grow resulting in a denser endometrium. In 1975, by describing the histology for each specific day after ovulation, Noyes et al. (444) established the classic endometrial-dating paradigm that still serves as the gold standard for clinical evaluation of luteal function. An endometrial biopsy is considered to be out of phase if it shows a difference of more than 2 days between the histologic dating and the actual day after ovulation (444).

Comparison of endometrial steroid receptors and proliferation index between natural cycles and GnRH-agonist/human menopausal gonadotropin-stimulated cycles for IVF have revealed distinct alterations in endometrial maturation. On the day of oocyte retrieval in stimulated cycles, a more advanced secretory endometrial maturation combined with reduced estrogen and progesterone receptors and a low proliferation index in glands and stroma has been found (445).

It is assumed that the supraphysiological levels of estradiol and progesterone during stimulation lead to a reduced

number of estradiol and progesterone receptors and a low proliferation index in glands and stroma. These endometrial alterations may adversely affect implantation.

### **Endocrine profile in IVF-cycles after oocyte retrieval, depending on the type of final oocyte maturation**

In stimulated IVF cycles, final oocyte maturation is a crucial step when planning to retrieve oocytes from preovulatory follicles. Human chorionic gonadotrophin is usually administered for final oocyte maturation to mimic the mid-cycle LH surge. In GnRH-agonist protocols final oocyte maturation is achieved by the administration of hCG. In recent years, the use of GnRH-agonist in GnRH-antagonist-protocols has become increasingly common. Human chorionic gonadotrophin and LH activate the same receptor, however, differ significantly in their half-life, <60 minutes for LH and >24 hours for hCG. Following hCG-administration for final oocyte maturation theca cell production of progesterone is sustained for at least 5 days attributed to both LH-activity and the half-life of hCG of more than 24 hours. In contrast, low levels of endogenous LH after GnRH-agonist trigger lead to low progesterone levels and subsequent luteal phase insufficiency (446).

### **Luteal phase support in ART**

To counterbalance luteal phase insufficiency after ovarian stimulation, luteal phase support (LPS) is recommended. Currently available formulations of progesterone include oral, vaginal, rectal, subcutaneous (SC), and intramuscular (IM).

### **Oral progesterone**

Natural micronized progesterone administered orally is subjected to first-pass pre-hepatic and hepatic metabolism. This metabolic activity results in progesterone degradation to its 5a- and 5b-reduced metabolites (441). Oral administration of progesterone yields poorly sustained plasma progesterone concentrations and its use as luteal phase support in IVF yielded very poor results. Parenteral administration (vaginal, rectal, SC, and IM) of progesterone overcomes the metabolic consequences of orally administered progesterone.

Dydrogesterone is an established oral retro-progesterone approved for the treatment of threatened and recurrent miscarriage (associated with proven progesterone deficiency), and infertility due to luteal phase insufficiency (447). Recently, a Phase III randomized controlled trial comparing the efficacy, safety, and tolerability of oral dydrogesterone as compared to micronized vaginal progesterone for luteal support in in vitro fertilization was published reporting comparable ongoing pregnancy rates (447).

### **Subcutaneous and intramuscular progesterone**

Progesterone is rapidly absorbed after IM injection, with peak concentrations achieved after approximately 8 hours. However, the IM application of progesterone may be painful and daily injections are required to sustain sufficient progesterone concentrations. In addition, swelling, redness, and even

sterile abscess formation may be experienced at the injection site. It has been reported that the use of IM progesterone may be associated with eosinophilic pneumonia, a severe morbidity in otherwise healthy young patients (448). Since the administration of vaginal progesterone is comparable to administration of IM progesterone for luteal phase support in assisted reproductive treatment, IM progesterone is not recommended as a first choice for LPS. A previous study evaluated the administration of SC progesterone as compared to vaginally administered progesterone for luteal phase support of IVF and reported that the SC route of administration was well-tolerated and comparable in efficacy to a vaginal insert (449). However, the conclusions of the study are limited to the progesterone dosing regimen studied and the duration of treatment for the patient population studied (449). Further studies are required prior to the implementation of the SC route of administration into routine clinical practice.

### Vaginal progesterone

Following vaginal application, serum progesterone concentrations reach maximal levels after approximately 3 to 8 hours and then fall continuously over the next 8 hours. In the majority of cases, 300–600 mg of vaginal progesterone are administered daily, divided into 2 or 3 doses. Vaginally administered progesterone is cleared from the circulation faster as compared to IM progesterone and serum progesterone levels are higher after IM administration as compared to vaginal gel administration (441). Independent from the measured serum progesterone levels, adequate secretory endometrial transformation is achieved following vaginal progesterone use. In addition, endometrial progesterone levels are higher after vaginal progesterone administration as compared to IM injection (441). Vaginally administered progesterone exerts a direct local effect on the endometrium and myometrium prior entering the systemic circulation. This is called first uterine pass effect. Until now, the mechanism of the first uterine pass effect is not fully understood and different modes of action are discussed: absorption of progesterone into the rich venous or lymphatic vaginal system and/or possibly direct drug diffusion through the tissues or even due to intraluminal transfer from the uterus to the vagina, similar to sperm transport. As the vaginal route is effective in providing sufficient luteal phase support and has minimal side-effects, it is the preferred route of progesterone administration. If the patient complains of vaginal irritation and discomfort secondary to discharge rectal administration may also be effective.

### Human chorionic gonadotrophin for luteal phase support

Following the administration of hCG, luteolysis will not occur as the corpora lutea will be stimulated and progesterone production will be maintained. For many years, hCG-administration was the standard form of luteal phase support. The disadvantage is the possible risk of ovarian hyperstimulation syndrome, particularly in those patients who have responded well to stimulation. Therefore, hCG should not be the first choice of luteal phase support in stimulated ART cycles.

### Timing of LPS with progesterone

Ovarian stimulation results in supraphysiological steroid concentrations with consequent inhibition of LH secretion by the pituitary via negative feedback at the level of the hypothalamic–pituitary axis resulting in declining and low progesterone levels after oocyte retrieval. To avoid a progesterone deficiency and therefore a negative impact on the endometrium, LPS has to be initiated before the endogenous progesterone levels are decreasing or low but pre-ovulatory exposure of the endometrium to progesterone may also have a negative impact on endometrial receptivity. Therefore, the timing of luteal phase support is critical to treatment success. A recent systematic review suggests that there appears to be a window for progesterone start time between the evening of the oocyte retrieval and up to day 3 following oocyte retrieval in hCG triggered cycles (450).

### Duration of luteal phase support

In early pregnancy, a deficiency of endogenous LH will be compensated for by rapidly increasing amounts of hCG produced by the developing embryo. The use of progesterone supplementation after oocyte retrieval is practically universal but the optimal duration of progesterone administration remains controversial. A meta-analysis evaluated the optimal duration of progesterone supplementation in pregnant women after IVF/intracytoplasmic sperm injection. The authors of this study reported that the currently available evidence suggests that progesterone supplementation beyond the first positive hCG test after IVF/intracytoplasmic sperm injection might generally be unnecessary, although large-scale randomized controlled trials are needed to strengthen this conclusion (451).

### Luteal support after GnRH-administration for final oocyte maturation

Administration of a GnRH-agonist for final oocyte maturation is becoming increasingly common, primarily as this approach significantly reduces the incidence of ovarian hyperstimulation syndrome (OHSS) due to rapid luteolysis after administration. Following the introduction of GnRH-agonist for final oocyte maturation in GnRH-antagonist protocols studies reported very poor reproductive outcomes. This finding was largely attributed to the induced severe luteolysis which cannot be counterbalanced using standard luteal phase support with progesterone “only” administration (452). To prevent luteolysis, administration of hCG or high doses of steroids are considered to be essential. Luteolysis will occur when LH support is withdrawn from the corpus luteum for  $\geq 3$  days but interestingly, corpus luteal function can be rescued if LH activity is reinitiated within approximately 72 hours (453).

Different treatment options have been described to maintain progesterone production from the corpora lutea and rescue the luteal phase. As GnRHa-induced luteolysis can be reversed by the administration of hCG, one treatment concept is the application of low-doses of hCG during the luteal phase. Despite low dosages of hCG, OHSS can still occur in some



patients. It has been shown that longer duration of stimulation, higher levels of progesterone on the day of final oocyte maturation and higher numbers of retrieved oocytes will result in higher levels of progesterone 48 hours after oocyte retrieval (454). Therefore, luteolysis after GnRH-agonist trigger is patient-specific and also luteal phase support requires individualization (454).

### Concept of individualized luteal phase support after final oocyte maturation with GnRH-agonist trigger

Over the last number of years, ovarian stimulation for IVF-treatment has become increasingly individualized based on assessments of each patients ovarian reserve such as by the use of antimüllerian hormone and ultrasound-based antral follicle count. However, until now LPS is performed uniformly in almost all patients.

Previously, it was demonstrated that GnRH-agonist trigger for final oocyte maturation would lead to severe luteolysis (446). However, luteolysis is not following the same pattern in all patients. To avoid unnecessary administration of hCG with the potential risk of late-onset OHSS-development, a new concept of luteal coasting has been developed. The concept of luteal coasting transfers the experience from follicular phase coasting for OHSS prevention to the early luteal phase for patients having fresh transfers (455). Daily monitoring of progesterone levels is required and a rescue hCG bolus can be administered, once progesterone concentrations decrease below 30 nmol/L (455). This novel approach reduces the risk of OHSS development in high-responder patients undergoing fresh embryo transfer without adversely affecting reproductive outcome (455).

### Conclusions

The cause of luteal phase defect in stimulated IVF cycles seems to be related to the supraphysiological levels of steroids. Luteal phase support with hCG or progesterone after assisted reproduction results in increased pregnancy rates. However, hCG is associated with a greater risk of OHSS. Vaginal, SC, and IM progesterone seem to have comparable outcomes. However, IM progesterone should not be the first choice due to possible side effects. Natural micronized progesterone is not effective, if taken orally. The duration of luteal phase support in stimulated IVF cycles does not appear to be mandatory beyond the day of a positive hCG test (451).

The new concept of personalized luteal phase support focuses on the luteal phase after final oocyte maturation with GnRH agonist in a GnRH antagonist protocol. Personalized luteal phase support demonstrates that the hCG dose and timing can be individualized based on individual luteolysis pattern (454). It appears that OHSS after GnRH agonist administration for final oocyte maturation can be avoided by individualization of luteal phase support and adopting the novel approach of luteal coasting in high responder patients planning fresh embryo transfer without adversely affecting their chance of achieving pregnancy (454, 455).

## OVARIAN HYPERSTIMULATION SYNDROME

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Daniela Galliano, M.D., Ph.D.

Ovarian hyperstimulation syndrome (OHSS) would probably never have appeared if assisted reproduction technologies (ART) were developed as a very successful approach to treat infertility from the very beginning. But, unfortunately, that was not the case. Although the first in vitro fertilization (IVF) cases were managed with natural cycles by Steptoe and Edwards, it soon became apparent from the work done in Australia that a greater number of oocytes collected provided a higher chance of getting more (and perhaps) better embryos, and controlled ovarian stimulation (COS) soon became a substantial part of an IVF cycle. Ovarian hyperstimulation syndrome is the most serious complication resulting from the medical manipulation of the ovary.

Soon after the introduction of ovulation induction (OI) in anovulatory patients with clomiphene citrate and gonadotropins in the early 1960s (456), it became apparent that OHSS was a potential complication reported to a mild degree of severity in OI. A couple of decades later, the introduction of aggressive COS in women undergoing ART who required the yield of a substantial number of oocytes resulted in OHSS as a routine and frequent complication in normo-ovulatory women.

Rarely, OHSS may occur in the absence of ovarian stimulation, generally appearing between the 8th and 14th week of spontaneous pregnancies. This is due to ovarian hypersensitivity to gonadotropins resulting from mutations in the follicle-stimulating hormone receptor; high levels of endogenous gonadotropins in molar pregnancies or multiple pregnancies; high levels of molecules structurally similar to gonadotropins, such thyroid stimulating hormone in hypothyroid states, occupying their receptors; or women with pituitary adenomas (457).

Since the description of OHSS, many investigators have tried to understand its pathophysiology, describing the symptoms and applying methods for management of already established full OHSS. Although this literature has been helpful in managing symptoms, it was not until 10 years ago that OHSS started to disappear from our terminology, as the risk factors have been fully described and preventative measures have been successfully employed.

The true incidence of OHSS has always been difficult to determine as there is no generally accepted definition of the syndrome. Moreover, it depends upon the clinical setting (OI or COS), the drugs employed, and the type of patient treated (either at risk or not at risk). When ovulation is induced with clomiphene citrate or aromatase inhibitors, either for timed intercourse or intrauterine insemination, a mild form of OHSS may occur, but moderate to severe OHSS is rarely seen (459). In COS, the overall incidence of moderate and severe OHSS is 3% to 6% and 0.1% to 2%, respectively (458). Hospitalization due to OHSS has been reported to range between 0.3% and 1.1% (458).



## Pathophysiology

OHSS results from multiple follicular development with gonadotropins and subsequent ovulation triggering with human chorionic gonadotropin (hCG). If pregnancy follows, trophoblast-derived hCG will worsen the symptoms. Also, it should be recognized as a phenomenon localized to the ovaries. In fact, oophorectomy restores normality and OHSS does not develop in experimental conditions in which ovaries are not present (459). Similarly, OHSS does not occur when many follicles develop as a result of COS with gonadotropins and hCG for ovulation triggering is withheld. This shows that hCG is the driver of all pathophysiological events in OHSS. hCG has no direct vasoactive properties, but vascular endothelial growth factor (VEGF) acts as the necessary hCG mediator that binds to its type 2 receptor (VEGFR-2). This in turn increases vascular permeability in the ovarian capillaries and leads to substantial vascular leakage and ascites formation (459).

Among women who display OHSS, the most important group are those with polycystic ovary syndrome (PCOS). Recent studies have provided information about the role of dopamine in ovarian physiology and its relationship with OHSS. Dopamine binds functionally to VEGF, avoiding phosphorylation and thus increasing capillary permeability. In other words, it acts to physiologically balance the action of VEGF on vessel permeability (459). It turns out that PCOS women display lower amounts of type-2 dopamine receptors in granulosa cells compared to normal individuals (460). In contrast, the secretion of 3,4-dihydroxyphenyl-acetic acid, the primary metabolite of dopamine, is significantly increased in PCOS, suggesting that the metabolism of dopamine is faster in PCOS than in normo-ovulatory women. All in all, it seems that PCOS women display an increased vascularization, which could be explained by higher serum and follicular VEGF levels compared to normal ovulatory subjects. However, the dopaminergic system is different than in normal individuals, which results in higher expression of the signs and symptoms of the syndrome (460).

## Clinical manifestations

The variety of clinical manifestations of OHSS is the consequence of the processes that define the syndrome. Enlarged ovaries may themselves produce abdominal discomfort. Increased ovarian vascular permeability leads to fluid accumulation in the abdomen and other body cavities, which then leads to abdominal heaviness and breathing difficulties due to limited diaphragmatic mobility (461). Furthermore, this shift in serum from the intravascular to the extravascular space causes hemoconcentration and reduced blood perfusion, resulting in reduced general organ perfusion. Oliguria and renal insufficiency may occur, and liver function may also be affected. Moreover, hemoconcentration increases the risk of thromboembolic events. In very severe forms, renal failure and reduced perfusion in other vital organs, such as the brain and heart, may have fatal consequences (461).

There are two clinical forms of OHSS, both hCG related: the early onset, occurring 3–7 days after hCG administration; and the late onset, occurring 12–17 days after hCG adminis-

tration, which is related to pregnancy-induced hCG production (462). The early onset is usually mild to moderate, while late OHSS is more severe as the rising hCG during pregnancy exacerbates the course of the syndrome.

In order to define an increasing degree of severity in the establishment of OHSS, different classifications have been published (461, 462) based upon the severity of symptoms, signs, and laboratory findings.

## Prevention of OHSS

An important step in the prevention of OHSS is to identify risk factors before COS is started. The PCOS phenotype is among the most relevant. Additional risk factors include women who have previously developed OHSS, black women, and younger patients (<35 y). The use of markers for ovarian reserve has improved the risk assessment of OHSS. A cut-off value of 3.36 ng/mL serum antimüllerian hormone levels has been considered a good predictor of OHSS. Levels >10 ng/mL are associated with a threefold increase in the incidence of OHSS. Antral follicle count (AFC) is also predictive of OHSS. The risk of OHSS increases from 2.2% in women with an AFC <24 to 8.6% with an AFC >24 (462).

For all of these patients, a GnRH antagonist COS protocol is strongly advised, with benefits including similar pregnancy rates compared to protocols employing long-GnRH agonist suppression, lower costs, and lower risk of developing OHSS. This appears to act at two levels: lower serum levels of estradiol during ovarian stimulation, and the possibility of triggering ovulation with a GnRH agonist by taking advantage of the flare-up mechanism. It is also worth stressing that the dose of gonadotropins used in women at risk should not be higher than 150 IU/day.

Human chorionic gonadotropin has been widely employed to mimic the mid-cycle surge of luteinizing hormone (LH) to trigger final oocyte maturation and ovulation for more than 60 years. Human chorionic gonadotropin has a longer half-life than LH and is known to initiate the cascade of events leading to OHSS. Human chorionic gonadotropin stimulates VEGF release in granulosa-lutein cells that bind to VEGF-R2, which increases vascular permeability in the ovaries (459).

Administration of a GnRH agonist is associated with an initial “flare-up” effect in which both serum LH and follicle-stimulating hormone are increased (463). It has been shown that a so-called GnRH agonist-induced surge of gonadotropins can last for 24–36 hours and induce oocyte maturation (463). An analysis of 17 RCTs that assessed GnRH agonist compared with hCG trigger found that the agonist resulted in a lower incidence of OHSS in fresh autologous cycles (odds ratio [OR]: 0.15, 95% confidence interval [CI]: 0.05–0.47), as well as in donor-recipient cycles (OR: 0.05, 95% CI: 0.01–0.28) (115) compared to hCG (462).

Once COS has started, a number of growing follicles >20 significantly increases the risk of OHSS. Serum estradiol concentrations >3,500 pg/mL are also associated with OHSS (462). Thus, in these patients it is imperative to avoid hCG administration using GnRH agonists for ovulation triggering. If this is not possible, it is recommended that the cycle be cancelled.

Adjuvant therapies during COS may also help to prevent OHSS. The incidence of severe OHSS can be reduced in patients who receive a daily dose of 100 mg aspirin compared to no treatment (462). For PCOS patients, Metformin at a dose of 500 mg three times daily or 850 mg twice daily during COS significantly reduces the incidence of OHSS (462).

Another possible approach is to substitute gonadotropin-releasing hormone (GnRH) antagonists with progestogens in COS with cycle segmentation. No randomized-controlled trial has tested the role of these stimulation protocols in preventing OHSS, but they are widely employed and the published experience using 4 to 10 mg/day medroxyprogesterone acetate simultaneously with gonadotropins shows good quality oocytes and pregnancy rates without LH surges or OHSS (464).

Retrieval of >15 oocytes significantly increases the chance of OHSS (463). In these cases, oocyte/embryo vitrification should be implemented after showing substantial and reproducible improvement in survival and overall health of newborn infants (465). This freeze-all policy or cycle segmentation is considered the best OHSS preventive measure as it avoids late-onset OHSS, the most dangerous form of the syndrome.

If the number of oocytes collected is <15, embryo replacement could be carefully considered in that cycle. Studies have reported that agonist trigger was associated with a lower live-birth rate (OR: 0.47, 95% CI: 0.31–0.70) in fresh autologous cycles due to a luteal phase insufficiency. That said, corpus luteum function can be sustained with a single 1,500 IU hCG injection administered the day of oocyte retrieval (466). If the patient is not at risk, a second 1,500 IU hCG injection can be administered five days later. However, some cases of late-onset OHSS have been described, highlighting the fact that embryo transfer is a major driver of OHSS regardless of the number of follicles developed (466). Therefore, the freeze-all policy should always be considered as a viable option in these cases.

It is advisable to measure serum LH on the day of triggering, as the chance of having a suboptimal response to GnRH agonist triggering 12 hours after administration (LH <15 UI/mL) can be as high as 25% if serum LH is undetectable, compared to 5% in the general population (467). The suboptimal response is particularly evident in women with anovulatory irregular cycles and when taking oral contraceptives for a long period of time.

Another option for overcoming a potential luteal phase defect is supplementing the luteal phase with both estrogens and progesterone according to standard steroid replacement protocols, which maintains similar pregnancy rates regardless of the triggering done with hCG or GnRH agonists (466).

Future alternatives to trigger ovulation without the risk of OHSS may include the use of kisspeptins, a group of hypothalamic peptides that are essential for normal human fertility. It has been shown that Kp-54 administration has a positive effect on egg maturation in women undergoing ART with a low risk of inducing OHSS (468).

Due to the relevance of VEGF and VEGF-R2 in the pathophysiology of OHSS, we introduced the concept of targeting VEGF by administering dopamine agonists (459). We showed that both cabergoline and quinagolide significantly reduced ascites formation and OHSS development in non-pregnant

patients, but were ineffective in patients who became pregnant after embryo replacement (459). This may be either because the amount of trophoblast-derived hCG was too elevated to be compensated by the doses of dopamine agonist administered, or because pregnancy stimulates other pathways that also affect vascular permeability irrespective of VEGF blockage. Whether a more aggressive intravenous administration of dopamine in women developing severe/critical OHSS will work has not been studied, but there are some reports showing that this strategy has successfully rescued renal output in compromised cases (469).

If oocyte/embryos are vitrified, letrozole can be employed as a luteolytic agent resulting in reduced incidence of ascites formation and overall moderate early-onset OHSS (470). Another luteolytic strategy could be to use a GnRH antagonist for 2–3 days.

Thus, today we have sufficient alternatives to avoid OHSS in most patients. Although the main driver of OHSS, the need of an important oocyte yield still stands, the measures presented herein developed over the last 40 years are a guarantee that this complication is almost gone from our medical nomenclature in ART. However, it is our task to educate our residents, fellows and patients in order to understand the potential complications of a high oocyte production and the measures available to avoid OHSS.

## ROLE OF ULTRASOUND IN IN VITRO FERTILIZATION

Matts Wikland, M.D., Ph.D.

In 1978, the same year as the first in vitro fertilization (IVF) child was born, the first study of the use of ultrasound for monitoring follicular growth was published (471). At that time the use of ultrasound imaging in early pregnancy and obstetrics was developing rapidly and became used more often in daily clinical work. However, ultrasound scanning as a diagnostic tool in gynecology was, in those days, not as widely appreciated as in obstetrics. However, with the introduction of clinical IVF around 1980 and the fact that ultrasound could be a potential tool for monitoring follicular maturation, the interest in utilizing the technique in IVF started to grow. With the rapid increase of IVF cycles there was a need for simplification of certain steps in the clinical procedure. One such step was oocyte retrieval, which in those days was performed as a laparoscopy procedure, and therefore had to be performed under general anesthesia at a hospital.

Knowing that ultrasound had been used for some years in aiding the puncture of cystic and solid tumors gave IVF groups the confidence to explore the use of the technique in oocyte retrieval. Rapid development of the technique led to the introduction of vaginal sonography, which turned out to be the ideal tool for guiding oocyte retrieval and made a contribution to other clinical steps in the IVF cycle.

### Oocyte retrieval

For the first five years of clinical IVF, oocytes in most patients were retrieved under the guidance of laparoscopy. However,

TABLE 10

Combination of different ultrasound scanning and puncturing routes for oocyte retrievals.

Scanning type	Oocyte retrieval	Reference
Abdominal	Transabdominal	Lenz et al., 1981 (473)
Abdominal	Transvaginal	Delenbach et al., 1984 (475)
Abdominal	Per urethral	Parsons et al., 1985 (476)
Vaginal	Transvaginal	Wikland et al., 1985 (479)

Forty years of IVF. *Fertil Steril* 2018.

there were some major disadvantages such as the necessity for general anesthesia, which made the procedure expensive and often time-consuming. Furthermore, laparoscopy exposes young women to a certain degree of risk. Thus, there was a need for a more simple and safe technique for oocyte retrieval.

Mature ovarian follicles are large (18–20 mm in diameter) cystic structures which can be imaged by ultrasound (471). As early as 1972, a Danish group (472) described the ultrasonically-guided percutaneous puncture of cystic and solid tumors. In the beginning of the 1980s, the time when clinical IVF started to grow, advances in ultrasound technology resulted in the production of real-time scanners of compact size, which provided excellent real-time imaging. These factors were important for the development of oocyte retrieval guided by ultrasound.

As Danish researchers had had an impact on the development of ultrasound guided puncturing procedures, it is not surprising that the first report of successful ultrasound-guided oocyte retrieval came from the Danish IVF group in Copenhagen in 1981 (473). Due to very open communication between the Danish IVF group and our IVF team in Gothenburg, Sweden, we adopted the practice of ultrasound-guided follicle aspiration quite early (474). The method both groups initially practiced was abdominal real-time scanning and transabdominal puncture. This technique utilized a full urinary bladder as an acoustic window. The method spread to other groups and alternative puncturing routes, such as the transvaginal and per urethral, were developed while still using abdominal scanning (475, 476). The different scanning and puncturing routes are listed in Table 10. As often happens with new techniques, there was initial skepticism. It was claimed that fewer oocytes were retrieved than when using laparoscopy. However, observational studies performed in the middle of the 1980s showed no significant differences between the outcome of IVF procedures which had used laparoscopy, versus transabdominal ultrasound, for oocyte aspiration. The first prospective randomized study was performed by Lewin et al. (477) in 1986 and they found similar outcomes in a comparison of the two methods. Still there was one main criticism of the technique and that was the pain patients experienced when the procedure was performed under local anesthesia. For this reason, general anesthesia was preferred and the advantages of transabdominal ultrasound over laparoscopy for oocyte aspiration were not widely appreciated. However, in 1983 our IVF group in Gothenburg started to explore the possibility of performing vaginal sonography using a small

sector scanner. The idea was to avoid scanning via the abdominal wall and the full urinary bladder, but instead to scan via the vaginal fornix using a small sector transducer placed in the vagina. This made it possible to get very close to the ovaries and obtain excellent real-time imaging. However, the available sector scanners in those days were not ideal for vaginal scanning so we started a collaboration with a Danish ultrasound company, Brüel and Kjaer, and developed a technique utilizing a specifically designed vaginal sector transducer to guide transvaginal oocyte retrieval. Besides the excellent imaging, the vaginal examination meant much less discomfort for the patient, since there was no need for a full urinary bladder. The advantages of vaginal scanning were described by Meldrum and co-workers at an early stage (478). As there was no need to puncture the abdominal wall and the urinary bladder and the puncturing distance was much shorter, the procedure could be carried out under local anesthesia using a para cervical block. It could now easily be performed as an outpatient procedure (479, 480).

Despite the lack of randomized controlled trials for transvaginal oocyte retrieval, under the guidance of vaginal ultrasound the technique proved to be simple and safe. It has been refined over the years and is today regarded as the gold standard. Complications are rare, but one must be aware that placing a needle in the pelvis and in highly vascularized ovaries can occasionally result in severe complications. Several studies have reviewed the complications after transvaginal ultrasound (TVUS) guided follicle aspiration. In a study following up more than 7,000 IVF cycles the severe complication rate was 0.08% (481). It is probable that the simplification which the transvaginal oocyte retrieval has meant for the IVF procedure has given more patients access to assisted reproductive technology than if the procedure had been performed by laparoscopy. Furthermore, development of the technique marked the start of the use of vaginal sonography, which since then has become such an important diagnostic tool in gynecology in general and in reproductive medicine in particular.

## Cycle monitoring

**Follicular monitoring.** In the early days of IVF, even though it was possible to achieve a full-term pregnancy utilizing the woman's natural cycle, it was soon realized that multiple follicular development could play a major role in its success. Different protocols for controlled ovarian hyperstimulation with gonadotropins became routine. In order to adjust gonadotropin dosage, ensure adequate follicular development and decide on the time for triggering the final oocyte maturation, it was necessary to monitor the woman's cycle. In the early days of IVF, the tool for monitoring was a biochemical assay of hormones such as estrogens, progesterone, and luteinizing hormone in the urine or blood. However, the work of Hackelöer and Robinson, showed that by measuring the growth of the mean follicular diameter by ultrasound it was possible to predict follicular maturation (471). With the introduction of linear array and sector real-time scanners, follicular scanning became much easier to carry out. The method is extremely practical since it is non-invasive and can be



performed by the clinician or the sonographer and gives an up-to-date status of the number and size of growing follicles. With the introduction of TVUS, monitoring follicular development in IVF cycles soon became routine. However, initial concerns were raised that there was a risk that ultrasound energy could have a negative influence on ovulation, or even on the oocyte. This claim has never been proved.

Some groups use the method as the only tool for monitoring while others combine TVUS monitoring with estrogen and progesterone levels. A Cochrane review was carried out, based on randomized controlled trials where cycle monitoring was performed either solely by two-dimensional (2D)-TVUS or in combination with estrogen during controlled ovarian hyperstimulation for IVF. No evidence was found that either of the methods was superior with regard to clinical pregnancy rate or the incidence of ovarian hyperstimulation syndrome (OHSS) (482). As good clinical practice, combining ultrasound and serum estrogen in women at risk of OHSS should be continued as a precaution.

Even though we know that decisions regarding ovulation triggering are based on a mean follicular diameter commonly set to 18–20 mm. However, in the literature there are no uniform recommendations for this measurement. In IVF treatment, the clinical decision regarding ovulation triggering is, however, based on a 2D-TVUS measurement of the size and number of follicles. Another interesting fact about the use of TVUS for monitoring follicular development is that there are no standardized protocols for the procedure. The size of the follicle, as measured by 2D-TVUS, is the mean of the two largest diameters perpendicular to each other. It is obvious that as the number and size of follicles grows, it becomes harder to measure them accurately and the method becomes increasingly time-consuming. This explains the variability of follicular measurements, both inter and intra observers. Problems using 2D-ultrasound in the standardization of follicular measurement and the development of three-dimensional (3D)-ultrasound, have led to the use of computerized algorithms for counting and calculating the volume of follicles. This information is used for clinical decision-making in IVF treatments (483). This automated measurement in 3D-TVUS is significantly faster than in 2D-TVUS, which is why some groups are supportive of this new technique. It is an interesting development which seems to have the potential for improving the accuracy of follicular measurements and subsequent decisions.

Doppler analysis of the follicular blood flow has also been tested as a tool for monitoring follicular development and maturation. However, the clinical value of the technique has so far not been appreciated, at least partly because even poorly vascularized follicles may contain oocytes that can result in a normal pregnancy.

**Endometrial monitoring.** Ultrasound imaging of endometrial thickness and pattern was described early as a possible clinical biomarker of estrogen levels in IVF cycles. Since then the clinical value of using sonographic imaging of endometrial thickness and pattern in IVF has been tested in several studies, but with conflicting results (484). Another uterine parameter that can be visualized by TVUS and which has been claimed to be valuable for IVF, is sub-endometrial

waves. The clinical value of this parameter is not currently clear and thus not routinely used.

There are many ultrasound devices which also make it possible to measure uterine blood flow by means of the Doppler technique. However, it has not yet been possible to show a clinical value for uterine Doppler analysis in IVF. Endometrial ultrasound parameters, as mentioned above, have so far not been proved to be practical clinical tools in the monitoring of the IVF cycle, and more studies combining and including new parameters are needed to fully explore their value.

## Embryo transfer

The pregnancy rate in IVF is dependent on multiple factors. One important factor is the embryo transfer technique. Traditionally it has been performed blind, using the clinical touch. By using ultrasound to guide the procedure, it became possible to visualize where the fluid bubbles containing the embryo/embryos were placed in the uterine cavity, which appeared to be of importance. Abdominal ultrasound-guided embryo transfer was first described by Strickler et al. (485). During the last 20 years numerous studies have been performed comparing the “clinical touch” and ultrasound-guided embryo transfer, sometimes with conflicting results. However, meta-analysis of randomized controlled trials has demonstrated the benefit of ultrasound guidance in pregnancy and live birth rates (486). Furthermore, prospective randomized trials comparing 2D-TVUS with abdominal scanning showed that the former technique seems to be superior in visualizing the catheter tip, so lessening the discomfort of patients. Thus, current knowledge strongly supports the use of ultrasound as a routine method for guiding embryo transfer.

## Pre-treatment assessment

During recent years, using TVUS to assess women prior to IVF has been shown to be useful. It can be used for identifying adnexal and uterine pathology and for assessment of the ovarian reserve. The latter information can be used as a possible indication of successful treatment as well as aiding in clinical decisions, such as which stimulation protocol to use.

**Antral follicle count.** Prior to IVF treatment, many doctors and patients would welcome a predictive test which could indicate the chance of success. However, no such test is currently available. Antral follicle count by TVUS, with the purpose of assessing the ovarian reserve, is a tool that has attracted much interest over recent years and several studies have been published. The test is based on counting small follicles (2–10 mm) in a 2D-ultrasound scan of both ovaries. An important limitation of the method is that it is not standardized. It appears to be of similar value to measurements of the antimüllerian hormone in the prediction of ovarian response, and results appear to be contradictory. Further studies are needed concerning the use of antral follicle count in examining ovarian aging and predicting pregnancy.

## Conclusion

Ultrasound has become a key instrument in clinical IVF. Its development started with the exploration of the



possibility of using ultrasound for guiding oocyte retrieval. This led to the introduction of vaginal sonography, which turned out to be the ideal tool for guiding oocyte retrieval and is a method which has become the gold standard.

Since then, vaginal sonography has also proved to be an extremely important tool for cycle monitoring and embryo transfer, and shows promise in the pre-assessment of the IVF cycle.

## MALE REPRODUCTION

### SEMEN ANALYSIS

Mark Sigman, M.D.

The analysis of seminal plasma has a storied history much longer than that of in vitro fertilization. The discovery of sperm begins with Johann Hamm, a Dutch medical student who, in 1677, brought Antonie van Leeuwenhoek a sample of pus mixed with semen; likely from a male with gonorrhea. Hamm reported that the sample was from “a man who had lain with an unclean woman.” He had examined it under a microscope and noticed many animalcules. Leeuwenhoek confirmed the student’s findings and then decided to examine his own semen. Due to religious restrictions against masturbation, Leeuwenhoek had intercourse with his wife, then collected and examined the sample. He found millions of tiny animalcules moving about and called them semen animals (spermatozoa). He eventually wrote his findings in a letter to the Royal Philosophical Society of London taking care to note that his semen was collected “without sinfully defiling myself, remains as a residue after conjugal coitus” (487, 488).

There was very little advancement in male fertility evaluation over the next several centuries due to many societal prejudices and assumptions such as the long held belief that the ability to copulate was evidence of fertility. Clinical evaluation began with post-coital testing which was advocated by Dr. Marion J. Sims to the New York County Medical Society in 1868. He noted the presence or absence of sperm on examination of post-coital cervical mucus. He also encountered much prejudice against examining semen from the press which reported that “dabbling in the vagina with speculum and syringe was incompatible with decency and self-respect” (489).

Modern quantitative counting of spermatozoa was developed by Macomber and Sander in 1929. They concluded that pregnancies could occur with sperm concentrations of <60 million/ml and that higher counts increased the chance of pregnancy. The first comprehensive approach to the study of male fertility incorporating the semen analysis was by Robert Hotchkiss in his 1944 book *Fertility in Men* (489). He itemized the primary parameters of the semen analysis as volume, viscosity, motility, sperm concentration, and sperm morphology. Of interest, motility was gauged by three parameters: the percentage of motile sperm, the quality of forward movement, and the change in motility over a 24-hour period. While the first two metrics remain as part of the modern analysis, the last is no longer reported as it has been found to have no significant clinical relevance. Without the Clinical Laboratory Improvement Act and Occupational Safety and Health

Administration regulations, simple measurement techniques were suggested such as the determination of viscosity by compression of a drop of semen between the index finger and thumb of the bare hand and of observing the tenacity of the drop when the fingers are separated (490).

The semen analysis was refined by John MacLeod, a Ph.D. and Rugh Gold (a statistician) who studied the semen parameters of Dr. Hotchkiss’s fertile and infertile couples over a period of decades from the 1940s up through the development of in vitro fertilization (IVF) in the 1970s. The methods of proper semen analysis were reported in the 1977 book *Male Infertility* which noted that while sperm morphology was related to the chance of conception, abnormalities of morphology had no relationship to abnormalities of pregnancy after conception (miscarriage, ectopic pregnancy, or stillbirth) (491). The authors also advocated for measurement of seminal pH as well as antisperm antibody assays. It is during the 1970s that the World Health Organization (WHO) became involved in the evaluation of semen. This stemmed from the organization’s interest in contraception development through the Male Task force of the WHO. It was initially chaired by Dr. Alvin Paulson a former president of the American Society for Reproductive Medicine (1980). The WHO was not particularly interested in the evaluation of the infertile male, but because male contraceptive development required the ability to perform proper semen analyses; the organization pursued the development of standards of analysis. This led to the publication of the 1st edition of *WHO Semen Manual* in 1980 which has been revised five times through the 5th edition in 2010. One problem that plagued the interpretation of semen parameters from the beginning is how to interpret the results. Up until the 5th edition, lower limits (often termed limits of adequacy) were used for parameters that were based on expert consensus. These stemmed from MacLeod’s work wherein he initially suggested that a lower threshold of sperm density of 60 million sperm/ml was appropriate. However, his analysis of semen parameters of fertile and infertile couples ultimately led him to recommend a lower limit of 20 million sperm/ml. His data showed that fewer fertile men than infertile men had counts below that limit. This limit was finally changed in the 5th edition of the WHO manual which utilized semen data from a large international cohort of men that conceived within 12 months to develop reference ranges identifying the lower 5% one sided confidence interval thresholds (492, 493). This approach, while statistically derived was quite controversial and confusing to many practitioners (494). While the WHO considers the ranges as reference ranges, not normal ranges, many clinicians interpret the thresholds as absolute

indicators of infertility, despite the fact that 5% of fertile men had parameters below the thresholds and many infertile men had values above the thresholds.

Over the last two decades, modern statistical analysis has been utilized to examine the metrics of the parameters of the semen analysis. Most modern diagnostic tests are evaluated by metrics such as sensitivity, specificity, positive and negative predictive values, and by receiver operating characteristic curves. When individual semen parameters are analyzed, what we find is that they fair quite poorly; area under the curve (AUC) for count, motility, and morphology are generally  $<0.7$  which indicates the semen analysis is not very robust assay when examining individual parameters (495). Have the last 40 years of data shown us we should discard the semen analysis as a test for male fertility? Until newer, more robust tests are developed, the semen analysis still remains a critical component of the male evaluation. However, what has also become apparent is that the more parameters (count, motility, morphology) that are below thresholds, the greater the chance of infertility. Over the last 40 years we have come to realize that the semen analysis needs to be taken for what it is: a set of multiple metrics that help evaluate the male partner but should be used as part of, not as a complete male evaluation. What we have gained over the years are more robust well determined threshold limits.

The story of sperm morphology scoring encompasses at least five different scoring systems developed over the last fifty years. All have struggled with defining what is a normal sperm and what is the threshold value of normal forms. In the 1960s, MacLeod promulgated a scoring system that focused on evaluating sperm heads and grouping sperm into a variety of abnormal categories with fertile men having at least 60% normal forms. This data emanated from his semen data on the fertile and infertile couples evaluated in his laboratory. While this classification system became the most commonly utilized one from the 1960s to 1980s, one obvious limitation of this approach is that tail and midpiece abnormalities, which may cause infertility, were ignored in this system. The paradigm of this approach was that a sperm was considered normal unless it fit a defined abnormal category. The early editions of the WHO manual primarily used this type of approach while adding tail and midpiece abnormalities as categories. Despite the wide use of MacLeod type categorizations, experts frequently could not agree on what was a normal sperm and as a result, thresholds were consensus driven with much disagreement among experts. This was apparent when the WHO changed the threshold from 60% to 30% in the 3rd edition of the WHO in 1992; a change that confused many people as it was not based on well-defined data sets. A major paradigm shift occurred in the late 1980s and early 1990s (496) when a group from South Africa reversed the long-held concept that sperm were normal unless they fit an abnormal category. They noticed that sperm in the upper endocervical canal all had a similar appearance and used these sperm to define normal with very precise criteria. Sperm that did not fit those criteria were classified as abnormal. We then dropped from multiple categories of sperm morphology to two: normal and abnormal. While to some degree this approach simplified sperm morphology

scoring, it led to an unintended consequence, one technician's normal, was another's abnormal. Survey results indicated a wide variability in scoring among laboratory technicians. The problem emanates from some of the natural subtle variations in the appearance of stained sperm heads. The classification still required a line to be drawn between what was normal variation and what was pathologic variation. To add to the confusion, many technicians unknowingly move the line and become stricter over time unless careful repeated training and proficiency testing was incorporated into the laboratory protocols.

With this new strict criteria, a lower threshold of the fertile population needed to be defined. While examination of endocervical sperm helped define normal sperm morphology, fertility data was needed to set a threshold. The initial publications identified a 14% limit for normal morphology based on pregnancy rates from IVF cycles. Further analysis suggested a middle category of 4% to 14% of reduced but still good prognosis with  $<4\%$  as having a poor prognosis. While many IVF laboratories began utilizing this method and threshold, the data was often misinterpreted, and limitations not understood. The initial data was from men with isolated sperm morphology defects (other parameters were normal). This did not represent the average infertile male's semen profile. In addition, the outcome was pregnancy by IVF, not intercourse. In the current 5th edition WHO manual, which utilized data from analysis of a large population of fertile men conceiving through intercourse, determined the lower one-sided 95% confidence interval limit to be 4%. Interestingly while the initial data using strict criteria came out of IVF, some IVF labs have found little predictive value to sperm morphology and with the advent of ICSI, morphology is not always predictive. This has led many to completely ignore sperm shape, while others over interpret mild defects. Thus, with well over 50 years of morphology scoring development, there remains much room for improvement in laboratory technique and interpretation.

The standard semen analysis has been and remains a labor-intensive assay. As computers evolved over the last 30 years, they have been applied to develop computer assisted semen analysis equipment (CASA). These have primarily been utilized to measure sperm density and motility. While early enthusiasm envisioned CASA machines rapidly performing clinical semen analyses in most laboratories, we have seen only a small fraction of labs incorporate CASA machines for clinical use. The machines offer the measurement of standard parameters as well as a whole variety of additional measurements such as straight line and curvilinear velocity. Unfortunately, there is little evidence those parameters add clinically useful information beyond what is obtained from the manual measurement of parameters. Also, inaccurate measurements of very low sperm densities have been a problem and the high cost was and remains prohibitive for many labs. Current machines offer all standard semen parameters while some also measure additional metrics such as morphology, sperm DNA fragmentation and rates of the acrosome reaction. If ease of use improves, measurement of all parameters (including) morphology becomes standardized and

reproducible, and costs drop, we may see more utilization of this interesting technology. Until then, the manual semen analysis remains the gold standard—unless of course testing moves out of the lab to be performed by home kits for use by patients. We now have home kits to determine if sperm counts are low and some utilize smart phone dongles and apps and examine motile sperm counts. While these are not made to replace the laboratory analysis and only measure limited parameters, this technology is in its infancy and only time will determine the limits of these innovative approaches.

In the 1970s and 1980s, almost anyone could perform semen analyses. In my residency, all residents performed manual semen analyses in the clinic. We utilized manual, mouth suction for pipettes—we did have a catch tube attached with rubber tubing—and safety was generally not a concern (HIV had only recently been reported), and there was nothing remotely resembling proficiency testing. Although the Occupational Safety and Health Administration was established in 1971, it had clearly not made it to the sperm lab. Universal precautions were not introduced until the mid-1980s when the transmission of HIV and Hepatitis C became national health concerns. The days of residents and medical students performing diagnostic semen analyses ended with the introduction of CLIA 88—the Clinical Laboratory Improvement Act of 1988. These regulations followed national concern about lax laboratory standards reported in a Pulitzer Prize winning 1987 article in the *Wall Street Journal* entitled “Lax laboratories: the Pap test misses much cervical cancer through labs’ error—cut-rate ‘Pap mills’ process slides using screeners with incentive to rush—misplaced sense of security” (497).

While the semen analysis has remained a cornerstone in the evaluation of the male, the limitations of the assay have encouraged the development of alternative measures of male fertility. This has led to what are often called sperm function assays—metrics that attempt to determine the fertility potential of sperm based on the ability to perform biologic functions more closely linked to natural fertilization. One of the first functional assays, the post coital test, originated in the 1800s as mentioned above. To remove some of the biologic variability of such an *in vivo* assay, variations were developed to make this a more robust measurement of pure male fertility. Thus, we have seen cervical mucus placed in capillaries with sperm penetration depth measured. The crossed mucus hostility assay utilized both patient and donor sperm and patient and donor mucus to identify the source of poor penetration. To completely remove the human female partner from the equation, bovine cervical mucus was utilized in another *in vitro* test. As with other functional assays, there were contradictory reports of the correlation with IVF results—some reporting good correlation, others poor correlation. While the ability of sperm to penetrate cervical mucus *in vivo* is clearly an important biologic function of *in vivo* fertility, the value of *in vitro* testing has lost support since many couples move to Intrauterine insemination (IUI) or IVF/intracytoplasmic sperm injection (ICSI) if they fail to conceive with intercourse regardless of the cervical mucus test results.

Further innovation with sperm function assessment brings us back to the beginnings of IVF when in 1976 the zona free hamster oocyte penetration assay (SPA) was developed (498). It had been noted that sperm binding to the zona pellucida was species specific. If the zona pellucida was removed, human sperm could penetrate hamster ova and undergo nuclear decondensation. This required sperm to be motile, be able to undergo hyperactivation, capacitation, membrane fusion, and nuclear decondensation. This truly measured a variety of actual sperm functions. The metric that was measured was the percentage of ova penetrated or the number of penetrated sperm per ova depending on the protocol utilized. Unfortunately, protocols often differed between laboratories and the false positive and false negative rates were inconsistent between protocols which limited the acceptance of this assay. However, it had been used for couples to determine if they should proceed with IUI if the test was normal or IVF, or ICSI if abnormal. The assay also gained attention when tabloid newspapers reported that the assay created hamster-human hybrids. This required clinicians to be clear with patients that this was not the case, despite the evidence presented by pictures in the tabloids. Other functional assays such as the hemizona assay were developed in the 1980s and 1990s. This particular technique measured the ability of sperm to bind to human zona pellucida; a process not measured by the SPA. While data indicated it did help predict IVF failure, issues such as access to human zona pellucida and the technical requirement of splitting human zona in half, precluded widespread use of this assay. Most of these functional assays went by the wayside with the development of ICSI which bypassed most steps that these assays measured.

The last decade has seen the development of newer assays such as testing for reactive oxygen species (ROS) and the measurement of sperm DNA fragmentation (SDF). Reactive oxygen species in small amounts are required for normal sperm processes such as hyperactivation and sperm capacitation. On the other hand, excess amounts of ROS can injure sperm plasma membranes and cause fragmentation of sperm DNA. There is a push and pull relationship between ROS production and ROS neutralization by seminal and cytoplasmic antioxidants. When production exceeds antioxidant capacity a pathologic state exists called oxidative stress. Both seminal leukocytes and immature spermatozoa produce ROS. It has been clearly shown that semen from infertile men has more ROS and oxidative stress than semen from fertile men. A variety of assays have been described to measure the amount of ROS and the antioxidant capacity of semen. Proponents hope this assay would identify patients in whom excess ROS is etiologic in their infertility and in whom treatment with oral antioxidants may reduce oxidative stress and improve pregnancy rates. As we have seen with other assays, there have been problems with lack of standardization between laboratories limiting the widespread acceptance of this assay. In addition, additional studies of well-defined populations with pregnancy as an outcome are needed for further acceptance of this approach. The limited evidence has not inhibited the marketing of multiple formulations of oral antioxidants, which are primarily mixtures of vitamins and nutraceuticals, over the internet to infertile couples. In 1980, the concept that

defective sperm chromatin structure was associated with reduced fertility was described by Evenson et al. (499). Thus, SDF testing was born. The measurement of the quality of sperm chromatin has been approached by a variety of techniques the most common being by flow cytometry, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), Comet, and sperm chromatin dispersion imaging. Much data exists indicating higher SDF in infertile populations than fertile populations. In addition, lower pregnancy rates by intercourse, IUI, and IVF, and higher miscarriage rates have been reported in couples in which the male has high rates of SDF. Interestingly the negative effect of elevated SDF on ICSI outcomes is mild (and controversial). What is not clear is why the effect of elevated SDF is muted with ICSI but more prominent with other approaches to conception. Over the last decade there have been multiple meta-analyses that have examined the data, many pro and con articles, as well as debates that discussed the value of the tests. While most would agree that lots of sperm DNA fragmentation is not a good thing, the role of these assays is still unclear with no consensus on the indications for utilizing these assays in the management of the infertile male. Currently the American Society for Reproductive Medicine does not recommend SDF testing as routinely indicated for infertile couples but suggests that the results may be informative in individual cases.

While the history of the semen analysis starts in 1677, the most changes have occurred over the last 40 years. As societal disapproval of analysis of semen was left in the past, techniques have been refined and standardized while thresholds better defined using modern statistical methods. We have also seen criticism of the value of the semen analysis continue. What has become clear is that it is not a perfect test for male fertility; it has many flaws, and many detractors. This has led to the development of a variety of other approaches to the analysis of sperm. Some, such as the SPA and hemizona assays are quietly resting in peace, having been abandoned by most laboratories. Others such as SDF testing remain hotly debated and continue to undergo refinements. However, of all the advancements that have occurred, the semen analysis, flaws and all, remains the only test that is indicated and recommended as part of the male evaluation for all couples. Careful examination of van Leeuwenhoek's animalcules has stood the test of time.

## ADVANCES IN DECISION MAKING FOR THE EVALUATION OF AZOOSPERMIA IN THE MODERN AND POST-MODERN ERA

**Richard A Schoor, M.D.**

Ever since the first embryo was created in the lab using in vitro fertilization (IVF) and later intracytoplasmic sperm injection (ICSI) the entire approach to management of the azoospermic male has changed. Prior to the advent of these amazing technologies, men with uncorrectable azoospermia were told to adopt or to use donor sperm. Now, in the current era, telling

a couple after a only a basic evaluation that they should simply use donor sperm or to adopt can be considered mean and uncaring and couples given this advice will often seek second opinions from urologists who are more willing to explore alternatives and pursue the most cutting edge management for azoospermia. Intracytoplasmic sperm injection, which requires only one sperm, in contrast to standard IVF which requires 10,000 or greater numbers of motile sperm, has particularly revolutionized the management of the most hopeless male fertility cases, because with ICSI, so long as the urologist could find just one sperm, even an a-motile one, the couple could have a biological child of their own. All of the sudden the game changed completely with no follicle-stimulating hormone level too high, no testis too small, no genetic anomaly unsurmountable (500). However, this is not exactly true as certain deletions on the AZF locus are associated with total absence of sperm within the testis. In addition, the diagnostic testicular biopsy, in the minds of a few progressive urologists and followed by an increasing number, became superfluous and unnecessary, and men were increasingly being counseled to undergo sperm retrieval surgery followed by ICSI and to forego the intermediary step of diagnostic biopsy.

The only problem with this approach was that in at least 50% of cases of idiopathic azoospermia, cases in which an obvious cause for obstruction was not apparent, no sperm found on testicular sperm extraction (TESE), and in the case of a TESE done in conjunction with a fresh egg retrieval, the couples would have undergone an IVF/ICSI attempt for, in the words of some patients, nothing. Coming out of a procedure room after a negative attempt at locating sperm on TESE is very unpleasant. Delivering such news to the hopeful couple is stressful to any caring urologist and devastating to the couple emotionally and often financially. Many urologists who practice advanced male infertility, such as myself, have spent entire careers refining our crafts just to avoid whiffing on sperm retrieval procedures. Thankfully, because of improved use of routine clinical data; improved ultrasound imaging and diagnosis; genetic diagnosis and counseling; data science/information technology; advanced surgical innovations and operative microscopy; sperm cryopreservation; and current concepts of informed consent and shared medical decision making, male infertility specialists whiff with much less frequency and have patients with high sperm retrieval and pregnancy rates and, for those we cannot help, few patient regrets regarding evaluations and treatments neither tried nor discussed. These advances are detailed in this article.

### Background

Azoospermia can result from blockages of the ductal system. When this occurs, the azoospermia is classified as obstructive azoospermia (OA). The cause of the majority of OA cases can be determined by history and physical examination alone, with history of prior vasectomy among the most common etiologies. Other conditions that can be determined on readily available clinical evidence include vasal obstruction from prior hernia repair, with or without mesh, congenital vasal agenesis, prior extirpative pelvic surgery, or ejaculatory duct obstruction.



When the testicles don't produce sperm at all or they do not produce sperm cells in sufficient number to get into the ejaculate, it is classified as nonobstructive azoospermia (NOA). Common conditions that cause NOA include current or prior usage of exogenous testosterone, history of undescended testes, history of malignancy and chemotherapy, current malignancy, genetic anomalies, environmental exposures, and pituitary dysfunction, among others.

The importance of differentiating OA from NOA is that sperm retrieval techniques are always successful in locating sperm from OA patients, but locating sperm from NOA patients is challenging in the best circumstances and can be impossible in some. In order for the urologist to give good advice to his patient/couple, it is vitally important to accurately differentiate OA from NOA and for those with NOA, to be able to give the patient a reasonable probability of a successful retrieval. Armed with the advice, patients can decide what is best.

### Advances in our use of routine clinical data

Under the care of any competent male infertility specialist, the etiology of a man's azoospermia can be diagnosed using little more than clinical data derived by a comprehensive history and physical examination with routine laboratory confirmation. In many patients, the etiology of a man's infertility is clear within minutes of meeting the patient and eliciting a history. All that is needed in such cases is confirmatory physical findings and diagnostic testing. In fact, the male infertility clinical evaluation can be considered checklist-like and all that the urologist needs to do is to identify items on the list. A well-designed electronic health record template can help. [Table 11](#) lists the pertinent clinical data that can be used to differentiate one category of azoospermia from another in the majority of patients.

**Endocrine evaluation.** The blood tests most likely to add diagnostic information are the gonadotropin levels, follicle-stimulating hormone (FSH), and luteinizing hormone (LH). Testosterone, while not very useful as an isolated test, is helpful in relation to FSH, LH, prolactin, and estradiol levels in determining the overall healthy function of the male reproductive gonadal-pituitary system. Analyses of FSH and LH levels, in and of themselves, have been shown to differentiate OA from NOA with a high degree of accuracy. With only testicular long axis and FSH/LH data, Schoor et al. ([500](#)) demonstrated that one could predict OA versus NOA with >95% accuracy. Of course, in clinical practice one has access to much more data than just FSH/LH and testicular long axis.

**Semen analysis.** Azoospermia is diagnosed by the lack of sperm cells in at least two properly prepared and centrifuged semen samples. While a semen analysis is, in most cases, a crude indicator of a man's fertility potential, the consistent finding of total absence of sperm on multiple analyses indicates that the man is 100% infertile. Semen parameters such as pH, semen volume, and the presence/absence of fructose add additional and useful clinical data. Clinical and endocrine data, will also establish a diagnosis of OA versus NOA in >90% of cases. Compared to other urologic conditions,

such as prostate cancer diagnosis, urologic pain disorders, and others, this small degree of diagnostic uncertainty is actually pretty impressive. Use of additional tools improves accuracy even more.

### Improved imaging technology

**Ultrasound.** Ultrasound has come a long way since the early 1990s when I was a medical student at the University of Pennsylvania and my radiology professor described abdominal ultrasound as "looking at the weather over Chicago." Resolution was so poor as to virtually be useless to all but the most skilled sonologists. In the early 1990s testicular ultrasound had limited utility and the major debate at that time was whether or not it could be used to reliably determine if the testis has vascular flow. As ultrasound quality improved and became more ubiquitous, male infertility specialists started detecting non-palpable testis tumors and varicoceles. Now what to do?! This forced clinician-researchers to determine the clinical significance of these findings.

Current ultrasound technology has become quite impressive in terms of resolution, Doppler and Power Doppler flow and wave-form analysis. Now, super-high frequency probes exist that can resolve individual seminiferous tubules and peri-tubular blood vessels and some urologists suggest that pockets of sperm production tend to be near such vascular areas (American Urological Association [AUA] Basic Ultrasound Course). Moreover, the cost of high quality sonographic units has come down and is now affordable to private, and even solo, practice urologists for use at the point of care. Having the ability to perform an ultrasound oneself, as an extension of the physical exam, enables the urologist to immediately relay information to the patient and investigate findings real-time, as needed, and very efficiently. Finally, current ultrasound images are stored digitally and the information embedded in the files will likely, in the near future, be available for datamining.

Though AUA best practice guidelines for the evaluation of azoospermic male do not recommend the routine use of ultrasound, this inexpensive, readily available, and safe study can improve diagnostic accuracy and change management for men with azoospermia ([501](#)). While the aim of this article is not to argue that ultrasound should or should not be routinely performed in men with azoospermia, information obtained by ultrasound can certainly be useful in differentiating between OA and NOA ([502](#)).

Testis measurements such as long axis length, width, and height are easily determined by ultrasound and from these measurements, volume can be accurately determined. Though using an orchidometer is certainly a less expensive way to measure testis volume, the ultrasound can measure volume as accurately, if not more so, because orchidometers rely upon best estimation by the clinician and not all clinicians are equally experienced in their use. Since the majority of a testicle's mass is comprised of seminiferous tubules, as opposed to Leydig cells, small testicles are more generally consistent with disorders of sperm production rather than obstruction ([503](#), [504](#)).

**TABLE 11****Clinical risk factors for obstructive azoospermia and nonobstructive azoospermia.**

OA				
Prior vasectomy	Prior Hernia	Vasal agenesis	STI history	Testis LA >4cm
FSH/LH between 3 and 7.5	Dilated SV	Midline prostate cyst	Dilated ejaculatory duct	Dilated rete testes
Hx CF	Fhx CF			
NOA				
History TRT	Testicular atrophy	FSH/LH >7.6		Testis mass
Prior malignancy	Hx chemotherapy	Heterogeneous parenchyma on sonogram	Decreased intratesticular vascular flow	Hx of torsion
Hx UDT	MicroY +	Abnl karyotype	Pituitary dysfunction	

Note: CF = cystic fibrosis; FSH = follicle-stimulating hormone; Fhx = family history; Hx = history; LA = long axis; LH = luteinizing hormone; NOA = nonobstructive azoospermia; OA = obstructive azoospermia; STI = sexually transmitted infection; TRT = testosterone replacement therapy; UDT = undescended testicle.

Forty years of IVF. *Fertil Steril* 2018.

Tumors may be found in 4% of men who present to the fertility center for evaluation and fifty percent of men with testis cancer are infertile/subfertile at the time of diagnosis (505). While the majority of testis tumors can be detected on physical exam, not all can, and an occult tumor can be associated with oligo and azoospermia, and of course occult tumors can also be malignant. Ultrasound is a great imaging modality for testicular parenchyma but, as the argument goes, it may be too good. Many of these occult tumors are benign and detecting one puts the patient at risk for unnecessary surgery, including orchiectomy at worst and testicular biopsy at best, and it always leads to anxiety of health in all affected individuals. However, for the purposes of detecting testis tumors that may be contributing to a man's azoospermia condition, occult or otherwise, ultrasound is useful.

Varicoceles are associated with male infertility and abnormalities in sperm production and function. Very few men with varicoceles as an isolated finding are azoospermic and clinically significant varicoceles are, by definition, palpable on physical exam. As such, the presence of a varicocele, clinical or subclinical, on ultrasound does not in and of itself add much useful information in the differentiation of OA from NOA. However, as part of a constellation of findings, confirming their presence on ultrasound has value.

Tubular ectasia is a dramatic sonographic finding of cystic dilation of the rete testes. This is a benign condition and its presence has been postulated to be associated with epididymal obstruction (506). The rete testes can be seen in proxy form by estimating the width of the mediastinum testis, which can be easily seen on ultrasound. Mediastinal testis width as a predictor of obstruction in men with persistent azoospermia post vasovasostomy is under investigation at present.

The epididymis is readily imaged on ultrasound and epididymal pathology can easily be determined. As the normal thickness of a human epididymal head is in the 7–8 mm range, enlargement can be an indication of an obstructive etiology (507). Missing segments of the epididymis or total absence of the epididymis on ultrasound suggest an obstructive etiology as well. Solid tumors of the epididymis, though rare, would also suggest an obstructive etiology.

Ultrasound is a great study to determine the vascularity and flow within the testicular parenchyma. Ultrasound with Doppler flow can be useful not just for the detection of torsion, but it can also be useful to determine whether or not a testis has suffered a prior vascular incident such as torsion or as a result of a severe infection. As genital tract infection can also cause epididymal obstruction, one must use the totality of ultrasound findings, rather than a singular finding, to be of use of differentiating OA from NOA. Findings of diffusely heterogeneous parenchyma with low testicular volume and low but present Doppler flow are indicative of an unhealthy testicle as opposed to a healthy but obstructed testicle (508).

**Transrectal ultrasonography.** Transrectal prostate sonography is an important tool in the evaluation of men with azoospermia. Information learned from this readily available study can be used in conjunction with that learned elsewhere to maximize diagnostic differentiation of OA versus NOA. Ejaculatory duct obstruction is an unusual and difficult-to-diagnose disorder characterized by low volume, acidic ejaculate and oligo, and on occasion, azoospermia. On transrectal ultrasonography one can often, but by no means always, see dilated seminal vesicles and a dilated ejaculatory duct. While there are no absolutely diagnostic features for this condition, when a patient has enough findings, obstruction is likely. Such findings include midline prostatic cyst, dilation of the seminal vesical, and seminal vesical agenesis.

**Magnetic resonance imaging of the pelvis.** Magnetic resonance imaging (MRI) has little role in the evaluation of male infertility in general and azoospermia in specific. Perhaps the only time to order such a test is in cases of suspected ejaculatory duct obstruction, though even this is debatable. Magnetic resonance imaging offers little additional information over transrectal ultrasonography, at least information that is clinically useful, and MRI is an expensive test that is very difficult to obtain from an insurance authorization perspective, as a purely pragmatic matter. If ejaculatory duct obstruction is suspected, rather than get an MRI, perhaps seminal vesicle aspiration is a more direct approach that will yield greater diagnostic information and potentially enable retrieval of viable sperm and their cryopreservation (509).

## Genetic diagnosis and counseling

Advances in genetic diagnosis has significantly impacted decision making for men with azoospermia. These tests are now readily available in most commercial diagnostic labs and are usually covered by insurance. Any man with azoospermia who does not have a clear reason for the azoospermia, such as prior vasectomy or use of exogenous testosterone, ought to have genetic evaluations that include karyotype determination, genetic evaluation of the Y-chromosome, and a cystic fibrosis panel, per the AUA best practice guidelines for the evaluation of the azoospermic male (500). The presence of one or another genetic defect will make the diagnosis. Men with 47-XXY (Klinefelter) will be NOA, while men with cystic fibrosis mutations are OA. The presence of a Y chromosome micro-deletion indicates NOA. In addition, certain deletions of the AZF region are associated with absence of sperm on advanced sperm retrieval procedures and these men should never be offered surgical sperm retrieval surgery (509). Genetic diagnosis has been a major advance in the evaluation of azoospermia since sperm can always be found in OA patients yet not always in NOA patients. In addition, the presence of deletion on the AZF B locus is 100% predictive of the absence of sperm anywhere within the testes. No other diagnostic blood test or imaging study can predict whether sperm can be found within the testes, even when given a thorough surgical search.

One additional item ought to be discussed in regards to genetic diagnosis. Prior to the advent of ICSI, such genetic abnormalities were never passed onto offspring. This is no longer the case and any genetic defect in sperm can be transmitted to the embryo. Couples must be counseled accordingly even though few couples refuse to proceed with assisted reproductive technology upon learning about transmissibility. In addition, preimplantation genetic diagnosis, testing the embryo for the presence of a gene, has become routinely available, even in community IVF centers, and armed with this information, the couple can decide which embryo to implant.

## Data science and information technology

Electronic health records (EHR) have been touted by many physicians as the bane of their existences and in studies on physician burnout, forced use of EHR technology is high on the list of causes of burnout, based on many physician survey studies. Physicians complain that EHR usage gets in the way of making eye contact with patients and impedes fostering a good doctor patient relationship. This was certainly true in my experience in 2000, when as a chief resident at the Chicago Lakeside VA Medical Center, an unhappy patient wrote a letter to my chairman stating, "Dr. Schoor entered the room, introduced himself, immediately went to the computer began typing then told me to drop my pants and bend over..." (Guilty as charged!). In addition to impacting communication between doctor and patient, many physicians cannot get through, in a timely manner, patient encounters due to all the clicking involved in documenting them. Enter the newest member of the medical work-force, the scribe.

The EHR is not all bad and, in my own opinion, the EHR might just be the best single advance in medicine in modern times! Electronic health records make datamining possible and possible at levels never before seen in world history. While EHR use results in legible documentation, fewer prescription errors, and efficiencies in billing and coding, it is the conversion of data from paper to digital that is the most important feature of EHR technology. Having a comprehensive male fertility history template in a check box fashion, or a similar one, as part of the EHR requires that the physician (or scribe) capture each and every pertinent item in the comprehensive male infertility assessment (Table 11). Not only that, all laboratory data and radiographic imaging data is now in digital format and accessible. Data captured in this way is granular and accessible for research. Multiply this by thousands of male fertility specialists in the U.S. alone and tens of thousands worldwide, and one can see that opportunity for big data research is awesome indeed.

While the majority of urologists have electronic medical records these EHR systems are disparate and not interconnected. Such *Tower of Babel* lack of interoperability has been a problem in the past but not so much anymore. Data from such disparate systems is already visible and readable on clearinghouses and other web-based data repositories. As such, datamining is possible and currently being performed in research settings. The AUA Quality Registry Program (AQUA) is a software system embeddable in almost any electronic health record software system and it can map any type data, whether it be in discreet, granular form or even if non-granular. Data can be aggregated and is currently being used for research purposes (510, 511).

Watson is a very sophisticated cognitive computing system that has a number of applications in a variety of industries, including health care. Using IBM's Watson, researchers are to perform medical research by analyzing data on a scale never before seen (512). Watson can access any digital data and analyze for patterns not apparent or obvious to human intelligence. In the culinary world, Watson is creating novel recipes that have been tested on human taste buds with good results. These very powerful tools may result in significant progress in our capability to predict non-invasively whether or not a man has OA or NOA and hopefully it will be able to predict with high probability that sperm will be present and retrievable in an NOA testicle. It is only matter of time until Watson, or something like it, will be able to guide the urologist caring for an azoospermic male.

## Advanced surgical innovations and operative microscopy

The two innovations detailed below have made negative sperm retrievals, if not a thing of the past, certainly less frequent of an occurrence. Most couples, in my own 17-year experience, want to know that everything that could be done medically has been done and only then can I refer them to donor sperm or adoption. This does mean that donor sperm or adoption are never mentioned earlier in the process, because they are mentioned, but it is that couples are not willing to realistically accept them as viable alternatives until



a thorough evaluation has been done. The testicular fine needle aspiration (TFNA) mapping and microdissection testicular sperm aspiration (TESA) are options for these very couples. At urology and fertility meetings that I have attended, Paul Turek and Peter Schlegel, the pioneers of the two innovations that will be discussed, have debated in open forum that one technique is better than the other. Nonsense! The TFNA mapping and testicular microdissection are complementary and when used appropriately and with expertise have enabled previously unattainable success rates for sperm retrievals and ICSI and thousands of couples to have babies that otherwise could not have done so. Absolutely incredible.

TFNA mapping is a diagnostic office-based procedure popularized by Turek et al. (513). The procedure, which will not be described here, is performed in an office setting under perivascular cord block. Critics of TFNA mapping state that it is a diagnostic-only-procedure, and as such, it does not directly improve the couple's ability to get pregnant. Committing a man to a TFNA mapping means that he might have one unnecessary procedure at worst, in the case of a mapping that shows no sperm, and at best he will need to have at least one, if not more than one, invasive procedures to harvest that sperm. The TFNA mapping is invasive itself and can result in injury and, at least in theory, loss of the testicle due to vascular injury. Proponents report that the TFNA mapping is quick for the patient, very well tolerated, and with little, but not zero, risk. Finding sperm on a TFNA mapping almost guarantees that sperm will be found in sufficient quantity to use for reproductive purposes during a future TESE. In addition, the approximate geographic location of that sperm can be determined in advance of a TESE, thus improving the efficiency of the TESE. In cases where no sperm is found on TFNA mapping, the couple can be counseled in advance that proceeding to standard TESE is ill-advised and that they would benefit either from testicular microdissection (mTESE) by a qualified doctor in a capable center or that they ought to proceed with donor sperm. This is perhaps the greatest advantage to the TFNA mapping: it can be done by community urologists with advanced fertility training yet they do not have access to reproductive endocrinology centers that are equipped to or are willing to handle mTESE and all that mTESE requires so that it can routinely be successful (514).

Testicular microdissection, mTESE, as developed by Schlegel, has been a revolutionary innovative technique that has enabled men with the most severe defects in sperm production to become biological fathers. While the details of the mTESE will not be discussed here, the essence of the mTESE involves making an extensive transverse opening of the tunica albuginea of the testis to expose all areas of seminiferous tubules for inspection under high power operative microsurgery, like turning a sock inside-out. Critics of the mTESE state that the very nature of the procedure puts the testes at risk for devascularization and permanent damage, though, at least in the hands of Schlegel and other experienced centers, this has not been found to be a significant problem (515). The main problem with mTESE is that it requires a significant and extensive level of expertise and infrastructure from not just the urologist but also from the REI centers and their embryology staff. As a result, mTESE is not practical in

many community settings, such as my own. Many patients with severe male factor conditions who lack the financial resources to get advanced reproductive care from mTESE centers of excellence cannot benefit from this procedure.

**Sperm cryopreservation.** The ability to reliably cryopreserve, store, and thaw sperm and then to show that the cryopreserved-thawed sperm function just as well as fresh sperm when used for IVF and ICSI has had an enormous impact on the management of azoospermic males. Prior to 2000, most reproductive endocrinology and infertility (REI) centers did not feel comfortable working with frozen-thawed sperm as they felt it would decrease their fertilization and pregnancy rates. They favored fresh sperm. Fortunately, ample research has demonstrated that fresh and frozen-thawed sperm from any source, ejaculated, testis, or epididymal, perform equally well in terms of fertilization and pregnancy rates for ICSI (516). While some REI centers still prefer to use fresh sperm, most have become quite adept at handling frozen-thawed sperm.

So why is this so important? There are several problems with using fresh sperm exclusively. One, demanding that the urologist be on-call for sperm retrieval is just not practical from a scheduling perspective. The fact that a woman may ovulate any day of the week does not work out well for a busy urologist's clinic or surgical schedule. Use of frozen and thawed sperm is perfectly okay for most circumstances, except, perhaps, for severe NOA cases in which sperm harvest may be so limited that no one is willing to risk any loss of sperm in the freeze-thaw cycle. Two, by using cryopreserved sperm, couples know going into the egg retrieval that they have sperm that can be thawed and used for fertilization. Three, multiple cryovials can be processed and stored, even in severe NOA cases, and thawed as needed, one by one, so that men rarely need to undergo more than one surgical sperm retrieval procedure. Four, cryopreservation of sperm is inexpensive as it costs several hundred U.S. dollars per year. Five, sperm can remain frozen for decades and be thawed as if nothing happened, like Rip Van Winkle, so long as they remained submerged in liquid nitrogen or within the vapor, undisturbed, at minus 196°C and the laboratory makes sure the levels of liquid nitrogen in the tanks remain adequate and monitored.

**Informed consent and medical decision making.** The 1990s saw a major change in how physicians spoke to and counseled patients; prior to the late 1980s/early 1990s, doctors said and patients did. There was a paternalistic attitude that doctor knew best and told patients what to do. I started medical school 1990 and we were taught differently, such as medical decision making was a joint process between patient and doctor; shared medical decision making. A patient's beliefs, desires, fears, biases, education levels, and ethics must be incorporated into a treatment plan, within reason. Such teaching became the norm in medical education by the early 1990s. Though not without problems of its own, shared decision making has had a major and a positive impact on the management of azoospermia.

The discussion between doctor and couple on how to proceed when faced with azoospermia is not a simple one. Many factors must be considered, such as the couple's ethical and religious directives; their financial and insurance issues;



concerns over success and failure rates; experience of the urologist and the REI center; feelings about donor sperm and adoption; concerns over complications and pain; and so on. The decision is certainly not, azoospermia then biopsy followed by TESE, but is much more involved with many decision points. Do we do TFNA mapping or mTESE or standard TESE etc., also do we obtain diagnostic tests such as endocrine profiles and genetic evaluation and imaging? Having a one-sized-fits-all approach is not acceptable and can create significant harm to patients, including financial harm, even bankruptcy, and yet not get the couple any closer toward having a child. The decision must be a shared one indeed.

### The future

This article has discussed the most significant advances in the past 50 or so years but we are really just starting. While the urologist's role in the management of azoospermia will remain important, it will change, as everything ultimately does. Replacement of urologists by artificial intelligence (AI) is unlikely in the near future though not impossible. However, what will likely happen is that AI will advance to the point of being useful to urologists caring for azoospermic men. In such a system, the urologist obtains all clinical data in an electronic format, as she does now, but she will submit the data to AI analysis. Probabilities will be calculated and location of sperm pre-determined. In nanoseconds, an AI can scour the individual's data, held in any digital environment, and compare it to data published in any journal or study or even any big data system (AQUA, registries, etc.), and then give a probability on likelihood of one diagnosis versus another and whether or not a TESE ought to be attempted. This is already functional and operational in the medical field (The Medical Futurist, <http://bit.ly/2rw0nr5>) and in other industries, such as recipe development (Watson) and is only a matter of time before it comes to ours. A health system, entity, or individual would own, or more probably license the use of such an AI. One can choose to fight AI or to embrace it like any other tool and use it to more effectively help men with azoospermia get the right treatment. Exciting times indeed.

### Summary

Adequate decision making in any endeavor requires access to accurate data and then good, high quality science and evidence-based interpretation of that data. The field of male infertility is data-rich and this has allowed clinicians to make tremendous advances in the evaluation and management of the infertile male. Within male infertility, the greatest strides have been made, in this author's opinion, in the areas discussed above. Moreover, as we are at the dawn of data science, cognitive computing, and artificial intelligence, perhaps this is most exciting time to be involved in the care of these men, whether it be at the level of the bench or the bed. Perhaps not-so-distant future, a human decision maker will be either replaced or more likely augmented by advanced computing and AI. Use of advanced cognitive computing and artificial intelligence will only serve to augment the clinician's decision-making. Urologist can choose to ignore the technology, to fight it, or to embrace it.

## MICROSURGICAL CORRECTION OF VARICOCELES AND OBSTRUCTIVE AZOOSPERMIA: HISTORICAL AND CURRENT CLINICAL PERSPECTIVE

Marc Goldstein, M.D. and  
Larry I. Lipshultz, M.D.

*Start by doing what's necessary; then do what's possible; and suddenly you are doing the impossible.*—Francis of Assisi

The first compound microscopes are attributed to the Dutch spectacle makers Hans Jansen, Zacharias Jansen, and Hans Lippershey. In the 1590s these inventors discovered that objects could be magnified using elongated telescope tubes. While during the late 19th century surgeons used magnification to help in tissue dissection, it was not until 1912 that the Zeiss Company introduced a relatively light binocular set of operating loupes worn like glasses. In 1923, the Leitz Company manufactured a prismatic loupe, which permitted beam splitting that allowed assistants to share the same view as the surgeon, thus facilitating teaching of surgical technique and photographic image capture. In 1921, an otolaryngologist named Carl Olof Nylén was the first surgeon to use a microscope in the operating room by using a monocular microscope rather than loupes. In 1946, the American ophthalmologist Richard Perritt borrowed a binocular operative microscope from his otorhinolaryngologist colleague for use during an eye operation. Subsequently, in the 1960s, as use of microsurgical techniques became widespread, surgeons innovatively improved existing surgical instruments to facilitate microsurgical approaches to a broader variety of operations. Needles and sutures were miniaturized by Swiss manufacturers under the indispensable influence of an orthopedic hand surgeon, Robert Acland. These surgeon-driven advances, along with the development of fiber optics, bipolar electrocautery, xenon lamps, and variable magnification microscopes have collectively maneuvered microsurgical approaches into mainstream use and improved patient outcomes.

The role of the operating microscope and microsurgery instrumentation has gained increased importance in urology over the past 30 to 40 years, especially as it pertains to both varicocele repair and vasectomy reversals. Herein we address these two procedures from a historical perspective, identify current state-of-the-art technical innovations and, importantly, describe the role of the varicocele repair in the era of in vitro fertilization with intracytoplasmic sperm injection (IVF/ICSI). Operative correction of the varicocele, when present, is by far the most common procedure for treating male infertility.

### Historical advances in urologic microsurgery

In 1979, Silber reported a case of irreversible azoospermia due to testicular artery ligation at the time of conventional varicocelectomy, and suggested that this was due to inability to identify testicular arteries without magnification (517). In 1983, Wosnitzer and Roth suggested use of optical

magnification and Doppler ultrasound to identify arteries during varicocelectomy, but did not present a technique or any cases. In 1985 at the Ferdinand C. Valentine Urology Essay Contest of the New York Academy of Medicine, Dr. Marc Goldstein's microsurgical artery and lymphatic-sparing technique for varicocelectomy using an inguinal approach was first presented by his resident, Jack Dwosh. In 1985, Joel Marmar and colleagues described a microsurgical subinguinal approach to varicocelectomy in 71 men. In addition to ligation, Marmar utilized injection of a sclerosing agent to occlude small veins (518). By allowing magnified, clear visualization of all spermatic cord vessels, selective ligation of veins with artery and lymphatic sparing could be achieved, resulting in excellent outcomes with lower recurrence and complication rates than non-microsurgical approaches. Goldstein et al. (519) reported 640 cases in 429 men in 1992 employing microsurgical inguinal varicocelectomy with delivery of the testis, facilitating acceptance of microsurgical varicocele repair as the standard of optimal surgical correction.

### Current state-of-the-art surgical innovations

Whether one uses an inguinal or subinguinal approach remains surgeon's choice, since outcome superiority studies of each technique have had inconsistent conclusions. Orhan and colleagues (520) retrospectively evaluated these new operative approaches: 82 microsurgical inguinal varicocelectomies and 65 subinguinal cases. The authors reported no significant difference between the two groups in operative time, semen improvement, or pregnancy rate, although the number of veins and arteries was higher in the subinguinal group. Goldstein reports that delivery of the testis provides direct visual access to every possible route of venous return, and in over 4,000 cases done by one of the authors (M.G.), the failure rate was <1% with minimal morbidity (521). Interestingly, in another much smaller non-randomized, retrospective study from the same institution, the results of microsurgical inguinal varicocelectomy with (N=55) and without (N=110) testis delivery were compared (522). At 1 year, the pregnancy rate was 40.0% for those with delivery and 55.0% for those without delivery. Finally, open microsurgical varicocelectomy has demonstrated lower recurrence rates and fewer complications, compared with laparoscopic or a high retroperitoneal approach, including a lower incidence of hydrocele formation (523).

One author (L.I.L.) has found several intraoperative techniques to be of significant benefit. The use of fine-tipped Gerald forceps allows for atraumatic grasping of veins for ligation. Permanent suture (i.e., 4-0 silk or microvascular clips) is essential to occlude the veins. Teasing the veins away from surrounding structures is facilitated with fine-tipped Jacobson clamps (ASSI) or the same micro-needle holders used for vasovasostomy (ASSI.B138). Identification of the internal spermatic arteries is greatly facilitated by using a micro-tipped Doppler (VTI Instruments), consisting of a 1mm tip and a zero white noise amplifier. These nuances of instrumentation can help with either surgical approach.

### The role of the varicocele repair in the era of assisted reproductive technology

Although many couples attempt IVF/ICSI, despite the husband having identifiable varicoceles, there are reasons, albeit somewhat controversial, for varicocele repair in the era of IVF/ICSI. In the early days of assisted reproductive technology (ART), Ashkenazi et al. (524) studied the effect of spermatic vein ligation on successful IVF and embryo transfer. This study evaluated 22 infertile couples who failed IVF. In each, the male partner had suboptimal semen quality and a varicocele. All male partners underwent varicocele repair before a subsequent IVF attempt. Postoperatively, the embryos showed statistically significant improvements in fertilization and cleavage rates as well as a 20% pregnancy rate ( $P<0.01$ ).

More recently, Esteves et al. (525) evaluated 242 infertile men with varicoceles undergoing IVF/ICSI. Prior to IVF, 80 (group I) underwent subinguinal microsurgical varicocele repair; the remaining 162 (group II) had an untreated varicocele present at the time of IVF/ICSI. Postoperatively, significant changes were noted between groups I and II in mean sperm density (34.9 vs. 10.6,  $P\leq .01$ ) and motile sperm count (15.4 vs. 5.1,  $P\leq .01$ ). Pregnancy and live birth outcomes also favored varicocele repair with an odds ratio (OR) of 1.82 (95% confidence interval [CI]: 1.06–3.15,  $P=.03$ ) for pregnancy and an OR of 1.87 (95% CI: 1.08–3.25,  $P=.03$ ) for live births. There was also a statistically significant decrease in rate of miscarriage after varicocele repair (OR: 0.433, 95% CI: 0.22–0.84,  $P=.01$ ).

Daitch et al. (526) retrospectively examined the effect of varicocele repair in the setting of intrauterine insemination (IUI). In a study of 58 infertile couples, 24 underwent 63 IUIs without treatment of varicoceles. The remaining 34 were treated with varicocelectomy and underwent 101 IUI cycles. When pre-wash semen parameters were examined, only the percentage of motile sperm was significantly different between the treatment and non-treatment groups (38.1 vs. 48.6,  $P=.02$ ). In the treated cohort, there were 12 (32.4%) pregnancies that resulted in 12 (32.4%,  $P=.01$ ) live births. In contrast, only four (16.7%) pregnancies were recorded in the untreated group resulting in one (4.2%) live birth. After controlling for variables known to affect outcomes, the study showed that varicocele repair increased the odds of pregnancy by 4.4-fold. Matthews and Goldstein first reported induction of spermatogenesis and pregnancy after microsurgical varicocelectomy in men with nonobstructive azoospermia. Over 50% of men had appearance of motile sperm in the ejaculate adequate for IVF/ICSI.

Kirby et al. (527) should be commended on their detailed meta-analysis addressing the impact on pregnancy and live-birth rates for both oligospermic and azospermic men undergoing varicocele repair before ART. The authors demonstrated improved outcomes for both IUI and IVF (OR of 8.36 and 1.76, respectively). The authors also illustrated that both men with low sperm counts and those requiring testicular sperm extraction did indeed benefit from a varicocele repair even in instances where ART is still required. These findings underscore the importance of a formal urologic evaluation

for such men and the opportunity to diagnose and treat a varicocele.

In the era of ICSI, however, it is unclear how many of these men with varicoceles are referred to male infertility specialists. The study by Kirby et al. (527) described some of the lost opportunities and foregone benefits associated with circumventing a complete male evaluation. Finally, it is clear that varicocele can have a detrimental effect on Leydig cell function as well as spermatogenesis, and repair improves serum testosterone, as well as sperm quality.

### Treatment of obstructive azoospermia

The first description of microsurgical vasovasostomy was by Fernandes, Shah, and Draper, published in the *Journal of Urology* in 1968 in dogs (528). They reported successful anastomoses in 19 of 20 using the microscope compared to 10 failures out of 12 non-microscopic anastomoses using wire splints. The first reported microsurgical vasectomy reversal using vasovasostomy in humans was Silber's description of the technique in 1976 and 26 cases in humans in 1977 (529). Silber was fellowship trained in microsurgery under the mentorship of the late Earl Owen, a world-renowned microsurgeon/plastic surgeon in Melbourne, Australia. Silber's project during his fellowship with Owen involved rat kidney transplantation. Silber discussed potential theoretical applications of microsurgical techniques in clinical urology when he returned to the University of California at San Francisco, published in *Urology* in 1975 and on a microsurgical technique for vasovasostomy in *Surgery, Gynecology & Obstetrics* in 1976. Earl Owen, who claimed that he taught Silber the technique, or gave Silber the idea for the technique, published his results in the *Australia and New Zealand Journal of Surgery* in 1977 (530), the same year that Silber reported a significant series in *Fertility and Sterility*. It is likely that they came up with the idea jointly and both deserve credit for pioneering microsurgical vasovasostomy. The first very large series of reversals was the Vasovasostomy Study Group of 1,469 reversals published in 1991 (531). Further advances in microsurgical vasovasostomy included development of a microspike clamp for securing the vas without the use of high pressure during vasovasostomy, the use of double-armed sutures (532) and, finally, a technique to address the marked discrepancy in vasal lumina often seen at the time of vasectomy reversal, the micro-dot technique (532). Currently, in the hands of experienced microsurgeons, vasectomy reversal results in return of sperm to the ejaculate in anywhere from 85% to 99% of men with pregnancy rates varying from 40% to 80%, depending on female age and time since vasectomy. Several studies have shown that, compared to going directly to IVF/ICSI, when female age is not a factor, vasectomy reversal is significantly more cost-effective than IVF/ICSI.

The next major advance in microsurgery for obstructive azoospermia came with the development of end-to-end specific tubule microsurgical vasoepididymostomy, first reported by Silber in 1978 (533). This technique resulted in a dramatic increase in return of sperm to the ejaculate from <20% with macroscopic techniques to over 50%. Further refinements in

vasoepididymostomy include the development of the end-to-side technique, first described by Wagenknecht in 1980 (534). Further description of this technique followed by Fogdestam, Fall, and Nilsson in 1986 (535). The end-to-side procedure was popularized by Anthony Thomas. The next major advance was the description of the intussusception technique by Richard Berger, initially a triangulation end-to-side technique, published in 1998 (536). The difficulty of dealing with six needles and making a well-defined opening between the triangulation created by the needles placed in the epididymal tubule, led to the development of a transverse technique using two needles with simultaneous placement of the two needles described by Marmar in 2000 and, finally, the technique currently accepted as the state of the art, the longitudinal intussusception vasoepididymostomy, which currently yields patency rates of up to 90% and pregnancy rates of over 40% (537).

In spite of these major advances in the microsurgical treatment of obstructive azoospermia, more and more patients are being shunted straight to IVF/ICSI, even though abundant evidence now suggests that microsurgical repair is more cost-effective and, clearly, the most appropriate first technique with young female partner age and desire for multiple children. At the time of vasoepididymostomy, since the failure rate is significantly higher than vasovasostomy when sperm are found in at least one vas, the authors strongly recommend intraoperative cryopreservation of epididymal and/or testicular sperm so that if the operation fails, another procedure will not be required to perform IVF/ICSI. Finally, since there is a significant late failure rate after initial appearance of motile sperm, we recommend cryopreservation of ejaculated semen as soon as motile sperm appear in the ejaculate.

### Key Points

Varicocele is the most commonly identified correctable cause of male infertility.

Varicocele repair is associated with improved outcomes of both intrauterine insemination and IVF/ICSI.

Current evidence indicates that bypassing varicocele correction with IVF/ICSI is not in the best interests of the infertile couple.

Finally, varicolectomy can prevent and treat androgen deficiency, which is a life-long health issue in men.

Microsurgical correction of obstructive azoospermia is highly successful in the hands of experienced microsurgeons, and is the cost-effective procedure of choice when the female partner is <35, and in couples who desire more than one child.

### Conclusion

Varicocele remains the most commonly identified correctable cause of male infertility. The exact mechanisms of the varicocele effect on testicular function have yet to be fully elucidated. Our understanding of the molecular effects of this



vascular abnormality continue to evolve. This increased understanding should lead to a concurrent increased awareness that bypassing varicocele treatment with IVF/ICSI may not be in the best interest of the infertile couple or the male. Microsurgery for the treatment of varicocele and obstructive azoospermia has resulted in steady and dramatic improvements over prior techniques. Of course, the success of these procedures is very much dependent on the surgeon's prior training and experience in microsurgery. These operations should not be performed by urologists who are not well trained and who do the procedures only infrequently. With the right choice of couples, microsurgical repair of varicocele and obstruction will continue to offer couples the opportunity to conceive with, and often without, IVF/ICSI using the male partner's own sperm.

## **microTESE: AN EVOLVED, EFFECTIVE PROCEDURE IN THE TREATMENT OF SEVERE MALE INFERTILITY**

**Peter N. Schlegel, M.D.**

The assisted reproduction treatment of in vitro fertilization was originally limited by abnormal sperm function (538). The development of micromanipulation techniques to enhance fertilization rates for men with abnormal sperm production and function revolutionized our options for treatment of men with severe male factor infertility (539). Although ejaculated sperm were first used for intracytoplasmic sperm injection (ICSI), men with obstructed and nonobstructive azoospermia (NOA) were also observed to have sperm (540). Sperm from men with obstructive azoospermia were used for ICSI with effective results (541). These observations overturned many longstanding tenets of male reproductive physiology. It was previously assumed that sperm in the testis, those that had not undergone epididymal maturation, were incapable of fertilization of oocytes and subsequent development of normal pregnancies. Intracytoplasmic sperm injection changed the concept of what sperm could be used to initiate pregnancy. With the evolution of assisted reproduction, a much broader focus on treatment of male factor infertility was possible.

### **Intracytoplasmic sperm injection with surgically retrieved sperm**

After demonstration of the effectiveness of ICSI using ejaculated sperm, men with obstructive azoospermia had sperm retrieved for use with assisted reproduction. Men with congenital bilateral absence of the vas deferens and other unreconstructable cases of obstructive azoospermia were previously only offered donor sperm or adoption for substitutive management of their infertility, so the ability to use testicular or epididymal sperm for in vitro fertilization (IVF) with injection of limited numbers of sperm with ICSI was a dramatic revolution in management of couples with azoospermia (542, 543). Once ICSI with surgically retrieved sperm was

recognized as a viable treatment option for male infertility, the potential for treatment of other severe forms of male infertility was opened.

### **Sperm identification in men with nonobstructive azoospermia**

The observation that sperm could be identified on testis biopsies of men with NOA was the other key observation that allowed the potential to develop a treatment of NOA men with a form of testis biopsy, commonly referred to as testicular sperm extraction (TESE) (540). Interestingly, although we made the observation that men with NOA had sperm on testis biopsy in 1993, the importance of the observation was not translated into treatment of men with NOA at our center until 1996. We initially were concerned that the morphologically abnormal and typically non-motile sperm seen during TESE could not be effectively used, even with ICSI. The success of sperm retrieval and subsequent ICSI-derived pregnancies demonstrated the functional capability of sperm from men with NOA (542, 543). Indeed, even the most dramatic forms of NOA, such as Klinefelter syndrome, were able to be effectively treated with TESE and ICSI.

### **Techniques for sperm retrieval in nonobstructive azoospermia**

Once the feasibility of obtaining testicular sperm from men with NOA was demonstrated, along with the functionality of those sperm using ICSI, refinement of the techniques for sperm retrieval were undertaken at a number of different centers. The use of fine needle aspiration was shown to provide limited numbers of sperm in men with nonobstructive azoospermia, and was recognized as an approach for sperm retrieval for these men (544). Multiple-biopsy sperm retrieval was also shown to be effective, but in some cases, up to 20 biopsies were required to identify sperm needed for ICSI (545). The rapid introduction of multiple-biopsy TESE, rather predictably, resulted in the interruption of testicular blood supply to the testis, since the vessels supplying the testicular parenchyma travel under the surface of the tunica albuginea, and multiple random biopsies resulted in compromise of testicular blood flow. We first reported devascularization of the testis as a consequence of a multi-biopsy TESE procedure done at another center in the mid-1990s for attempted treatment of nonobstructive azoospermia (546). This observation led us to consider that a multi-biopsy approach would create risk of damage to the testis. Based on previous observations by Jarow et al. of the human testicular blood supply as it courses under the tunica albuginea, we began to use an operating microscope during the incision of the tunica albuginea for TESE. The use of the operating microscope has been proposed by Goldstein et al. (547) to enhance a wide variety of procedures at Weill Cornell. At this point, I observed that seminiferous tubules within the testis, as seen through an operating microscope, had varied morphologies. The tubules that were larger obviously contained more spermatogenic cells and were therefore more likely to contain sperm. The use of an operating microscope to identify the sites of sperm production was easily identified as a more effective way to find sperm



compared to multi-biopsy TESE, and provided sperm with less tissue removed (548).

### Anatomic principles of microTESE

The technique of microTESE (mTESE) was thereby born. Informal polls at more recent scientific meetings demonstrate that this procedure is used as part of the management of men with non-obstructive azoospermia by a majority of experts in male reproduction. microTESE refers to the procedure of opening the testis widely, followed by dissection of testicular tissue in parallel to the blood supply of the seminiferous tubules. Anatomical studies dating back to the 1600s have demonstrated that the highly coiled seminiferous tubules originate and terminate in the center of the testis (in the mediastinum or intratesticular rete region), traveling out to the periphery of the testicle. Each tubule is separated by a typically very fine filamentous septum with blood vessels running parallel to the tubules. This organization allows dissection deep within the testicular parenchyma, so a surgeon can directly observe nearly every region of the hundreds of seminiferous tubules within the testis.

The distribution of testicular sperm production can be most effectively “mapped” and sperm efficiently retrieved using a mTESE approach. It was initially (inaccurately) proposed that sperm production was multifocal and diffuse throughout the testicle (549). As microsurgical identification of the sperm production sites was effectively mapped with mTESE, we were able to see that focal individual areas of sperm production are not predictable and do not reliably occur in any specific geographic location within the testis. Although sperm can be identified on initial wide opening of the tunica albuginea (65% of cases), deep dissection within the testicular parenchyma is needed to identify the focal sites of sperm production in the remaining cases, and examination of the contralateral testicle yields sperm in the remaining cases, even when no sperm was found after dissection of an entire testis (550). Comparative studies of sperm retrieval approaches have shown that mTESE is about 1.5-fold more likely to identify sperm than conventional TESE, and TESE is 2-fold more effective than fine-needle aspiration/mapping or testicular sperm aspiration procedures at sperm retrieval (551).

### Variability of microTESE procedures with different surgeons

Recent studies where repeat mTESE has found sperm after a prior failed attempt at sperm retrieval after medical or other intervention reflects that an ineffective initial sperm retrieval procedure (incomplete microdissection) rather than the medical therapy prior to sperm retrieval is the reason why sperm are found after these repeat sperm retrieval attempts. Of the many surgeons who have come to observe the procedure of mTESE at Weill Cornell, it is evident that the technique performed varies greatly in different surgeons' hands, despite use of the term mTESE to reflect the procedure. Unfortunately, a limited incision, with limited or no dissection beyond the initially exposed surface of testicular tissue is often done by

these surgeons. Limited exposure of tissue adversely affects the efficacy of a sperm retrieval procedure. It is unfortunate that in some cases, the mTESE procedure is limited either because of surgeon or institutional time-constraints, fear of damage to the testis, or a lack of understanding of the importance of extensive dissection to identify sites of sperm production. When mTESE involves extensive dissection of testicular tissue following the anatomy of the testis, limited removal of tissue and careful management of intratesticular structures, there are limited effects on testosterone production and testis loss or severe damage to the testicle is not a measurable risk of mTESE in our experience (552).

### Confirmation of azoospermia: role of extended sperm preparation

One of the critical factors for management of men with non-obstructive azoospermia is the re-evaluation of an ejaculated semen specimen on the day of planned sperm retrieval. We have routinely done sperm retrieval on the day before oocyte retrieval in a programmed IVF cycle, in part because 5% to 10% of men with presumed azoospermia can have rare sperm found on careful evaluation of a semen specimen, using an extended sperm preparation technique; thereby avoiding an unnecessary sperm retrieval operation. This is a very favorable result for a man who previously thought that surgery was needed, and sperm from the ejaculate was available for ICSI.

It is important to remember that azoospermia is defined by a lack of sperm identified in the neat semen specimen followed by centrifugation of the semen specimen down to a smaller volume that is then directly examined. The centrifuged semen specimen may have a volume of 60–300  $\mu$ L, and the specimen examined after centrifugation may be only 5–10  $\mu$ L in volume. Obviously, rare sperm may be present in the residual portion of the total centrifuged 60–300  $\mu$ L volume pellet. Extended sperm preparation involves examination of additional microdroplets of this total pellet (under oil to prevent evaporation) so that the entire specimen is examined, not a limited part of the sample. We have found this type of analysis to be particularly helpful for men with nonobstructive azoospermia associated with maturation arrest. Often these men have normal or near-normal follicle-stimulating hormone and nearly normal or normal volume testes. However, rare sperm can also be found in “azoospermic” men with elevated serum follicle-stimulating hormone levels of 20–60 IU/L.

### Simultaneous sperm retrieval with in vitro fertilization

As demonstrated in the last paragraph, there are reasons to prefer simultaneous sperm retrieval with a programmed IVF cycle. Sperm retrieval is obviously unnecessary in some men who have previously been treated with intentional cryo-TESE (freezing of sperm). In addition, many of the men who have sperm found with mTESE may not have enough sperm to freeze, or may not have the sperm survive freeze-thaw. Finally, even for those men with nonobstructive azoospermia who have sperm frozen and survive freeze-thaw, we have

observed lower pregnancy rates at our center than parallel cases where sperm from men with NOA were used fresh with IVF. Since sperm are the limited resource for couples where the man has nonobstructive azoospermia, every effort to maximize the use of those sperm is worth considering.

### Processing of microTESE samples

MicroTESE allows detection of the rare sites with sperm production. In most cases, sperm production is present throughout the entire length of the enlarged tubule identified during mTESE. If only focal dilation/enlargement of the tubule is seen, then just the enlarged region is excised for sperm sampling. In some cases of focal enlargement of tubules, the tubule may be artifactually enlarged because of Leydig cell nodules near the tubule. The amount of tissue removed is typically very limited in men with NOA undergoing mTESE. The delivery of the maximum number of sperm from limited tissue to the IVF laboratory enhances the ability of the laboratory personnel to identify rare sperm in these tissues. An additional intervention that helps to identify these rare sperm is aggressive mechanical dispersion of these tissues prior to an attempt at examination of the tissues. Prior to handing tissues off the operating field (or with processing on a back table), we will cut the tubules with scissors and confirm the disruption of tissue by passing the tissue suspension through a 24-gauge angiocatheter. The tissue is typically in 300–500  $\mu$ L of sperm wash fluid. A small aliquot of this suspension is then examined under a phase contrast microscope on a glass slide with cover slip at  $\times 200$  magnification for detection of sperm. Mechanical processing with this approach enhances the number of sperm seen by nearly 300-fold (545). Sperm are typically immotile immediately after retrieval but often acquire at least twitching motility by incubation in sperm wash medium, including incubation overnight in that medium with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer removed and a Plasmate (Bayer Corp.) protein source.

### Use of testicular sperm for intracytoplasmic sperm injection

Sperm retrieved from men with NOA are routinely abnormal in appearance with 0% normal morphology. There are no specific morphological findings that preclude the chance of pregnancy of ICSI using NOA sperm, despite some IVF center's concern with use of these sperm. There are statistically lower chances of pregnancy if sperm have not acquired motility by the time of ICSI. Similarly, sperm with severe acrosomal abnormalities are also less likely to affect fertilization and pregnancy. We have seen that even men with sperm that have marked morphologic abnormalities, including testicular sperm with short tails (elongating spermatids) can initiate pregnancies. Every effort to freeze residual sperm after ICSI is recommended, since NOA men have severely limited sperm production.

### Results of intracytoplasmic sperm injection in nonobstructive azoospermia

Of men who underwent initial sperm retrieval for non-obstructive azoospermia using TESE at our center, sperm

are identified in the operating room in 55% of cases. For the cases with sperm retrieved, a clinical pregnancy is detected with a fetal heart beat on ultrasound in 45% of cases, with an average female age of 32 years at our center.

### Etiology of nonobstructive azoospermia: results of microTESE

Men with complete deletions involving the AZFa, AZFb or AZFb+c regions of the Y chromosome have no measurable chance of sperm retrieval. We also do not attempt sperm retrieval after a prior failed mTESE attempt at our center. On the other hand, men with AZFc deletions alone often have sperm in the ejaculate, and even those azoospermic AZFc-deleted men with have a 70% sperm retrieval chance. Men with NOA and a history of cryptorchidism have a good chance of sperm retrieval (67%–74%). Similarly, men with Klinefelter syndrome have a 68%–70% chance of sperm retrieval, whereas men with a history of prior alkylating agent chemotherapy and azoospermia have a 30% chance of retrieval. Men with idiopathic NOA (no Y microdeletions, no significant history and no karyotypic anomalies) have a 48% sperm retrieval chance.

### Learning curve for microTESE

MicroTESE appears to be a difficult procedure to learn. During early experience, trainees commonly provide too much pressure on the testicular tissue that may separate tubules from their blood supply and devascularize regions of the testicle. Similarly, microdissection and direct examination of the tissue may be hard to do when the surgeon is struggling to maintain the tissue in focus under an operating microscope. The higher the magnification used under the operating microscope, the more effectively the surgeon is able to detect subtle differences in seminiferous tubule size. We have seen that surgical times for mTESE may vary from 1–7 hours with a slow, progressive decrease in operating time with hundreds of cases of surgical experience. Similarly, sperm retrieval rates trend upward with surgeon experience over time, when controlling for the cause of NOA, an observation that we have most clearly demonstrated for men with a predominant pattern of Sertoli cell only.

### Conclusions

In summary, development of the technique of mTESE has optimized sperm retrieval for men with NOA, providing sperm from a majority of men with this severe defect in spermatogenesis for ICSI. This procedure is a more effective technique for sperm retrieval than prior, random approached at identifying sperm. This microsurgical technique is anatomic, following the normal structure of the testis. Although mTESE is a more involved and sometimes tedious technique, the long-term safety of the procedure is well-demonstrated over time for effective treatment of men with nonobstructive azoospermia.

## ENDOCRINE STIMULATION FOR SPERMATOGENESIS IN THE AZOOSPERMIC MALE

Alayman Hussein, M.Sc., M.D.

According to the 2010 World Health Organization manual of semen analysis, men with a sperm count <15 million/mL, forward progressive motility <32%, and strict morphologically normal sperm <4% have a lower chance to naturally impregnate their partners. Assisted reproductive technology (ART) help infertile men who are beyond, or not responding to, available medical or surgical treatment to father children. To reach the expected probability of successful outcome, intrauterine insemination requires the availability of 5 to 10 million motile sperm in the ejaculate. In vitro fertilization is introduced to help men with semen below this level of total motile sperm count and requires the availability of 50 to 100 thousand motile normal sperm per ovum. In a key step in the field of ART, intracytoplasmic sperm injection (ICSI) is introduced and requires the availability of only one viable sperm per ovum. Intracytoplasmic sperm injection opened the door for cases of severe oligospermia and azoospermia to have a chance of becoming a father if very few sperm were available in their ejaculate or could be retrieved from their testes.

In all cases of obstructive azoospermia and some cases of nonobstructive azoospermia enough sperm could be retrieved from the testes to be used for ICSI. Sperm retrieval rate in conventional testicular sperm extraction (TESE), in cases of nonobstructive azoospermia, ranges from 16.7% to 45%. Recent studies to increase testicular sperm retrieval rate in nonobstructive azoospermia employ two different and often complementary strategies. The first strategy is to improve the technique of testicular sperm extraction to be able to retrieve as much as possible from the available testicular sperm. Microdissection TESE succeeded to increase sperm retrieval rate to the range of 42.9% to 63% (553). The second strategy is to increase the available number of sperm in the testis by increasing testicular sperm production. Basically, if we stimulate spermatogenesis before TESE, more sperm will be produced and we will have a higher probability of finding sperm in TESE. Understanding spermatogenesis and its hormonal regulations is the fundamental of this proposed line of management.

The details of spermatogenesis and the action and requirements of the hormones which are responsible for its regulation are not very clear. However, genetic and pharmacological studies have demonstrated many clear facts. The primary role of follicle-stimulating hormone (FSH) is initiation of spermatogenesis and stimulation of Sertoli cell proliferation and determining the number of germ cells at the time of puberty (554). Follicle-stimulating hormone is also essential for maintenance of normal 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 gonadotropin-releasing hormone antagonist. FSH treatment increases all spermatogenic

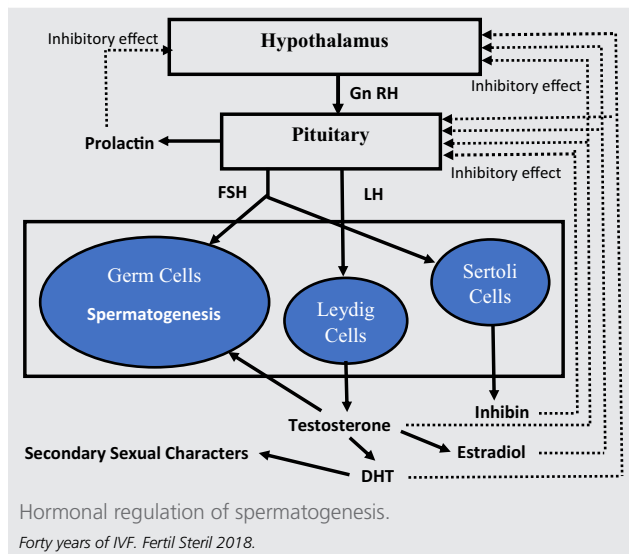
cells prior to elongated spermatids. Lack of FSH results in defective chromatin packaging and reduction in acrosomal glycoprotein content of sperm. In addition, FSH may synergize with testosterone by stimulating the synthesis of the androgen receptors. It was suggested that FSH has a role in facilitating the transport and localization of testosterone within Sertoli cells (555).

Testosterone is required for maintenance of spermatogenesis and is responsible for maturation of round spermatids into mature sperm. It keeps the adhesion between germ cells and Sertoli cells, as testosterone withdrawal leads to premature release of round spermatids. High intratesticular levels of testosterone is essential for spermatogenesis. For normal spermatogenesis, higher levels of testosterone are required for androgen receptors in the testis compared to those in other androgen dependent tissues.

In conclusion, both FSH and testosterone are required for initiation and maintenance of normal spermatogenesis. So, it is axiomatic that patients presented with delayed puberty and diagnosed as hypogonadotrophic hypogonadism are azoospermic. Successful hormonal replacement with human chorionic gonadotropin (hCG) and human menopausal gonadotropin (hMG) in these patients restore the normal level of FSH and luteinizing hormone (LH) and stimulate Leydig cells in the testis to produce testosterone and start spermatogenesis as long as there is no testicular insult (556). The use of pulsatile injection of gonadotropin-releasing hormone is an alternative protocol for restoring the normal level of FSH and testosterone and successfully produces sperm in men with hypogonadotrophic hypogonadism (557).

To start an endocrinal treatment to azoospermic men without features of hypogonadism it is necessary to understand the hypothalamo-pituitary testicular axis that controls the serum level of FSH and testosterone. Figure 27 demonstrates that FSH and testosterone are directly involved in stimulation of spermatogenesis and the other hormones namely; LH, prolactin, and estradiol contribute by their role in regulations of the level of FSH and testosterone. Accordingly, it is recommended to estimate serum FSH and testosterone as an initial and basic step in evaluation of azoospermic men. Additional hormone analysis, including LH, estradiol, and prolactin, is particularly recommended in cases with low or low normal serum testosterone based on the likelihood of their abnormality and potential impact on treatment choices.

The requirement of FSH and testosterone to normal spermatogenesis and understanding the hypothalamo-pituitary-gonadal axis are the rational for the first use of clomiphene citrate in nonobstructive azoospermia in a 2005 multicenter study (558). Clomiphene increases endogenous gonadotropin-releasing hormone secretion from the hypothalamus and gonadotropin hormone secretion from the pituitary, thus increasing Leydig cell synthesis of testosterone and intratesticular testosterone concentration which is a fundamental requirement for normal spermatogenesis. Clomiphene is chosen in this study because it is known to be successful in some cases of oligospermia to improve sperm production and might be useful in nonobstructive azoospermia to produce enough sperm for ICSI either by resulting in sperm identified

**FIGURE 27**

in the ejaculate or potentially improving outcomes of TESE. Clomiphene citrate was administered to 42 azoospermic patients excluding patients with Sertoli cell only syndrome and testicular malignancy from the study. The dose of clomiphene was titrated according to the serum level of total testosterone which is measured every 2-3 weeks keeping it between 600 and 800 ng/dL. After clomiphene citrate therapy, 27 (64.3%) of the patients demonstrated sperm in their semen with a mean sperm density 3.8 million/mL, mean motility was 20.8%, and mean total motile count was 2.6 million. One partner achieved spontaneous pregnancy (3.7%). Sufficient sperm for ICSI was retrieved by testicular sperm extraction in all of the patients who remained azoospermic after clomiphene citrate therapy.

These results were encouraging and opened the door for further studies evaluating the value of clomiphene citrate and other endocrinal treatment options for nonobstructive azoospermia prior to testicular sperm extraction. When clomiphene is applied to all nonobstructive azoospermic patients prior to TESE without any histopathological studies, excluding patients with baseline FSH more than one and half the upper limit of normal, it is noticed that 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 the maximum dose of clomiphene is used. Some patients respond to clomiphene treatment by an obvious increase in FSH without increase in testosterone. Few patients respond to clomiphene by an unexpected decrease in testosterone which is also manifested by decrease in sexual desire.

Based on these findings, a new protocol for treatment of nonobstructive azoospermia prior to TESE was developed (559). The principle of this protocol is to reach the level of FSH and testosterone in the testis to an adequate level for stimulation of spermatogenesis with the use of Clomiphene

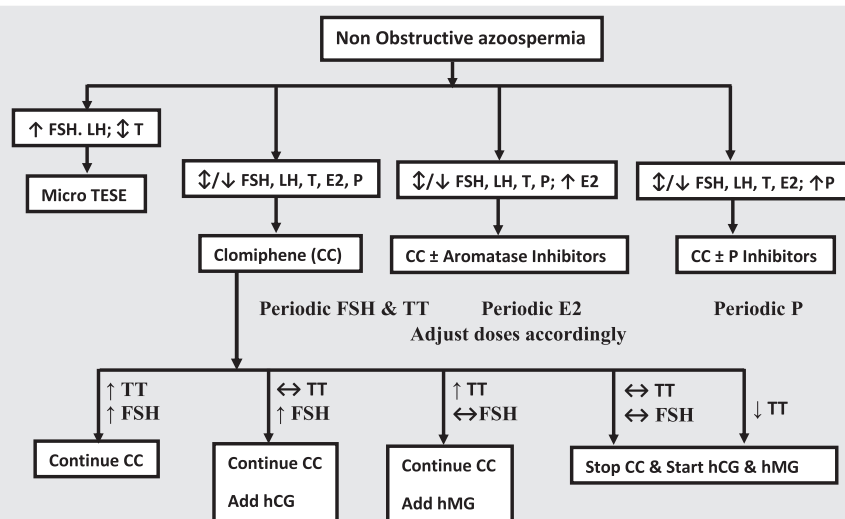
Citrate, HCG, HMG or a combination of them. Human chorionic gonadotropin, which is analogous to LH, stimulates the Leydig cell secretion of testosterone. Human menopausal gonadotropin (hMG) has both LH and FSH activity. A study including 612 patients with nonobstructive azoospermia evaluating this protocol demonstrated clearly that clomiphene citrate, hCG and hMG administration, when resulting in an increased level of FSH to one and half the upper limit of normal and total testosterone between 600 and 800 ng/dL, may result in producing sperm in the ejaculate and increased likelihood of successful micro-TESE. Hormonal optimization using clomiphene, hCG and hMG according to this protocol, might be useful in producing sperm in semen and avoid testicular surgery in 11% of cases. If the patient remains azoospermic after treatment, the probability of finding sperm in TESE is increased by 1.7-fold.

It is clear in this study that optimizing the levels of FSH and total testosterone is the base of the success of treatment and not the type of the used drug. It is necessary to optimize testosterone level by stimulating its endogenous secretion and avoid administration of exogenous androgens. The administration of exogenous testosterone and other androgens have a negative feedback on hypothalamic pituitary gonadal axis and inhibit the secretion of FSH and LH from the pituitary gland and consequently decrease intratesticular testosterone and inhibit spermatogenesis.

Many studies reported variable degrees of success of the empirical use of different drugs in the treatment of nonobstructive azoospermia. Exogenous gonadotropin treatments include the use of hCG, hMG, and recombinant FSH. The reason for gonadotropin administration in idiopathic azoospermia is based on its observed efficacy in the treatment of hypogonadotropic hypogonadism. However, their effectiveness for treating normogonadotropic azoospermia is less clear and uncertain. It is believed that the administration of hCG and hMG in men with normal level of FSH and LH is useless with a disappointing outcome. But, Selman et al. reported a 32-year-old man with Y chromosome microdeletion who received recombinant FSH and hCG and the few thousands of sperm that were demonstrated in his ejaculate were enough for a successful ICSI (560). Similarly, in the Efesoy et al. study, sperm in the ejaculate of 2 of 11 azoospermic men with maturation arrest were reported after treatment with recombinant FSH (561). On the other hand and in another study, Selman et al. treated 49 men, who had no sperm in the pre-treatment testis biopsy, with recombinant FSH and HCG and reported that all men remained azoospermic at the end of treatment, however, sperm were found in the biopsies of 22% men after treatment (562). Similarly, Shiraishi et al. reported that sperm were successfully obtained at the second micro-TESE in 21% of men who had negative initial micro-TESE and received hCG and recombinant FSH, whereas no sperm were retrieved from untreated men and noticed that the success at the second micro-TESE was more likely if histology at the first micro-TESE showed hypospermatogenesis (563). Also, Ramasamy et al. reported improvement in the outcomes of primary TESE following gonadotropin therapy in men with nonobstructive azoospermia and Klinefelter's syndrome (564).



FIGURE 28



Periodic Semen Analysis 3 - 6 months after hormone optimization

Micro TESE for patients remaining azoospermic

Endocrinal stimulation of spermatogenesis based on the initial and periodic hormonal levels.

Forty years of IVF. Fertil Steril 2018.

Targeting the imbalance between the circulating levels of testosterone and estradiol has been investigated as a potential therapeutic tool in men with nonobstructing azoospermia. Aromatase enzyme converts the circulating testosterone and other androgens into estrogen within fat cells, liver and testes. In markedly obese men, there may be an excessive endogenous conversion of testosterone into estrogen that results in a reversible imbalance in the testosterone/estradiol ratio. Estradiol suppresses pituitary secretion of FSH and LH and consequently inhibits testosterone biosynthesis in the testes and impairs sperm production. Aromatase inhibitors correct the suppressive effect of estrogen on gonadotropins and testosterone production and by restoring their normal levels it is supposed that spermatogenesis is improved. Raman and Schlegel used the aromatase inhibitors anastrozole and testolactone in 140 infertile men with low testosterone and a low testosterone/estradiol ratio and found a significant increase in testosterone level, sperm count and motility in oligospermic men (565). Pavlovich et al. studied the use of testolactone in men with nonobstructive azoospermia and in severe oligospermia and reported significant improvements in cases of oligospermia regarding total sperm counts and motility. But, none of the azoospermic men showed any return of sperm in the ejaculate (566). Many studies argue against the beneficial use of aromatase inhibitors in nonobstructive azoospermia to show sperm in the ejaculate. However, a potential role in improving the quality or quantity of testicular sperm is suggested, thus improving the outcomes of sperm retrieval in testicular sperm extraction (564).

The success of endocrinal stimulation of spermatogenesis depends mainly on the functional capacity of the testis which is manifested by the serum levels of FSH and testosterone. So,

in addition to complete history and full examination, hormonal evaluation is mandatory before starting any endocrinal treatment of spermatogenesis. When obstructive azoospermia is diagnosed, the choice of treatment will be genital duct reconstructive surgery and/or testicular sperm extraction without any kind of prior medical treatment. High levels of FSH and LH with low or normal testosterone in azoospermic men indicate a primary testicular failure and there is no role of medical treatment. The only option for these cases is micro-TESE and ICSI.

In cases with low testosterone in azoospermic men with normally developed sexual features, we need more hormonal evaluation to reach the specific cause of the decreased testosterone level. Assessment of serum level of FSH, LH, prolactin, estradiol, and thyroid hormones are required to reach the etiology and select the type of hormonal treatment. Figure 28 summarizes the available lines of endocrinal stimulation of spermatogenesis based on the initial and periodic hormonal levels.

It is preferred that the patient continue on endocrinal stimulation for six months which is equivalent to two spermatogenic cycles and semen analysis is advised to be done monthly starting at the third months. If the patient remains azoospermic after 6 months of treatment with achievement of the target levels of FSH and serum testosterone, it is recommended to schedule him for micro-TESE and the patient is advised to continue treatment up to the day of micro-TESE.

The disadvantage of medical treatment prior to TESE is the delay of ICSI for 6 to 9 months which is particularly important in old aged partners. On the other hand, it is also a waste of time if a patient, who is eligible for endocrinal stimulation, had a negative micro-TESE without a prior

endocrinal therapy. The advisable choice in this case is to start hormonal treatment and schedule the patient for a second microTESE. The success of sperm retrieval decreases with the repetition of TESE and the outcome of endocrinal stimulation for spermatogenesis is expected to be much lower in patients with one or more previous testicular surgeries that possibly cause testicular devascularization and fibrosis minimizing the response of treatment (567).

## THE GENETIC BASIS OF MALE REPRODUCTIVE FAILURE: EARLY DISCOVERIES AND CLINICAL CONSEQUENCES

Robert D. Oates, M.D.

The nascent field of male reproductive genetics has seen tremendous progress in the last 30 years, paralleling the advances in treatment options and opportunities for the most severely infertile men. No less important is the tremendous advancement in the understanding of the inner workings of the human genome. In this contribution, we will review the three most relevant clinical conditions by briefly discussing the phenotype and genotype of each and why and how this biological connection came to be known.

### Congenital bilateral absence of the vas deferens

On the shoulders of the pioneers of male reproductive medicine and surgery, coupled with the revolutionary development of in vitro fertilization, new basic science investigators and clinical practitioners came enthusiastically and collaboratively into the field of male infertility in the mid- to late-1980s. The first blend of genetics and novel clinical treatment strategies involved the disorder of congenital bilateral absence of the vas deferens (CBAVD). In 1990, Silber and colleagues first described harvesting sperm from the epididymal remnant of men with CBAVD, using the sperm in conjunction with in-vitro fertilization with the achievement of pregnancy (568). Ultimately, Silber et al. realized that successful pregnancy rates were markedly enhanced when the individual-retrieved spermatozoa were directly micro-injected into the oocyte (intracytoplasmic sperm injection [ICSI]) (569). To finish the clinical saga, shortly thereafter, Oates and colleagues imagined that, since ICSI was such a powerful tool to compensate for extremely deficient sperm numbers and motility, frozen-thawed epididymal sperm could be used with equivalent efficacy to freshly retrieved epididymal sperm. They indeed published the first paper detailing the intentional, planned approach of harvesting sperm with immediate cryopreservation into several aliquots/vials and then using a single vial as the sperm source in a later ICSI cycle. Their novel strategy separated in time and space the male and female procedures, greatly relieving the logistical pressures on the couple and the treating clinicians (570). Parenthetically, this group also published the first paper on intentional cryopreservation of testis sperm for nonobstructive azoospermic (NOA) patients, a strategy for both

epididymal and testis sperm that is now routinely and commonly employed (571).

As we were learning how to treat CBAVD more and more effectively, the genetic underpinnings of the condition were also being elucidated in an effort to prevent our ability to treat CBAVD from outstripping our knowledge of its genetic basis. The cystic fibrosis (CF) gene was isolated, characterized, and investigated for mutations in 1989, described beautifully in a series of three papers in *Science* (572–574). Based upon prior knowledge that males with clinical CF (pulmonary and pancreatic dysfunction and disease) had the very unusual coexistent finding of bilaterally absent vasa, Oates and colleagues posited in 1990 or so that males with CBAVD may also have mutations in their maternally and paternally inherited CF genes such that CBAVD could be considered a primarily genital form of CF. Indeed, this was true and immediately expanded dramatically the clinical phenotype on the more mildly affected end of the CF mutation disease spectrum (575). They also realized that it was the combination of mutations and their effect on ultimate cystic fibrosis transmembrane conductance regulator (CFTR; the protein product of the CF genes) quantity and quality that determined the clinical and phenotypic consequences in an individual. Genotype and phenotype were linked. This finding led to three very important corollaries. The first was for the patient himself. This not only explained why he had bilateral vasal absence but also why he may have chronic sinusitis or episodes of bronchitis, for example. It offered a new understanding for him and his physicians about the reasons for these associated conditions, potentially changing his treatments and management strategies for them with the new knowledge that those conditions were consequences of CFTR dysfunction. The second was about the couple and their offspring. Prior to undergoing sperm aspiration and ICSI, both partners need CF mutation analysis carried out to determine if the female partner is a carrier (an asymptomatic simple heterozygote). If she is, preimplantation genetic diagnosis should be considered to avoid transfer of an embryo that is destined to be an individual with life-shortening, life-altering clinical CF. Finally, the male with CBAVD was often the first in his family to have a CF mutation detected and so family counselling and screening became important to help others, especially his siblings who were often also of reproductive age, to learn of their carrier status and to use that information proactively.

Through the 1990s, different investigators confirmed this insightful discovery and provided more and more correlation between the genotype detected and the phenotype displayed. While acknowledgment of the CFTR dysfunction/CBAVD causal link was becoming rapidly known and accepted, Oates et al., as well as others in the field, appreciated that not all men with CBAVD had evident CFTR mutations, especially those with associated unilateral renal agenesis (576). Based upon an understanding of the embryology of the mesonephric duct and its role in both ureteral and reproductive duct development, it was easy to intuit a primary defect in bilateral mesonephric duct morphogenesis prior to week 7 of gestation that would result in harm to all 4 derivatives of the 2 mesonephric ducts. The most severe expression of this putative

genetic mishap would be bilateral renal agenesis coupled with bilateral vasal agenesis. However, those so afflicted do not survive much past birth. But if the phenotype expressed is just a little bit less pronounced and one ureteral/renal unit develops, that male may present later on in life with infertility secondary to CBAVD and be found to have unilateral renal agenesis. Cystic fibrosis transmembrane conductance regulator mutation analysis will not be informative. The genetic basis of CBAVD/unilateral renal agenesis has been elusive.

### Y chromosomal microdeletions

As our creativity increased vis-à-vis what male infertility conditions could be adequately treated with ICSI, we learned that even sperm harvested from the testis tissue itself would affect fertilization, that embryos would grow and mature, and that babies would be born (577). Individual spermatozoa could actually be retrieved from the seminiferous epithelium of approximately 50% of cases of NOA and employed successfully with ICSI, even frozen-thawed as previously mentioned. Were we being too zealous and not cautious enough in the use of our new found abilities to create biological fatherhood; where was the concern for the offspring and their health, both overall and reproductive? It was not enough to just inspect newborns and hope that they would be fine throughout life; it was necessary to try and determine, if possible, the genetic basis underlying these drastic conditions. Heretofore, nature and evolution had rarely permitted transmission of these genetic defects but for many men, our technology had overcome these limitations without a comprehensive understanding of the risks to the offspring. During these clinically advancing years in the early 1990s for men with NOA, research was being conducted in a parallel basic science field on the Y chromosome. What was the molecular geography of the Y chromosome, were there any genes of consequence along its length, where was the putative testis determining region located, and why was there spermatogenic failure when the long arm of the Y was cytogenetically visualized to be abnormal, as noticed by Tiepolo and Zuffardi (578). The Page laboratory at the Whitehead Institute, Massachusetts Institute of Technology, under the directorship of Dr. David Page was at the forefront of trying to answer these vexing questions with clinical material (blood) and clinical characterization of patient subjects being provided by Oates and Silber (579, 580).

In the mid to late 1990s it became clear that there were genes necessary for optimal sperm production sprinkled about the length of the long arm of Y, that there were several long palindromic sequences within which some of these genes resided, and that on rare occasions microdeletions would occur within these palindromic stretches (especially P5 though P1). Meshing the molecular findings of an individual patient with his clinical parameters, a picture slowly evolved of the relationship and bond between an absence of certain segments on Yq and the consequent adverse effect on spermatogenesis. Other laboratories and investigators also began to explore these causal associations and add to the growing evidence. The most common of the missing pieces was termed AZFc and is not uncommonly found in the severely oligosper-

mic or NOA male (581, 582). This early collaboration and synthesis of molecular biology and clinical medicine finally led to a commercialization of the Y chromosomal microdeletion assay, a routine test in the work-up of severe oligospermia or NOA, from laboratory to bedside in just a few short years. The reasons this discovery was so important were, and are still, quickly apparent. First, certain microdeletions discovered upon testing such as AZFb/c predicts that sperm will not be found on testicular sperm extraction (TESE), even microTESE described first by Schlegel and colleagues (583). If an AZFb/c microdeletion is detected, there is no reason to subject the patient to a surgical sperm retrieval procedure as, unfortunately, sperm do not exist and will not be detected, no matter how much invasive searching takes place (584). Secondly, an AZFc microdeletion, on the other hand, portends a much better prognosis for sperm retrieval; it can either be found in the ejaculate in very low numbers or in the testis tissue upon TESE in approximately 70% of men so afflicted (585). It is almost always a de novo event. Even though the sperm work well when used in conjunction with ICSI, every single male offspring that may result will inherit this Y chromosome and its AZFc microdeletion (586). That male, that son, that adult will be destined for, and burdened by severe, if not total, reproductive deficiency. There is certainly no ethical guideline to offer couples in these circumstances but with this knowledge at hand, they may decide to transfer only female embryos to have reproductively fit daughters, as an example.

Finally, even more aberrantly deformed Y chromosomes, e.g. isodicentric Y chromosomes, have been revealed and their complex developmental history and the implications in terms of spermatogenesis and offspring phenotype become understood (587).

### Klinefelter syndrome

Klinefelter Syndrome (KS) provides yet another example of the intersection of biological, genetic, and clinical insights that led to amazing advancements for couples and their many resultant children. Klinefelter, Reifenstein and Albright originally described the interesting phenotypic constellation of gynecomastia, hypogonadal levels of virilization, small testes and azoospermia, later shown to result from a 47,XXY karyotype (588). It was thought that all KS boys and men presented exactly that way, sterile with eunuchoid body proportions. Their testes were atrophic, seminiferous tubules were sclerotic and hyalinized and their Leydig cells were hyperplastic. "Unfortunately, we cannot help you as your testes do not make sperm," a common piece of devastating news we conveyed to our desperate couples.

As for other known and unknown reasons for underlying NOA, the questions became would the testes of KS men harbor sperm and, if so, would that sperm actually work. The answer to the first part came from Tournaye et al. in 1996, followed rapidly by the answer to the latter part from Palermo et al. in 1998 (589, 590). Sperm could indeed be found and could generate viable, healthy live births and those babies were 46,XY or 46,XX. These two dramatic and seminal papers initiated a resurgence in interest in KS. What a dramatic turn

of events for the 1:600 males born with KS; from absolute sterility to possible biological fatherhood. Numerous programs have since replicated that success and insights into the basic biology of the limited spermatogenesis in the 47,XXY testis have shown us that the spermatozoa found are almost all 23,X or 23,Y and arise from 46,XY spermatogonial clones that can complete meiosis and spermiogenesis. What a comforting thought for prospective parents that the genotype and phenotype of their offspring will be effectively the same as for couples in whom the male factor infertility component is not caused by a 47,XXY karyotype.

We learned of the tremendously wide phenotypic spectrum of KS, ranging from complete failure of pubertal progression in the adolescent to fully virilized but infertile in the adult. This difference is based simply on how much ability the atrophic testes (and their Leydig cells) have to produce testosterone. That is, virilization is a direct phenotypic manifestation of Leydig cell function /dysfunction. While the path offered to the adult male and his partner presenting for infertility is clear (microTESE and ICSI), what of the adolescent discovered to have KS? Damani et al. were first to describe the unknowns and the issues involved in these cases; would there be sperm present, should we be performing microTESE in this age group to preserve fertility, and what will the long term outcomes be (591)? Great debate ensued and, although not fully resolved, led to a new and innovative line of research by Paduch et al., as well as numerous others, into the associated consequences of having an extra X chromosome, especially the endocrinological ones (592). As we pay attention and learn about these other issues of sex chromosomal aneuploidy, we realize, vis-à-vis Klinefelter Syndrome, it is not just about the sperm.

## Conclusion

We must strive to continue to understand the conditions we are more and more successfully treating; we always should consider the offspring's health and welfare as well and information we can provide to the couple is how we can best do that. This pertains to spermatozoal defects such as globozoospermia, dysplasia of the fibrous sheath, and primary ciliary dyskinesia, to name but a few. The marriage of clinical medicine and basic science research, in terms of elucidating the genetic basis of conditions such as these, needs to be strong and stable. It has to grow and thrive. This will apply to not just genetic but also epigenetic etiologies of male factor dysfunction.

## FERTILITY PRESERVATION IN THE MALE

Robert E. Brannigan, M.D. and  
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### Fertility preservation: the emergence of a field

Over the course of a male's lifetime, he has an approximately 50% chance of being diagnosed with cancer. This diagnosis

can be truly devastating to a patient, but with advancements in oncologic therapies, dramatic improvements have been realized in survival rates over the last 40 years. More specifically, for males between 0-44 year of age, the five-year cancer survival rate is an impressive 78.3% (593). The vast majority of males diagnosed with cancer will thus survive their cancer, and the medical literature clearly indicates that most of these patients will want the opportunity to biologically father a child with their own sperm during their lifetime.

Cancer is a complex disease process, and the multi-pronged therapies commonly used to treat it are also often elaborate and involved. Even before the initiation of oncologic treatments, cancer itself and the body's immune response to cancer can adversely impact reproductive potential. Tumors involving the hypothalamus and pituitary gland can impair the secretion of gonadotropins, which are essential for the initiation and maintenance of normal spermatogenesis. The testicles are the site of spermatogenesis, and primary testicular tumors (i.e. seminomatous and nonseminomatous germ cell tumors) and tumors metastatic to the testicles (i.e. lymphoma) can have significant adverse effects. These tumors can replace normal testicular tissue, disrupt sperm production, obstruct the rete testes and/or efferent ductules, and block the egress of sperm from the testicles. A number of oncologic processes can involve the structures comprising the excurrent ductal system, including the epididymis, vas deferens, seminal vesicles, and the ejaculatory ducts within the prostate gland. Similarly, processes affecting the sympathetic nervous system, particularly within the retroperitoneum and pelvis, can disrupt seminal emission and ejaculation. Beyond these direct tumor effects, a patient's immune response to cancer can negatively impact fertility. Cytokines have been implicated in disrupting the normal function of the hypothalamus and pituitary gland, resulting in a relative hypogonadotropic hypogonadism state in some patients. Additionally, germ cell stem cells and their lineage cells progressing through the stages of spermatogenesis can be damaged by cytokines and other mediators of the immune system, causing massive germ cell loss and decreased fertility potential.

Beyond the effects of cancer itself, cancer therapies can similarly have a profound and deleterious impact on fertility. While a full review of the effects of specific chemotherapeutic agents is beyond the scope of this manuscript, these medications are well characterized in terms of the potential threat that they each pose to male reproductive potential. That being said, the specific effect that a particular drug or regimen will have on a given patient is difficult to reliably predict, as observed by Green et al. in their studies of survivors of pediatric cancer who had previous chemotherapy with alkylating agents (594). Radiation therapy is a mainstay treatment modality for many cancers. Radiation delivered directly to or even in the vicinity of the hypothalamus and pituitary gland can impair gonadotropic secretion, and radiation to the spinal cord, retroperitoneum, and pelvis can injure the sympathetic nervous system and thus disrupt seminal emission and ejaculation. Finally, radiation to the testicle, either primary or in the form of "scatter," can have lasting negative effects on germ cells. This is an important point, because while Leydig



cells do not usually exhibit functional decline or apoptosis when  $<20$  Gy of radiation are administered, the germ cell line is much less resilient. Doses as low as 2 Gy commonly result in permanent azoospermia. In addition to chemotherapy and radiation therapy, surgical interventions such as craniotomy, retroperitoneal lymph node dissection, orchiectomy, radical prostatectomy, and radical cystectomy can result in anatomic and/or functional disruption of key components of the male reproductive system. The hypothalamus, pituitary gland, sympathetic nervous system, testicle, epididymis, vas deferens, seminal vesicle, and prostate gland are all susceptible to damage from certain operative procedures. Therefore, when discussing operative therapy with patients, a careful review of the potential for injury or possible need to remove of part or all of these structures should be made if the structures are possibly at risk.

For decades, cancer survivors often had to be content with a fundamental tradeoff: fertility potential in exchange for survival. In the late 1990s and early 2000s, several important studies were published that highlighted the breakdown in communication surrounding this issue. On the provider side, a publication by Pryor et al. revealed that high percentages of oncologists reported discussing the fertility risks of cancer therapies with their patients (595). A subsequent study by Schover et al. revealed that only 60% of male patients age 14–40 years were informed of potential fertility issues associated with their cancer therapy, and only 51% of men desiring to have children in the future were informed of the option of sperm cryopreservation (596). The sharp disconnect between the physician reported data and the patient reported data led to a groundbreaking 2004 report by the President's Cancer Panel. This document provided transformative insights when it stated that communication breakdown regarding fertility loss and fertility preservation was a common event during oncologic counseling. The panel called for the use of cultural- and literacy-sensitive educational materials when counseling patients, and it also recommended that information be reviewed with patients both verbally and in writing. In 2006, the American Society of Clinical Oncology published a landmark article with recommendations regarding fertility preservation in cancer patients (597). This true milestone paper provided a roadmap for clinicians to follow as they diagnosed, counseled, and treated patients with cancer. Over the ensuing years, a large increase was seen nationally in awareness of fertility preservation and delivery of fertility preservation care. Many centers created formalized fertility preservation programs, and key themes emerged in the literature that integrated care and optimized interdisciplinary communication were important characteristics of effective programs (598). Furthermore, the creation of a patient navigator position, to oversee the coordinated delivery of fertility preservation care in the midst of oncologic testing and treatment, proved to enhance outcomes. Despite many successes, the field of fertility preservation is still hampered by lingering issues. For example, at this time there is no clinically proven fertility preservation treatment for prepubertal males. Their lack of spermatogenesis prevents them from banking gametes prior to cancer therapy. Several centers currently have experimental protocols in

place for cryopreservation of testicular tissue from these patients, with the hope that one day science will advance to the point where germ cell stem cells from within this tissue can be isolated and used to create mature, functional sperm. (599). Another group lagging in the delivery of effective fertility preservation care are adolescents. (600) While these patients are every bit as susceptible to the deleterious effects of cancer and cancer treatments as males  $>18$  years old, an abundance of literature has clearly shown that this group is offered fertility preservation care at markedly lower rates than men  $>18$  years old. Reasons for this gap in delivery of care include a lack of familiarity with reproductive medicine, discomfort among pediatric oncology providers in discussing reproductive issues with minors, and a simple lack of access to fertility preservation care. Clearly, adolescent males comprise a group of patients that we can and should be doing a better job caring for, as adult survivors of adolescent cancer reflect on missed opportunities for fertility preservation with both regret and anger. For this adolescent group, strides can be made in improving treatment through the education of pediatric health care professionals regarding fertility preservation care and enhancement of access of pediatric health care centers around the world to fertility preservation providers.

Research initiatives that successfully tackle challenging clinical problems are an important hallmark of fields that are growing and advancing. Over the last 15 years, numerous federal government (National Institutes for Health, National Cancer Institute, etc.) and private nonprofit (American Society for Reproductive Medicine, Livestrong Foundation, etc.) organizations have provided funding to help nurture this emerging field in multi-faceted ways. Project topics range from defining optimal approaches in physician-patient communication to basic science studies aiming to bring germ cell transplantation and in vitro spermatogenesis from the bench to the bedside. These latter initiatives have to potential to be truly transformative not only for cancer survivors left infertile after oncologic therapies, but also more broadly for large numbers of men with infertility due to other causes.

### **Sperm cryopreservation: the bedrock of fertility preservation**

One of the greatest impacts of the advancing technologies in in-vitro fertilization during the past 40 years has been in making paternity possible in males with azoospermia. Forty years ago, only men with azoospermia due to anatomic obstruction amenable to microsurgical reconstruction could potentially be treated and father children. Now, not only can men with obstruction intractable to surgery be remedied with testicular sperm extraction and intracytoplasmic sperm injection, but those with azoospermia due to spermatogenic dysfunction may be managed with the same strategy and similar success.

Frozen ejaculated sperm had been successfully used with intrauterine insemination and in-vitro fertilization for pregnancy, so it was only natural to ask the question whether testicular derived sperm could be utilized in a similar manner when intracytoplasmic sperm injection, without its requirement for a motile sperm, was demonstrated to yield

fertilization, pregnancy, and live birth. With cryopreserved ejaculated sperm, the convenience of not needing to have the male physically present to give a fresh sample was obvious. However, with testicular sperm and its attendant needs for procedures ranging from inserting a needle into the testicle to opening the scrotum, incising the testis, and later with microsurgery, the convenience of having cryopreserved testicular sperm in advance of the intracytoplasmic sperm injection cycle was doubly manifest. Cryopreservation of testicular sperm should it be possible and effective thus conferred certain significant advantages for the couple, the in-vitro fertilization laboratory, and the physicians involved in reproductive care. One would be that it could be scheduled in advance for the male, and both partners in the couple need not undergo invasive procedures on the same day allowing such small but important possibilities as each being present for the other's procedure and driving each other home. Another advantage was that should one doctor perform the in-vitro fertilization procedure and another perform the surgical sperm extraction procedure, the problematic logistics of timing two busy physicians' schedules was eliminated. But without doubt, the most compelling gift given by cryopreservation of testicular sperm was knowing whether or not sperm could be obtained and male gametes from the intended biological father were available for in vitro fertilization. This knowledge allowed the couple the psychological ability to consider well in advance what they wanted in the case that sperm was not available rather than having to decide on the same day as in-vitro fertilization, when both were undergoing invasive procedures at the same time.

Consequently, cryopreservation of testicular sperm was in many ways even more desirable than that of ejaculated sperm, but could it be done, and if it could, would it yield the same outcomes as that of fresh sperm in intracytoplasmic sperm injection? That was a compelling question in the 1990s, the decade after the introduction of this new, in many ways surprising form of in-vitro fertilization. As early as 1995, investigators described the successful use of sperm obtained from testis tissue in intracytoplasmic sperm injection, and many reports would soon follow in the ensuing years (601). Like many groups and labs involved in male reproductive medicine, we became very interested in cryopreserving testicular sperm and developed and published laboratory techniques that we observed to be optimal (602). One of the pressing questions during the advent of cryopreserving testicular sperm was whether sperm from a failing testis fared worse than sperm from an obstructed one. From the beginning, we observed that should strict laboratory protocol be followed, pre-freeze and post-freeze viability, and fertilization, implantation, and pregnancy rates were highly similar in both groups (602). The moral of the story was, if a sperm could be found in a testis and frozen, regardless of whether the testis was obstructed or dysfunctional, that sperm would fare the same in intracytoplasmic sperm injection.

One unsurprising observation we made was that testicular sperm was mostly non-motile and only occasionally twitching. In the years since and even today, that fact has confounded embryology technicians who intuitively desire a motile sperm to select prior to its injection into the ovum. Of course, the tech-

nician will render the sperm immotile as a necessary prerequisite to intracytoplasmic sperm injection, typically today by means of mechanical disruption with micropipette immediately after its identification as the sperm to use. Motility prior to injection was seen as some sort of proof of sperm viability for intracytoplasmic sperm injection, having precious few other means of demonstrating that the best sperm was selected. In an often overlooked but key study from 2005, investigators set out to ask whether motility mattered in predicting intracytoplasmic sperm injection outcomes, and observed that in fact it did not (603). What we're left with is the simple truth that at the present time, we don't have the means to determine in a non-destructive manner whether for a single sperm its proteome, metabolome, genome, and epigenome is up to the task of fertilizing an egg that will implant and lead to pregnancy and live birth. What we do know is that motility doesn't help in sperm selection. In fact, when we were developing the optimal laboratory techniques for cryopreserving testicular sperm, we observed impaired outcomes in intracytoplasmic sperm injection when agents such as pentoxifylline that are known to induce increased sperm motility were added. It was as if these agents served as toxicants for the delicate in-vitro fertilization cell culture. Certainly, pentoxifylline and other phosphodiesterase inhibitors and calcium ionophores could demonstrably induce motility, but induced motility did not help in selecting the best sperm for intracytoplasmic sperm injection, and in fact worsened outcomes. A sperm chemically induced to wiggle might please the embryology technician, but that sperm's real job would be impaired. This counter-intuitive result explained why certain embryology laboratories observed worse results with cryopreserved testicular sperm compared to that obtained surgically and then used immediately. Cryopreserved sperm was more likely to be bathed in chemicals such as pentoxifylline that induced motility after thaw but befouled embryo culture. For the same reason, this also explained why certain laboratories observed better results for sperm from men with azoospermia due to obstruction than from spermatogenic dysfunction where the occasional sperm didn't twitch as in obstruction and was more likely to be subjected to motility stimulants.

In the years following the initial description of cryopreserving testicular sperm, study after study observed similar outcomes with frozen and thawed tissue compared to that obtained on the day of surgical sperm extraction (604). A recent meta-analysis demonstrated no discernible difference between fresh and cryopreserved sperm derived from the testis when used in intracytoplasmic sperm injection in fertilization or pregnancy rates (604). The promise of a convenient method to obtain sperm from the testis that did not require substantial overhead in schedule for patients or physicians, and most importantly allowed the couple to know if paternal gametes were available prior to the in vitro fertilization cycle, was realized.

Challenges in cryopreserving testicular sperm certainly remain. While certain chemicals such as pentoxifylline may be toxic to embryo culture, others awaiting identification may promote or improve sperm function after freezing. But perhaps the greatest challenge is that cryopreserving sperm is an inefficient system with fewer sperm retrieved from the

storage system than the amount inserted. This effect becomes highly problematic when very few sperm are extracted from testis tissue, when sperm can be found in tissue but none retrieved at the time of thawing for in-vitro fertilization. While this is a problem for ejaculated sperm as well, the inva-

sive nature of surgery and that it is best performed only once creates a compelling need for a solution. Numerous approaches are under investigation in improving storage systems for cryopreserving very small amounts of testicular sperm.

## THE BIG PICTURE

### LEGAL AND ETHICAL ASPECTS OF IN VITRO FERTILIZATION

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Guido Pennings, Ph.D.

#### Legal aspects of in vitro fertilization

The success of in vitro fertilization (IVF) in 1978 has made it possible to create families in ways not previously imagined. The legal presumption that a child can only have one mother and father no longer exists, raising a multitude of novel legal issues. What is the legal definition of an embryo and who decides how it may be used or disposed? How do you establish parentage to a child born via third-party reproduction? Can a child have more than two parents? Can frozen gametes/embryos be used post-mortem? Are fees paid to donors and/or surrogates taxable? The list goes on and on.

Until very recently, attorneys in this field had little guidance from case law or legislation and few colleagues with whom to collaborate. It was not until the late 1980s that the American Bar Association formed the first national reproductive law committee. The American Academy of Assisted Reproductive Technology was formed twenty years later, in 2009, followed shortly thereafter by American Society for Reproductive Medicine's Legal Professional Group. Over the last ten years the legal community has experienced exponential growth with more lawyers practicing in this area, and more local and international reproductive law organizations emerging around the world. Currently, in the U.S. alone, there are four national legal conferences each year dedicated solely to assisted reproductive technology, and numerous additional state, local, and international meetings focused on assisted reproduction and the law.

Despite the recent growth in the legal community, laws addressing assisted reproduction have evolved slowly through the years. As is often the case, medical technology develops first, and eventually the law follows (often in reaction to an unfavorable or unanticipated legal outcome). In fact, if it is true that the first attempts at intrauterine insemination occurred with Henry the IV in 1455, it was centuries later before any case law began to develop (605).

The first set of reproductive cases (from the early 20th century) arose in the context of heirship, holding that children born by donor insemination were illegitimate, and the mothers of such children were accused of adultery, even when the husband was infertile and the insemination pro-

ceeded with the husband's consent (606). By the 1970s, public sentiment began to change and many states passed laws acknowledging that a husband and wife using donor sperm were the legal parents of the child. Many of these state laws were lifted in part or whole from the Uniform Parentage Act (UPA) of 1973 (607), which included a section on artificial insemination, but only applied to married heterosexual couples working with a physician for the donor insemination. Although these state laws (several of which are still in existence) seemed progressive at the time, today they are viewed by many as outdated, discriminatory and in violation of federal law. Single persons, same sex couples and unmarried heterosexual couples are excluded from legal protection. The UPA was updated in 2000 and amended in 2002 (608) to include a section on assisted reproductive technology which recognized egg donation and removed the necessity that the parties be married, but continued to apply only to heterosexual couples. It was not until 2017 that the UPA was revised to apply equally to same sex couples (609). Notably, only a handful of states have adopted the 2000 UPA (amended in 2002) and only the state of Washington has enacted the 2017 UPA.

Most American states have not addressed egg donation, and although parentage laws typically consider the woman who gives birth to a child as proof that she is the child's mother, in all but a handful of states an intended mother has no legal guarantee that she will be considered the legal mother of the child (610). Similarly, until very recently few states had surrogacy statutes, and even states with favorable law, or where surrogacy arrangements were occurring regularly and supported by local courts (absent specific legislation or case law), it has been burdensome for same sex couples to be deemed the legal parents to the resulting child. The 2017 update to the UPA may prompt more protective legislation in this area, as should the recent Supreme Court decisions of *Obergefell* (611), holding that laws barring marriage between two people of the same sex are unconstitutional, and *Pavan* (612), holding that states have to treat married same-sex couples and married opposite-sex couples the same with respect to putting a spouse's name on a birth certificate. However, lawyers must still proceed with caution. For example, notwithstanding the recent Supreme Court decisions, the administrative act of placing a parent's name on a birth certificate does not create an irrebuttable presumption of parentage (one state is not required to recognize another state's birth certificate). Because certain states have tried to create legal obstacles for same sex parenting, same sex couples should still be advised to pursue a co-parent adoption

and receive a court order (which must be recognized by all other states).

Another relevant issue in third party reproduction is whether children conceived from donor gametes have the legal right to know the identity of the gamete provider. While most states are silent on this issue, at least eleven European countries now require disclosure of the donor's identity upon the request of the child (613). Although the recently approved 2017 UPA does not require disclosure, it does require that the donor be asked whether he/she would like his/her identity disclosed, and also requires the gamete bank to make a good faith effort to disclose the donor's non-identifying medical history information to the child or intended parent(s) upon request. Whether states will start legislatively requiring donor identity disclosure, or leave it up to the parties to make these decisions is yet to be known.

The growing practice of reproductive tourism has led to other unanticipated legal issues. Many countries have laws specifically forbidding surrogacy and other forms of third party family building, especially if compensation is involved. Unable to build families at home, intended parent(s) travel to the U.S. or other surrogacy friendly countries to access third party reproduction, resulting in complicated immigration cases. Although most attorneys entered this field intending to practice family law, many are now tasked with also understanding immigration matters as children born in other countries must return home and gain citizenship. In a few recent cases, children born via surrogacy were initially stateless, as the home country (where the intended parents reside) did not recognize surrogacy as a means of parenthood and would not grant citizenship, and the intended parent(s) could not obtain citizenship or extended visas in the country where the child was born. For example, intended parents from Germany entered into an arrangement with a surrogate from India, resulting in the birth of their twins in 2008. Because surrogacy arrangements are strictly forbidden in Germany, the German government refused to recognize the intended parents as the legal parents and would not issue a German passport. Because the twins were born to a surrogate, India did not recognize the children as Indian nationals and refused to issue an Indian passport. These children were stateless for almost 2 years, until after extensive litigation they were granted Indian identity documents and the German government issued visas to allow them to travel to Germany (614).

In sum, the creation of IVF has led to a developing area of law much broader and more complicated than most lawyers practicing in this area could have imagined. As medical technology continues to advance the laws will continue to follow, and the attorney's role will continue to be to guide their clients, and create legal protections for intended parent(s), their offspring, and the gamete providers/surrogates who make third party reproduction possible.

### Ethical aspects of in vitro fertilization

Over the years, the ethical issues in IVF have changed considerably. One of the most contentious aspects of IVF at the start was the creation and destruction of human embryos (615). Since IVF was practiced in countries with largely Christian

dominated cultures, the embryo was attributed the status of a (potential) person and as a consequence had to be respected and protected. However, these concerns have died down to make place for the recognition that surplus embryos and embryo destruction were an inevitable part of IVF. This is remarkable since the main reasons for creating more embryos than necessary (meaning: more than could be replaced in the fresh cycle) were pragmatic. In fact, it would have been possible (and still is) to practice IVF without violating the rule of respect for human life in the form of embryos. Some countries, like Italy and Germany, regulate IVF in a way that many fewer surplus embryos are created. This evolution is all the more remarkable as it is linked to the major adverse reaction to IVF: multiple pregnancy. After a period of quasi-unlimited transfer, it was acknowledged that the fixation on pregnancy rates was having disastrous effects (616). Although the single-embryo transfer movement has been around for two decades, the change in practice has been limited. In countries without a legally imposed limit on the number of embryos for transfer, multiple pregnancy rates remain high.

The general practice of IVF cannot be understood independently from the society in which it was and is practiced. The original idea was to give a heterosexual married couple the possibility to become pregnant. Gradually, the indications for IVF were broadened: from blocked fallopian tubes over low sperm quality to unexplained infertility. Simultaneously, the categories of people eligible for treatment increased. This evolution was triggered by the increasing acceptance of previously discriminated groups in society, such as lesbian couples. This led to an expansion of the definition of infertility. While one originally needed a clear medical cause in order to justify an intervention, one moved further and further away from the medical need. Social infertility now constitutes a large part of all applications: single women, lesbian and gay couples, older women, etc. This evolution was inextricably connected to another modification of the original scheme: the use of donor gametes. Although the primary goal of assisted reproduction remains the creation of a child with the intended parents' own gametes, this goal cannot be reached in all circumstances. In some cases, there may be either a complete absence of gametes or an absence of functional or suitable gametes. Still, practitioners of medically assisted reproduction did and do everything possible to keep the use of donor gametes to a minimum. The introduction of intracytoplasmic sperm injection is the main illustration of this endeavor. Much research is directed at finding ways to overcome barriers that at present can only be solved by using donor gametes: in vitro maturation of oocytes, stem cell derived gametes, elective freezing of oocytes, mitochondrial transfer to rejuvenate oocytes, and even reproductive cloning. Each of these techniques requires extended manipulation of the gametes and embryos and thus introduces possible health risks for the child. Many ethical problems in the field demand the balancing of risks for mother and offspring against the wish to have a genetically related child.

The practice of gamete donation is an ethical mine field of its own. It started with the donor insemination within a heterosexual couple with a male partner with poor sperm quality.



Around the 1980s, oocyte donation came along. At the moment, about one in five IVF cycles are performed with third party gametes (617). At the start, practitioners advised patients not to tell the child or anyone else in their social network. In the last decades, openness has increased slightly but not spectacularly. The majority of heterosexual couples maintain non-disclosure of the use of donor gametes. The present impression of openness is largely due to the participation of patient groups (single women and lesbian couples) for whom secrecy is not an option. At the moment, the pendulum has gone completely to the other side with legislators and counselors pushing or forcing patients to be open about the donor conception to their child, even though no evidence is available that this benefits the children (618).

The second change is related to donor anonymity. In the last decade, some countries have abolished donor anonymity. The pressure for this change came from a small group of donor conceived people who argued that they needed to know the identity of their donor to complete their identity. This claim was and is rarely critically assessed or analyzed. In the meantime, the claim has been broadened: it now not only includes the possibility to find out the identity of the donor but also the possibility to contact the donor, to have regular meetings with the donor, to be informed about donor siblings and their identity. This evolution is completely based on a geneticization of identity, relationships, and family. "I know who I am when I know where my genes come from. We are family because we are genetically related." As such, this focus on genetics is diametrically opposed to the whole practice of gamete donation where the emphasis is precisely on intentional, social and psychological parenthood.

Finally, the entire field of gamete donation has been commercialized. There is a marked difference here between the U.S. and Europe: payment for gametes has been accepted from the start in the U.S. Over the years, the exorbitant amounts offered to certain categories of oocyte donors have drawn attention from the media. Europe holds more strictly to the prohibition on payment for body material in general but the shortage of gametes (and especially oocytes) has led to increasing amounts of money for 'reimbursement' (619). Moreover, also other aspects of the practice show the commercialization: the availability of catalogues of donors on the internet, the growing industry organized in private sperm and egg banks, the websites advertising eggs and sperm, the outsourcing of recruitment to large sperm and egg banks, and the increasing role of brokers. This evolution goes hand in hand with the trend in medically assisted reproduction in general where commercialization is rife. After 40 years, access to treatment is still a serious ethical problem. If infertility is an illness that should be included in the basic health care package of every citizen, reimbursement through either public or private health insurance would be appropriate. However, justice is not only a task for the government but also for the practitioners. As long as practitioners make no effort to lower costs, governments will be very reluctant to intervene to increase access.

In sum, medically assisted reproduction is a never-ending source of ethical challenges. People frequently hold very strong views and intuitions on matters related to life, repro-

duction and family building. To change these views, one needs not only time and rational debate but also a supporting society into which these techniques can be integrated.

## IN VITRO FERTILIZATION AND THE PSYCHOLOGY OF REPRODUCTION: OPPORTUNITY AND HOPE

Susan C. Klock, Ph.D.

The birth of Louise Brown marked a new era of opportunity and hope for infertile couples as the possibility of a successful pregnancy became a reality with in vitro fertilization (IVF). The psychological shift that accompanied this medical advance was significant. Instead of grief and sadness, there was the possibility of hope for couples to achieve their dream of having a child. Instead of being blamed for their infertility because it was assumed there was a psychogenic cause, infertile women could try IVF and in the course of their treatment a physical cause could be discovered and/or their goal of getting pregnant could be realized. The change in paradigm regarding the relationship between a woman's psychic state and her fertility changed with the invention of IVF.

In addition to the psychological paradigm shift after the discovery of IVF, the ancillary applications of IVF techniques to oocyte donation, gestational surrogacy and fertility preservation, both medical and social, have affected the psychology of infertility (620). By differentiating the three components of motherhood (oocyte source, gestating woman, and rearing mother) IVF technologies prompted an examination and refinement of the definition of who is a mother (621). In separating the roles associated with motherhood, IVF and its related technologies have introduced a host of changing family constellations (620). The psychological implications of these changing roles is an emerging area in the psychology of reproduction.

A third way in which the psychology of reproduction has changed due to IVF is the impact of fertility preservation techniques, both medical and social, on the opportunity for parenthood. Fertility preservation has broadened the course of the developmental and physical limits of the reproductive time span. Cancer patients can preserve oocytes or embryos and realistically hope to become a biological parent after gonadotoxic treatment. Unpartnered women can freeze oocytes to use later in life when they feel the circumstances are right for them, allowing them to pursue other life goals and minimize the biological pressure to reproduce. Transgendered individuals can pursue biological parenting in ways that were unavailable a generation ago. Embryos can be created and cryopreserved for years, prompting psychological, social and ethical challenges to decisions related to embryo disposition. IVF technologies have expanded the boundaries of reproduction and, as such, have expanded the scope of the psychology of reproduction. This review will explore these three issues primarily as they have affected women. This is due to the fact that the preponderance of psychosocial theory and research on infertility and IVF has focused on women, that women bear the preponderance of the treatment burden in IVF and that the changing definition of motherhood is specific to women.

## Change in paradigm

Prior to IVF infertile men and particularly women, were subjected to numerous psychoanalytic theories and interventions to “cure” their infertility. References from the 1950s, 1960s, and 1970s are framed in psychodynamic terms that posit a psychogenic cause of infertility and suggest that once the woman’s “conflict” with femininity and motherhood were solved, the woman would be able to become pregnant (622). The medical knowledge created from developing IVF was a milestone because it could provide a medical explanation for what had previously been attributed to a psychological cause. As IVF successfully treated the previously infertile, the utility of a psychogenic theory of infertility became obsolete. Blaming the infertile individual for their infertility due to psychological reasons no longer had a place in the psychology of reproduction. This paradigm shift is reflected in the publications that emerged in the post-IVF era (623). Mental health professionals noted that instead of psychological distress causing infertility; infertility and its treatment caused psychological distress. This simple but important shift in thinking advanced the field of reproductive psychology into a new era.

A new focus of psychosocial research investigated the psychological aspects of infertility and its treatment. Guided by stress and coping theory, studies investigated the stress levels of women undergoing infertility treatment, prevalence of depression and anxiety, ways of coping and marital adjustment to name a few. It was found that infertility was as stressful as other major medical disorders. Numerous studies indicated that rates of depression among women undergoing IVF were as high as 40% and up to twice as high as the rates of depression in the general population for both men and women (624). Rates of clinically significant anxiety in women undergoing IVF were also higher than the general population with estimates of 15% of women. Stress levels vary across the course of IVF treatment with women who subsequently did not get pregnant reporting higher levels of stress during the monitoring and waiting periods. Moreover, retrospective recall of stress tends to be higher than the daily ratings of stress during treatment. Also, for men, infertility related stress and depression increases over time, while sexual functioning decreases. In terms of coping, avoidance coping has been consistently associated with poorer adjustment to infertility and dispositional optimism is correlated with better adjustment (625). Last, qualitative and quantitative studies have demonstrated that a subset of patients (25%–50%) have reported strengthened marital relationships during the course of infertility and its treatment. In summary, the paradigm shift since the development of IVF has focused on the psychological consequences of infertility, its treatment and its impact on psychological well-being.

## Definition of motherhood

The development of parenting via IVF related methods of egg donation and gestational surrogacy have prompted consideration of the different components of motherhood—genetic, gestational and relationship/rearing or social motherhood (620, 621). As adoption has expanded, the category of motherhood to include mothering a child defined solely by the relational

connection, egg donation, and gestational surrogacy have allowed for further consideration of the genetic and gestational facets of motherhood by separating the roles of the woman providing the oocyte and the woman gestating the pregnancy. Previously, the woman providing the egg or the woman carrying the pregnancy was defined as the mother of the child. Since IVF and these related treatments, the definition of motherhood has changed by making the woman with the intention to parent the mother, subjugating the genetic and gestational roles to ancillary transactional roles. These changes have prompted new psychological challenges to all parties.

Clinically, intended mothers describe a multitude of thoughts and feelings. They are thankful for being able to get pregnant, they worry about how they will feel about their infant, they wonder, “Will she/he look like me and Will I love him/her?” There have been both qualitative and quantitative studies of parenting after oocyte donation describing the themes that women identified and worked through as they became mothers via oocyte donation. Mothers via egg donation acknowledged a strong desire for motherhood coupled with the realization of their infertility and the traumatic loss that infertility represented. As they made the decision to use oocyte donation to conceive, these women reported coming to terms with using donated oocytes to become pregnant and characterized the advantages of motherhood via oocyte donation as being able to experience pregnancy, to feel normal after the infertility diagnosis, and to nurture and establish a bond with a child. Golombok et al. have conducted several longitudinal studies addressing the adjustment of mothers after oocyte donation. These authors have concluded that the mother and child interactions up to age 14 years do not differ significantly from those of mothers with naturally conceived children (626).

Oocyte donors’ psychological experiences have also been studied. Studies of post-donation adjustment among oocyte donors have demonstrated that donors consistently show an altruistic motivation to help others become parents and most donors wanted to know the results of their donation but only a minority actually found out the result. In terms of the donor’s role in helping create a pregnancy, Jadva et al. (627) found that half of their sample of donors had concerns about having children they would never see or know. It is noteworthy that there are no long-term follow-up studies of donors to date that explore their subsequent family building plan, adjustment or contact with their adult offspring and this is an area in need of further study.

For gestational carriers, a review of the available literature found that there were few difficulties during the gestation process (628). Post-delivery relationships between the surrogate and the intended mother was generally good with clear understanding of the motherhood boundaries. In summary, the changing definition of motherhood prompted by IVF and its related procedures has led to further refinement of the primacy of the social/relationship aspect in defining motherhood.

## Expanding the possibilities of parenthood

Medical and social fertility preservation, with cryopreservation of oocytes or embryos, has been made possible by the

development of IVF techniques and as such has contributed to the ability of women to preserve their reproductive potential in the face of illness or personal circumstance. Medical fertility preservation has allowed women who have cancer or other life threatening illnesses to bank their oocytes or embryos, undergo potential gonadotoxic treatment, and remain able to have a child using their own gametes (629). As the number of cancer survivors grows due to advances in detection and treatment, the issues of fertility among cancer survivors has received increased attention. Many female cancer patients are interested in the possibility of having children and that the possible loss of fertility due to cancer treatment is a serious concern, in fact the possibility of having a child can be an important motivator for recovery. And while not all women who are counseled about fertility preservation decide to undergo it, there is evidence that women are less likely to regret not undergoing fertility preservation if they have been counseled about it by their oncologist and a fertility specialist (630). For those women who undergo fertility preservation, initial studies suggest that 13% to 23% return to use their cryopreserved tissue and attempt pregnancy (631). The expansion of medical fertility preservation has led to improved quality of life and renewed hope of having a child where it was previously unavailable.

Fertility preservation for nonmedical reasons is also growing as employers are offering oocyte freezing cycles as a benefit to their female employees or women choose to self-pay to undergo oocyte cryopreservation. The expanded use of oocyte cryopreservation allows women to delay childbearing due to career, relationship or other life circumstances (632). Oocyte cryopreservation can represent reproductive insurance for women who delay childbearing. Although it has been argued that oocyte cryopreservation's success has been overstated and represents a false sense of reproductive security, it is nevertheless an increasingly common treatment among those women who can afford it. Psychologically, it presents another avenue of choice and hope for women regarding control over their reproductive lives.

Development of IVF technology has also provided an avenue for gay, lesbian and transgender individuals to have options for family building. Gay male couples using donor oocytes and a gestational carrier are a growing segment of the third party reproduction field (633). Lesbian couples can share reproductive roles with one partner providing the egg, the other gestating the pregnancy for a new constellation of pregnancy creation. Transgender individuals are also utilizing fertility preservation and assisted reproductive technologies to maintain their options for having genetically related children after their transition. All of these options are innovations that provide opportunity and hope to individuals previously unable to create the type of family that they envisioned for themselves.

As a consequence of the advances since the development of IVF, the role of the mental health professional in reproductive medicine has also expanded. Mental health professionals work with the treatment team to educate and provide support for couples undergoing IVF and other assisted reproductive technologies before, during and after treatment.

In summary, since the creation of IVF and the techniques associated with it, the psychology of reproduction has changed and expanded. What was once considered impossible 40 years ago is a daily occurrence now because of IVF. This has required the concomitant expansion of psychological theories, research and evidence based interventions in reproductive psychology. The interaction of technology, medicine and psychology continues to expand and redefine the role of parenthood and has made parenting possible for those previously unable to attain it. IVF and its associated techniques have created opportunity and hope for the possibility of parenthood in new and expanded ways.

## EUROPEAN SOCIETY OF HUMAN REPRODUCTION AND EMBRYOLOGY AND THE ADVANCE OF ASSISTED REPRODUCTION

Simon Brown M.A. and  
André Van Steirteghem, M.D., Ph.D.

Despite its worldwide acclaim, in vitro fertilization (IVF) made only slow progress in the first years after the birth of Louise Brown. Moral objections seemed as great after her birth as before, and, according to Robert Edwards, such opposition would largely explain the hiatus between Louise's delivery in 1978 (at Patrick Steptoe's state hospital in the north of England) and the opening of their private clinic at Bourn Hall near Cambridge in 1981. "Human conception was considered sacred," Edwards would later say, "something that should not be tampered with" (634).

Progress was also hindered by the sheer scientific difficulty of IVF. Edwards himself had first described the technique in 1969 (635) and throughout the succeeding decade continued to report experiments in sperm capacitation and oocyte maturation from his Cambridge laboratory. This challenging work was also undertaken without specific funding as Britain's Medical Research Council turned down Edwards's first grant application in 1971 (636).

The world's third IVF baby, Candice Reed, was born in Australia in 1980, where progress in IVF had been openly documented and relatively encouraged. Her birth followed a report from the Melbourne group in 1973 of two biochemical IVF pregnancies which had been lost after <1 week (637). Thus, by the start of the 1980s, there were two groups leading the way in IVF, Edwards and Steptoe at Bourn Hall and Carl Wood and Alan Trounson in Melbourne, each with live births and a long track record in the biology of both human and animal reproduction. By now there were also several other groups in Europe and the U.S., invariably led by gynecologists and biologists determined to start their own programs.

In the U.S., any success had been frustrated first, by ethical objections to assisted conception, and then by the false scent of IVF in a natural cycle, set by the precedent of Louise Brown. Thus, Howard and Georgeanna Jones at their newly established clinic in Norfolk, Virginia, had begun in 1980, as Edwards advised, with the natural cycle but, after 41



TABLE 12

**Chairmen of European Society of Human Reproduction and Embryology, 1985-2018.**

Chairman	Country	Term
Robert Edwards	Great Britain	1985-1987
Jean Cohen	France	1987-1989
Pier Giorgio Crosignani	Italy	1989-1991
André Van Steirteghem	Belgium	1991-1993
Klaus Diedrich	Germany	1993-1995
José Egozcue	Spain	1995-1997
Basil Tarlatzis	Greece	1997-1999
Lynn Fraser	Great Britain	1999-2001
Hans Evers	Netherlands	2001-2003
Arne Sunde	Norway	2003-2005
Paul Devroey	Belgium	2005-2007
Joep Geraedts	Netherlands	2007-2009
Luca Gianaroli	Italy	2009-2011
Anna Veiga	Spain	2011-2013
Juha Tapanainen	Finland	2013-2015
Kersti Lundin (SE)	Sweden	2015-2017
Roy Farquharson	Great Britain	2017-2019

*Forty years of IVF. Fertil Steril 2018.*

laparoscopic oocyte retrievals, had cleavage in just 13 patients and no pregnancies. Only at the end of 1980 did they return to the stimulated cycle and a pregnancy in their 13th attempt (638).

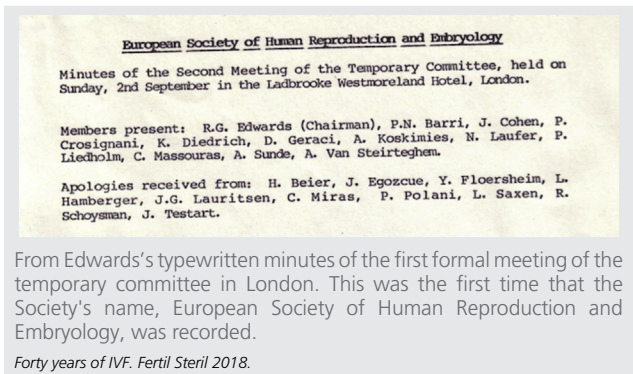
In this same report, Jones recalls how in 1981 he and Georgeanna attended a workshop hosted by Edwards and Steptoe at Bourn Hall at which “a small IVF mafia-type group” met to exchange experience. Among the guests were most of the few then making real advances in IVF: from Scandinavia (Hamberger, Wikland), Australia (Johnston, Trounson, Lopata), and France (Testart, Frydman, Plachot, Cohen). And it was in the friendship and helping hand offered by Edwards to the French gynecologist Jean Cohen where the origins of ESHRE lay.

By then, IVF in France had devolved into two publicly funded programs, the one led by René Frydman and the biologist Jacques Testart, and the other by Cohen. The former delivered its first IVF baby, Amandine, in February 1982, and the latter a few months later. However, despite these successes, both groups were perplexed by high rates of implantation failure and ectopic pregnancy. So, in early 1984, Edwards phoned Cohen with an offer to come to Paris and help his biologists Plachot and Mandelbaum.

During his stay, Cohen would later write, “...he also came occasionally to dinner at my home and there we would often discuss the prospects for reproductive medicine in Europe.” So it was here that they raised the need for a European society similar to the American Fertility Society, with its own agenda, annual meeting and, most importantly, its own journal. Reproductive medicine and science had so far been dominated by the American journals but now, Edwards would say, “...when so many of the advances in IVF were made in Europe, it occurred to me that we needed a European journal to serve as a forum for this work.”

So it was, that on a May morning in 1984, Edwards and Cohen were found sticking posters to the walls of the Finlandia Hall in Helsinki, where the 3rd World Congress of IVF was

FIGURE 29



in progress. The posters announced plans for a European society in reproduction and an exploratory meeting the next day. Around twenty attendees turned up, some just curious, others more committed, to hear four items on the agenda: the society's name, its aims, its constitution, and the election of officers. Edwards not surprisingly was voted chairman, but all other decisions were deferred to a follow-up meeting in London in September. And it was here at this first meeting of the temporary committee, held at the Westmoreland Hotel that the society was formally founded and the name ESHRE (European Society of Human Reproduction and Embryology) was first recorded.

There was much debate over the name, but implicit in its final format were both clinical medicine (human reproduction) and reproductive science (embryology), a unanimous recognition of the two integrated disciplines in human fertility. Today, that tradition initially laid down by Edwards and his committee colleagues persists in the disciplines of ESHRE chairmen alternating from one two-year term to the next between basic science and clinical medicine.

Today, one cannot over-exaggerate the energy Edwards put into ESHRE. His papers in ESHRE's archives in Belgium are testimony to boundless energy and gentle persuasion, evident in a deluge of correspondence which one day in 1984 ran to 37 typed letters on ESHRE business. Thus, by the time of that September meeting in London, Edwards himself had drafted by-laws which, with amendments at the time, are largely the constitutional arrangements of ESHRE today. The minutes of that meeting note that those present in London included Cohen, Crosignani (Italy), Diedrich (Germany), Sunde (Norway), and Van Steirteghem (Belgium), all of whom in time would follow the lead of Edwards and become chairmen of ESHRE (Table 12, Fig. 29). Edwards himself wrote up the minutes and noted, “It was felt that the scope should be restricted in general to the study of gametogenesis, conception, the first trimester of pregnancy, but with the inclusion of associated topics of relevance to the main subjects of interest, e.g. the birth of children conceived in vitro, ethics, the use of DNA libraries and other preparations.”

There is much more besides the name, constitution, and scientific scope which endures from those earliest days. It was at this time that proposals for both an Annual Meeting



and a journal were made, but there was one other initiative, raised at the temporary committee's fifth meeting in Brussels, which would have real lasting consequences. This was the formation of an ethical committee, ESHRE's first subcommittee, under the chairmanship of Jean Cohen. Of course, it's no surprise that Edwards looked to this new society to take a judicious position on what he knew from experience would be the controversies likely to dog the scientific progress of IVF. Indeed, in this same year of 1984, a UK government report under the leadership of Edwards's Cambridge colleague Mary Warnock had formally looked into the social and ethical implications of IVF, and its findings, reflecting the pluralism that IVF implied, would eventually form the basis of the UK's Human Fertilisation and Embryology Act. Thereafter, IVF would be subject to legal statute in the UK (from 1990), with licensed IVF centers led by a Code of Practice, and with it the greater acceptance of IVF among the public, all of which Edwards had supported from the beginning.

The position statements produced by ESHRE on ethics and law continue to this day, with subjects under discussion increasing in complexity with advancing technology. The first reports examined topics of basic debate, the moral status of the embryo, cryopreservation, and gamete donation, but in later years were added assisted reproductive technology (ART) in single and lesbian women, sex selection for non-medical reasons, and elective oocyte freezing. Even from the outset, many of ESHRE's original members cited consensus on the ethics of IVF as their principal reason for joining. Indeed, Paul Devroey, a later chairman of ESHRE, named ESHRE's activities in the production of guidelines and position papers as landmarks in ESHRE's history, and a cornerstone of everything which ESHRE went on to build. The ethics of IVF were included as a paper in the first issue of the Society's journal *Human Reproduction* and in the program of its first Annual Meeting in 1985. Even today, many of the clinical subjects pursued in the Society's hugely successful training workshops do so from an ethical and psychological angle.

If ethical consensus on the implications of assisted reproduction has been a running theme through ESHRE's history, its real universal impact lies with the journals and the Annual Meeting. A journal for Europeans was, after all, the main ambition of Edwards and Cohen in their plans for a European society. It was thus on the very first agenda of the temporary committee and unanimously agreed by the third that ESHRE should run its own journal, with Edwards proposed as editor. Despite his protest that he was overcommitted, Edwards agreed, but only on the understanding that an editorial committee might be formed to help him along. It was, of course, a momentous decision and one which would absorb Edwards for the next 15 years of his life, and immediately stamp ESHRE as a society committed to the science of reproduction. It would also, in greater measure than anyone could imagine, fulfill those first pioneer ambitions of founding a journal in which European scientists and clinicians would find a welcome home for their work and where the eyes of the editor would look warmly and diligently on any paper submitted.

Planning the first issue of *Human Reproduction* proved a tour de force for Edwards, with a pilot issue slated for distribution at the first Annual Meeting in 1985. Edwards went into

overdrive, setting a deadline for the first issue of January 1986, and seven further issues that year. Over the next several years Edwards never lost any opportunity to convert meetings into manuscripts or to canvass his contacts for papers for the journal, often stressing urgency and a need to increase the submission rate. "We need four or five papers a week as a minimum to provide a good base," he wrote in January 1986.

Edwards edited *Human Reproduction* until 2000, when a heated dispute over publication (self or commercial) and a strained relationship between editor and publisher finally proved the end of Edwards's glorious partnership with ESHRE. It was a turbulent time for the Society. Edwards would eventually go his own way, setting up *Reproductive Biomedicine Online* as a self-published journal, while ESHRE took a deep breath and extended its commercial contract with the Oxford University Press. Since then, the journal has had a succession of editors-in-chief, led initially by the Glasgow gynecologist David Barlow (2001–2006) and followed by André Van Steirteghem (2007–2012) and Hans Evers (2013–2018).

As submissions to *Human Reproduction* increased during the 1990s and pressure on page space intensified, Edwards had taken a somewhat unilateral decision in proposing two additional titles to the ESHRE stable, *Human Reproduction Update* (launched in 1996) and *Molecular Human Reproduction*. By then, in the nine years since *Human Reproduction*'s launch in 1986, total subscriptions had risen dramatically (from 351 in 1986 to more than 2,000 in 1994), along with annual page totals and revenue (from £30,000 to almost a half million by the mid-1990s). In addition, its impact factor had slowly risen year after year (and before the 1990s were over *Human Reproduction* would reach number 1 in obstetrics and gynecology), with the quality of papers published increasing all the time (as reflected in an increase in rejection rate from 25% to 40%) (Table 13).

Both *Update* and *MHR* were proposed to siphon off review and basic science manuscripts from the main journal, and both inevitably were edited by Edwards, a huge commitment with copy editing and page layout then performed at Bourn Hall. In time, *Human Reproduction Update*, under the editorships of Bart Fauser (2001–2006) and John Collins (2007–2012) would achieve unprecedented impact factors in the categories of obstetrics and gynecology and reproductive biology, in 2014 breaking the elusive double-digit barrier under the editorship of Felice Petraglia.

Not least among the landmark papers published by *Human Reproduction* in the 1990s were those from the Brussels group of Devroey and Van Steirteghem, describing intracytoplasmic sperm injection (ICSI) and results from their early experimental work. Intracytoplasmic sperm injection was without doubt a European initiative, and *Human Reproduction*'s growth in the early 1990s rose in parallel to the emergence of ICSI in Europe.

Brussels reported its first live births with ICSI in 1992 in the *Lancet*, but thereafter all reports were published in *Human Reproduction*. It took only a very short time for ICSI to prove a true revolution in reproductive medicine, as monumental as that of IVF itself, with most types of infertility, both male and female, now amenable to treatment. There was a clamor from centers around the world to know more, and both the

**TABLE 13****Impact factors for *Human Reproduction* and *Human Reproduction Update*.**

Year	Hum Reprod	Hum Reprod Update
1997	2.421	2.642
1998	3.650	3.651
1999	3.003	2.297
2000	2.997	2.887
2001	2.987	2.969
2002	3.253	3.710
2003	3.125	3.731
2004	3.365	4.194
2005	3.669	5.449
2006	3.769	6.793
2007	3.543	7.257
2008	3.773	7.590
2009	3.859	7.042
2010	4.357	8.795
2011	4.475	9.234
2012	4.670	8.847
2013	4.585	8.657
2014	4.569	10.165
2015	4.621	11.194
2016	5.020	11.748

Forty years of IVF. *Fertil Steril* 2018.

Vrije Universiteit Brussel hospital in Brussels, and ESHRE itself were instrumental in arranging hands-on and live video training workshops. ESHRE began the first of its registry audits with ICSI, to be followed a few years later with the ongoing European IVF Monitoring registry and the Preimplantation Genetic Diagnosis Consortium data collections. Data collection and analysis would in time become hugely important components of ESHRE's ongoing activities.

Despite widespread interest, Brussels was never complacent about ICSI. With ICSI a much more invasive technique than routine IVF and drawing on apparently impaired sperm, questions about its safety were and are still asked (and studied) today. The evaluation of pregnancies and children was a condition of ICSI's approval by the Brussels ethical committee, and over the past 25 years, follow-up studies have found a slight increase in fetal chromosomal abnormalities after ICSI, and a congenital malformation rate slightly higher than after natural conception, but otherwise no outcome differences. These results are reflected in the national registry studies of the Nordic countries.

Van Steirteghem, who in 1991 had become the fourth Chairman of ESHRE, reported Brussels' first ICSI results to a full house at ESHRE's ninth Annual Meeting in Thessaloniki, Greece. More than 1,500 members attended, and this congress, like the journal, was on a steady upward curve of popularity and authority. Just 650 had attended the first Annual Meeting in Bonn in 1985, while in recent years more than 10,000 regularly attend. The meeting is billed as the world's leading event in reproductive science and medicine, and a global presence is indeed an ambition of the modern ESHRE. The Society's reach today extends via guidelines, data collection and reports, certification programs, position papers, training, and of course the authority of its journals, to present ESHRE as a point of reference for all working in reproduction.

It's also clear from its history that ESHRE has never been afraid to grasp a nettle, however politically or scientifically uncomfortable that may be. ESHRE prominently opposed introduction of the infamous Law 40 in Italy in 2004 (banning embryo freezing and embryo selection) and more recently supported open ART legislation in Poland. ESHRE has also been consistent, even if controversially so, in its position on new introductions and adjuvant treatments in ART, repeatedly calling for a strong evidence base before everyday application. This has most prominently been seen in an approach to aneuploidy testing in embryos, where ESHRE has repeatedly doubted the strength of evidence for improved delivery rates. The ESHRE Study Into the Evaluation of Oocyte Euploidy by Microarray Analysis study, a randomized trial designed and supported by ESHRE, found no difference in outcome between aneuploidy tested oocytes (by polar body analysis) and controls. Interestingly, at a meeting marking the 20th anniversary of the ESHRE Preimplantation Genetic Diagnosis Consortium held in late 2017, the only commonly agreed claims for aneuploidy testing were in a reduced rate of miscarriage and a reduced time to pregnancy.

The challenges facing ESHRE today according to its most recent chairmen are reflected in the diversity of ART and its social, political and personal implications, for which ESHRE recognizes a public responsibility. In its earliest years, Edwards and Cohen proposed ESHRE to the Council of Europe as the legitimate representative of reproductive medicine in Europe. Today, such representation continues with renewed effort, to make sure that the directives and positions of a political Europe recognize ESHRE's members and the professional fertility sector in which they work. It is also clear that common standards of practice can be set and improved by guidelines and training, and these too remain an important priority for ESHRE. Some preconference courses at ESHRE annual meetings regularly attract more than 500 participants. Membership of the Society now stands at more than 7000 and, as fertility treatment continues to gather public acceptance, those numbers are expected to grow, along with the 7 million babies whose conception has been achieved by IVF since the birth of Louise Brown.

**Acknowledgments:** This article is written on behalf of the past chairmen of ESHRE and with the approval of the immediate past, present, and chairmen-elect.

## FROM AMERICAN FERTILITY SOCIETY TO AMERICAN SOCIETY FOR REPRODUCTIVE MEDICINE: THE SOCIETY AND ADVANCES IN REPRODUCTIVE MEDICINE

Robert W. Rebar, M.D. and  
Andrew R. LaBarbera, Ph.D.

We have been asked to end this issue on the occasion of the 40th anniversary of the first birth resulting from in vitro

fertilization and embryo transfer by recounting the development and transformation of the American Society for Reproductive Medicine (ASRM) over this same time period. To do so requires just a brief discussion of the earlier history of the Society.

### Beginnings of the society

The Society was first organized as the American Society for the Study of Human Sterility (with the word “Human” dropped in 1946) under the direction of Dr. Walter W. Williams as the President in Chicago in June 1944 (639). The Society was incorporated in the state of California by one of the founding members, Dr. Pendleton Tompkins. At that time societies in general were comprised of members with narrowly defined interests but not necessarily with special skills in any specific area. Societies were more like gentlemen’s clubs (as almost all physicians in the U.S. were men) where individuals with like interests might meet and share information to improve their knowledge and skills. The Society was organized for the express purpose of improving “... methods of diagnosis and treatment, and the dissemination of reliable information concerning fertility and sterility” (639). The original body of active members was defined as, “25 clinicians and scientists who have made meritorious scientific contributions to the subject of fertility” (639). This was the first professional society devoted to reproductive medicine and remains the largest such society today.

Volume 1, Number 1 of *Fertility and Sterility* was issued in January 1950 as the, “Official Journal of The American Society for the Study of Sterility,” with Pendleton Tompkins as editor. Subsequent editors included M. Edward Davis, Luigi Mastroianni, Roger Kempers, Alan DeCherney, and the current co-editors Craig Niederberger and Antonio Pellicer. The very first article in that issue proved the value of the new journal being, “Dating the Endometrial Biopsy,” by Noyes RW, Hertig AT, and Rock J (640). This journal continues to the present time as one of the most important journals in the medical sciences.

From the outset, the Society encouraged international membership and jointly sponsored the First World Congress on Fertility and Sterility, New York City, May 1953. Led by the Americans, 1,800 experts from 48 countries attended the five-day congress (639).

### Continued growth and development of the society

Through the 1950s, 1960s, and 1970s, the Society continued to grow and to focus on both fertility and sterility. Especially prominent early members included individuals such as John MacLeod, Irving Stein, W.T. Pommerenke, Alan Guttmacher, John Rock, Gregory Pincus, M.-C. Chang, Celso-Ramon Garcia, Luigi Mastroianni, S. Leon Israel, Robert B. Wilson, David Danforth, Herbert Thomas, Melvin Taymor, Richard Blandau, Kamran Moghissi, Veasy Buttram, Vernon Stevens, Edward Wallach, Russell Malinak, S. Jan Behrman, Georgeanna Seegar Jones, Bruce Stewart, Pierre Soupart, Edward Tyler, Roger Kempers, Robert Visscher, William Andrews, Sheldon

Segal, John Sciarra, and Alvin Paulsen. It was in 1965 that the Society changed its name officially to the American Fertility Society (AFS).

Of particular note in the history of in vitro fertilization (IVF) is the fact that Dr. Rock and his Harvard colleague Dr. Miriam Menkin are credited with the first in vitro fertilization of a human egg. Starting in the mid-1930s, Dr. Rock scheduled his surgeries to be performed at about the time of ovulation. Ovaries removed were examined and mature human oocytes painstakingly identified and isolated by Dr. Menkin. These harvested eggs were then mixed with human sperm and incubated in the laboratory. After nearly six years of trying, Dr. Menkin found an egg divided into two cells, but it was lost in the preservation process. However, just a few days later she found another fertilized egg and successfully preserved it (639, 641, 642).

Expansion of the Society in the 1970s and 1980s was probably facilitated by the decision of the American Board of Obstetrics and Gynecology to develop subspecialties in obstetrics and gynecology. The Society continued to diversify, developing a number of affiliated societies and professional and special interest groups. Quickly the AFS became a society of societies, recognizing that the Society was more influential as a coordinated group supporting all of reproduction in males as well as in females. In 1977, the Society opened its Public Affairs Office in the headquarters of the American College of Obstetricians and Gynecologists in Washington, D.C., in its continuing efforts to inform and influence public policy. The Society moved into its own new office headquarters building in Birmingham, AL, in 1984.

Early advances in reproductive medicine highlighted in both this journal and at annual meetings focused on the hormonal suppression of ovulation for contraception; ovulation induction in anovulatory women; microsurgical approaches to the treatment of endometriosis, fibroids, and pelvic adhesions; and the use of cryopreserved sperm from donors to assist couples in achieving a pregnancy. Fertility treatment did not advance appreciably until the advent of in vitro fertilization in the late 1970s.

### Changes facilitated by the advent of in vitro fertilization

In 1985, fully seven years after the birth of Louise Brown, the IVF Special Interest Group formed and then reorganized itself into the Society for Assisted Reproductive Technology (SART) in 1987. This Society, together with the AFS, pioneered development of the first U.S. medical registry, which reported clinic-specific IVF outcome data. Later SART and the AFS collaborated with the Centers for Disease Control and Prevention to publish the registry results in compliance with the Fertility Clinics Success Rate and Certification Act (Wyden Act) of 1992. Registry data have continued to be systematically analyzed and published, with the data compiled by SART arguably the most detailed and complete summaries of human assisted reproduction outcomes in the world. Contemporaneously the AFS constituted its Ethics Committee to consider the philosophical and ethical issues arising from



the ability to collect, store, and fertilize gametes in vitro. This Committee plays an important role in formulating ethical guidelines for IVF clinics and for the management of reproductive issues while the Practice Committee promotes evidence-based standards for clinical and laboratory practice.

### A name change on the 50th anniversary

In 1994, at its 50th anniversary meeting, at the behest of President Leon Speroff, the Society officially changed its name to the American Society for Reproductive Medicine (ASRM) because of the recognition that the Society encompassed all of reproductive medicine, and not just fertility. Under the guidance of Dr. Herbert Thomas and then Dr. Benjamin Younger, Executive Directors, and with the leadership of successive presidents, the Society continued to expand in scope and influence. American Society for Reproductive Medicine initiated partnerships with other national and multinational medical societies in reproductive medicine, including the International Federation of Fertility Societies (IFFS), the nascent European Society of Human Reproduction and Embryology (ESHRE), the Asociación Latinoamericana de Medicina Reproductiva (ALMER), the Middle East Fertility Society (MEFS), and the Asociación Mexicana de la Reproducción (AMMR). Several prominent ASRM members have served as President of IFFS and helped solidify that society's financial basis, including Dr. William Andrews, Dr. Roger Kemper, and Dr. Joe Leigh Simpson.

### Expansion in the 21st century

Early in the 21st century, the administrative stewardship of the Society changed, with Dr. Robert W. Rebar assuming the position of Executive Director, Nancy Frankel, the position of Chief of Operations, and Dr. Andrew LaBarbera, the position of Scientific Director. Sean Tipton continued as Director of the J. Benjamin Younger Office of Public Affairs in Washington. Their charge from the Board of Directors was clear: modernize the administrative and educational functions of the Society to better fulfill the needs of the members and enhance the global stature of the Society. ASRM reformulated its mission statement, proclaiming itself a "multidisciplinary organization dedicated to the advancement of the science and practice of reproductive medicine." The Society emphasized that its primary purpose was education and in a statement of purpose noted that its intent was to advance reproductive medicine through education, research, and advocacy. The Board outlined five specific elements in its strategic plan:

**Visibility:** Expand ASRM's recognition beyond its preeminence in infertility and assisted reproductive technology.

**Education:** Broaden the quality and value of educational values.

**Research:** Encourage and facilitate the funding and visibility of research.

**Membership:** Preserve and enhance the vitality of membership.

**Finance and Administration:** Sustain and improve financial integrity and organizational structure.

To increase visibility, the Society undertook a series of initiatives during the first decade of the 21st century. In this brief overview, only a superficial listing of these endeavors is possible. The Society assumed editorial responsibility for the *Journal of Assisted Reproduction and Genetics*, selected Dr. David Albertini as its first editor, and rebranded the Journal as one devoted to basic and applied science in the field of gamete, embryo and stem cell biology. This Journal's impact has increased significantly under ASRM's guardianship and Dr. Albertini's vision, providing scientists a unique forum to share advances in stem cell research. The Society formalized exchanges of scientific program speakers with national and international professional organizations: the Society for the Study of Reproduction (SSR), the American Association of Gynecologic Laparoscopists (AAGL), the Society for Reproductive Investigation (SRI), MEFS, the Chinese Society for Reproductive Medicine (CSR), the Indian Society for Assisted Reproduction (ISAR), ALMER, the Asia Pacific Initiative on Reproduction (ASPIRE), the International Menopause Society (IMS), and AMMR. The Society broadened its annual meeting program to include emphases on contraception, menopause and reproductive surgery while at the same time reducing the burden of formal meeting activities to encourage more informal interaction. The Society organized a series of workshops with the Association of American Law Schools to facilitate interdisciplinary discussion of the legal issues surrounding ART. The Society redesigned and modernized its website, created a second website targeted to the general public, and developed an active presence on social media. Jointly with ESHRE, the Society co-sponsored three consensus workshops on polycystic ovary syndrome and published the findings simultaneously in the journals of the respective societies. Moreover, ESHRE and ASRM initiated annual, now biennial, joint "Best of..." conferences alternating between Europe and North America. The Society joined and actively participated in the meetings of the Council of Medical Specialty Societies (CMSS), an organization of 42 medical specialty societies representing nearly 800,000 U.S. physicians, whose mission is to promote continuous professional development and guide federal and state healthcare policy. The Society played a more active role in collaboration with U.S. and international government agencies, putting on conferences together with the Centers for Disease Control and Prevention, Food and Drug Administration, the National Institutes of Health (NIH), the National Academy of Medicine, as well as with the World Health Organization (WHO) and the Canadian government. Interactions with the WHO ultimately led to the recognition of ASRM as an official Non-Governmental Agency (NGA) of the WHO in early 2014. To solidify its impact in Washington, D.C., the Society expanded the Office of Public Affairs and advocated more actively with the American Medical Association and the American Congress of Obstetricians and Gynecologists (ACOG) on national issues of importance in reproductive medicine.

The 21st century saw an explosion in new educational initiatives. A fundamental shift in emphasis of the Society's educational program was the transition to interdisciplinary education in recognition that reproductive care depends on the collaborative partnership of all disciplines in the healthcare team: physicians, nurses, laboratory scientists, mental health professionals, lawyers, and practice managers. Moreover,



ASRM has expanded educational activities for medical practitioners at different levels of training. For example, the Resident Reporter Program and medical student subsidies to attend the annual congress encourage education about reproduction for budding reproductive health professionals. The Society invested significant resources in the development of a robust on-demand distance-learning program for continuous professional development. The program includes online modules and courses branded as “ASRM eLearn” and its successor “ASRM airLearning.” Other popular learning modalities are periodic webinars in reproductive endocrinology, nursing, reproductive ethics, and mental health. These activities incorporate ample opportunity for interactive discussion and user participation. The Society also produced a multitude of new patient education materials, including fact sheets, booklets, and films that are readily available at no cost to the general public at [www.ReproductiveFacts.org](http://www.ReproductiveFacts.org). Many of these materials have been translated into Chinese, Spanish, and Portuguese. Educational materials for professionals and patients continue to rely on published scientific evidence. The Practice Committee further refined its procedures for conducting rigorous systematic reviews of published evidence to formulate guidelines that comply with the standards of the National Guideline Clearinghouse of the U.S. Agency for Healthcare Research and Quality (AHRQ). These enhancements in guideline development have strengthened the ASRM recommendations for clinical practice and led to ASRM’s participation in the Choosing Wisely campaign, an initiative of the American Board of Internal Medicine Foundation in partnership with Consumer Reports. More recently, ASRM has joined other surgical societies in expanding its focus on skills-building training with the invention and deployment of the ASRM Embryo Transfer Simulator.

The Society embarked on major new research initiatives, devoting significant financial resources to funding research projects of high priority to the advancement of reproductive medicine and biology. The Society now awards grants to research proposals deemed worthy by the ASRM Research Committee. Presentations of the results of these projects are given special recognition at the Scientific Congress. In addition, the Congress includes special prize paper sessions. The Society for many years has recognized outstanding accomplishment in scientific research through the Distinguished Researcher and the Ira and Ester Rosenwaks New Investigator awards. To foster the entry of new clinician-researchers into participation in clinical research projects, ASRM has partnered with the Eunice Kennedy Shriver National Institute of Child Health and Human Development (NICHD) and Duke University to administer the Clinical Reproductive Endocrinology Scientist Training (CREST) Program. Graduates of this program for physicians in the practice of reproductive medicine contribute to scientific knowledge in the field. Finally, the Society also has supported and helped develop NICHD workshops, Gordon Conferences, Testis Workshops, and the 2016 “Summit on Environment, Reproductive, Nutritional and Genetic Factors Affecting Reproduction” organized jointly with the SSR.

To promote the involvement of and interaction among members, the Society installed and recently upgraded interactive association management software. Many of the special interest groups, professional groups, and affiliated societies

have taken advantage of new opportunities for engagement with online bulletin boards, newsletters, and eblasts. Online voting increased membership involvement in leadership decisions. The Society also became one of the societies awarding the Gold Humanism Award to a member making special contributions in the field.

Expansion of the administrative staff and remodeling of the physical facilities were necessary to implement the initiatives described above. The move to a virtually “paperless” scientific congress and pre-congress courses greatly increased efficiency and timeliness and reduced expenses. In recognition of the industry-wide decline in commercial support for educational activities not targeted to product promotion, the ASRM initiated a long-term Fund Development Program. During the first five years of this program, the Society established over \$4 million in endowments to help ensure the future success of the Society’s mission. Interestingly, as reproductive medicine in the U.S. evolved into the domain of physicians specifically trained in reproductive endocrinology or reproductive urology, general obstetrician-gynecologists, and urologists drifted away from the Society and membership declined. However, the recognition by WHO that “Reproductive rights rest on the recognition of the basic right of all couples and individuals to decide freely and responsibly the number, spacing and timing of their children and to have the information and means to do so, and the right of all to make decisions concerning reproduction free of discrimination, coercion and violence” (643) has served to make the provision of reproductive services of importance worldwide. This has provided new global opportunities for ASRM.

## Final thoughts

The future of reproductive medicine is bright. Recent advances in genetics and stem cell biology suggest that research advances in this field will only accelerate in the years ahead and will almost certainly result in novel approaches to treating infertility in males and females. Assisted reproductive technologies have advanced our knowledge of gamete biology and of early embryonic development. There is still much to learn, and it seems safe to predict that the annual scientific congresses of the ASRM will continue to be exciting meetings presenting new scientific advances in the field.

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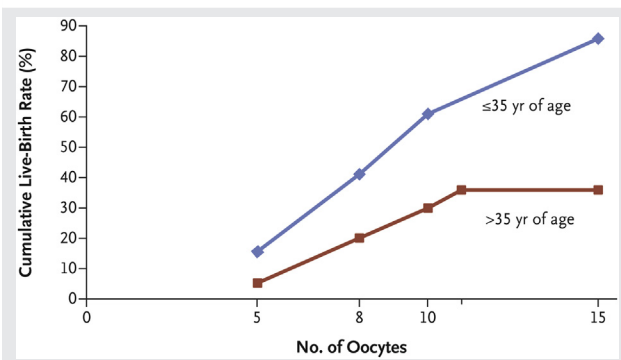
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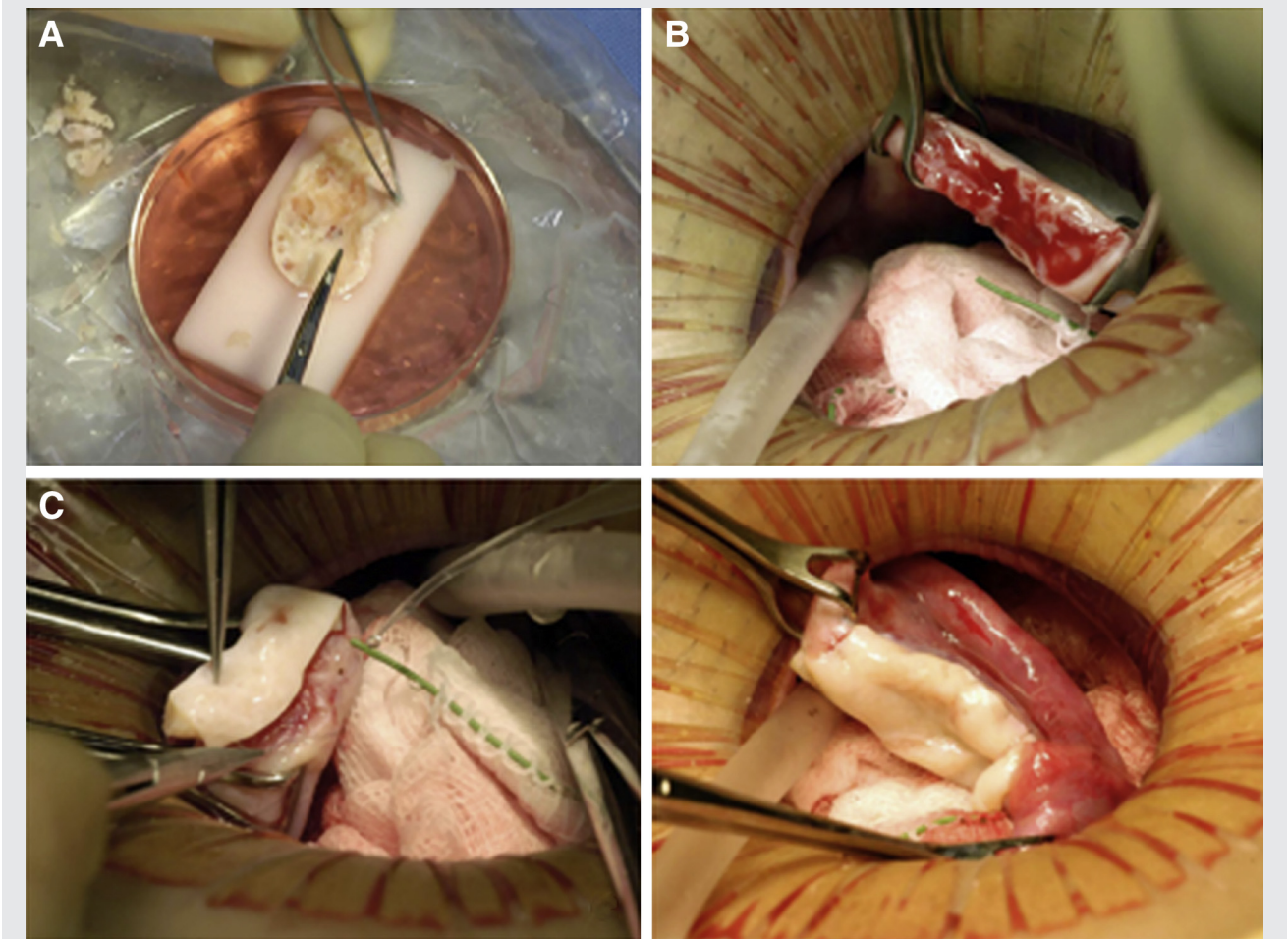
## SUPPLEMENTAL FIGURE 1



Cumulative live birth rates (CLBRs) with 5–15 oocytes according to age (from Donnez J, Dolmans M, N Engl J Med 2017;377:1657–65 and adapted from Cobo et al., Fertil Steril 2016;105:755–64).

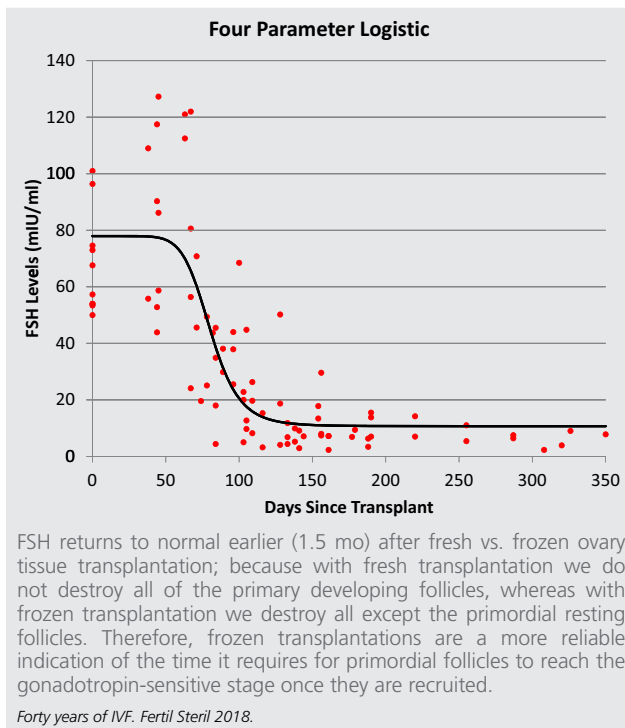
Forty years of IVF. Fertil Steril 2018.

SUPPLEMENTAL FIGURE 2

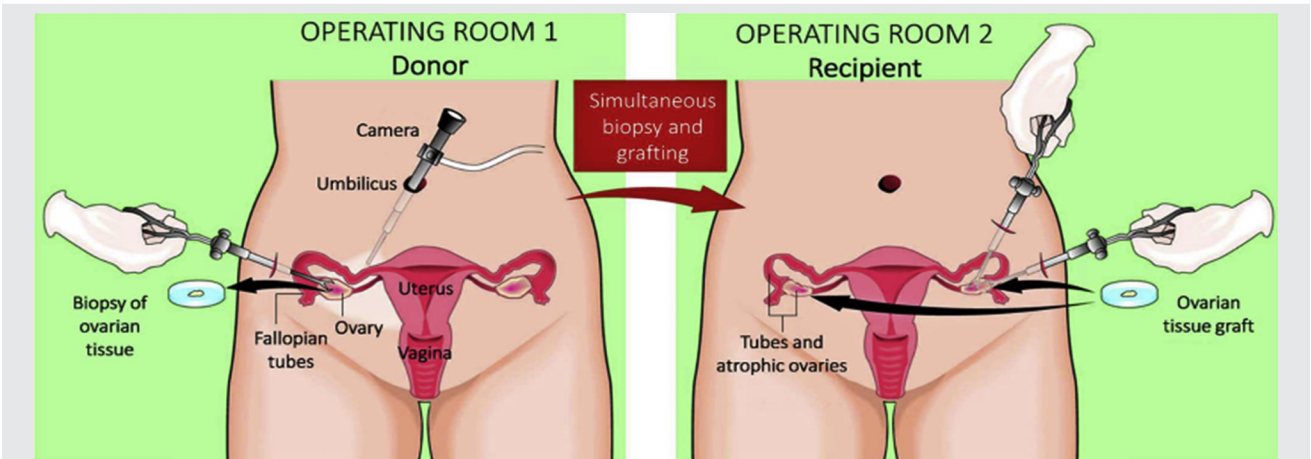


According to Silber: technique of fresh ovarian tissue transplantation (between monozygotic twins). The ovarian graft is attached with the use of interrupted 9-0 nylon sutures under optical magnification.  
*Forty years of IVF. Fertil Steril 2018.*

## SUPPLEMENTAL FIGURE 3



SUPPLEMENTAL FIGURE 4



Two genetically different sisters were operated on simultaneously in contiguous operating rooms. Ovarian tissue was laparoscopically removed from the donor's ovary and immediately sutured to the recipient's ovarian medulla (from Donnez et al., *Fertility Sterility* 2011;96:1407–11).  
*Forty years of IVF. Fertil Steril* 2018.



**SUPPLEMENTAL TABLE 1****Indications for fertility preservation.**

- A) Malignant diseases most frequently requiring gonadotoxic chemotherapy and/or radiotherapy or bone marrow transplantation:
- Hematologic diseases (leukemia, Hodgkin lymphoma, non-Hodgkin lymphoma)
  - Breast cancer
  - Sarcoma
  - Some pelvic cancers
- B) Benign conditions for which fertility preservation is indicated:
1. Nononcologic systemic diseases requiring chemotherapy/radiotherapy and/or bone marrow transplantation
  2. Nonmalignant ovarian diseases
    - Bilateral ovarian tumors
    - Severe and recurrent ovarian endometriosis
    - Risk of ovarian torsion
  3. Risk of premature ovarian insufficiency
    - Family history
    - Turner syndrome
- C) Social reasons:
- Age
  - Childbearing postponed to later in life

*Forty years of IVF. Fertil Steril 2018.*