E Scott Sills *Editor*

Screening the Single Euploid Embryo

Molecular Genetics in Reproductive Medicine

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In Memoriam

Professor Robert P.S. Jansen (1946–2014)

It is with great sadness that we acknowledge the passing of Professor Robert Jansen.

Even while he was not well, Robert continued to guide and advise on research directions, with his contributions to this book being among his final writings. The impact of his scholarly work continues to have a global reach and will be felt for many years.

Robert played a pivotal role in reproductive medicine and the development and progress of assisted conception in Australia over the last three decades. The founder and leader of Sydney IVF (now Genea), he led advances in clinical IVF application as well as the culture and incubation of human embryos. His contributions include the introduction of transvaginal ultrasound techniques in IVF, the development of MINC incubators and culture media which now enjoy global usage, PGD undertaken at the blastocyst stage, and many other groundbreaking developments. In an effort to improve pregnancy outcomes nearly 20 years ago, Robert used his own clinic as a research base to demonstrate that single embryo

transfers were not only a feasibility but also a practical reality, and patient outcomes need not suffer in the process.

A true visionary in his outlook, Robert published in areas as diverse as reproductive surgery, endometriosis, patient advocacy, ethics in reproductive medicine, as well as more applied areas of mitochondria and the aging oocyte, the environment of the early embryo in vivo, and blastocyst biopsy as an appropriate approach to efficient PGD. Holding a personal Chair at the University of Sydney, he actively taught both science and medical students, predominantly at postgraduate level up to and beyond his retirement 3 years ago. Professor Jansen was on the editorial board for two journals and served as a referee for many others.

Along with other founders in the IVF field in Australia, Robert was instrumental in achieving recognition for and developing the Reproductive Endocrinology & Infertility (REI) subspecialty within RANZCOG and was chair of the CREI subspecialty committee for many years.

He coordinated and led one of the largest and most successful educational meetings ever held in Australia, the World Congress of IVF and Human Reproductive Genetics in 1999. Robert was made a life member of FSA in 2012 and was a board member of PGDIS for several years during its early developing stages.

Medical specialist, physician, scientist, researcher, ethicist, advocate, philosopher, author, entrepreneur, wine and food connoisseur, poker player, sailor, surfer, teacher, Porsche enthusiast, art collector, husband, father, and grandfather; the untimely departure of Professor Robert Jansen is a great loss to family, friends, and colleagues throughout the scientific and medical communities.

Sydney, NSW 2000, Australia Don Leigh, Ph.D.

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About the Editor

E Scott Sills received his undergraduate degree from Vanderbilt and holds a PhD (molecular biology) from London's University of Westminster. Dr. Sills was awarded the MD degree from University of Tennessee in 1992. After a gynecology residency at NYU Downtown Hospital, he completed his subspecialty training in reproductive endocrinology at Cornell University. Having authored more than 100 peer-reviewed publications, Dr. Sills served as editor-in-chief for the *Journal of Experimental and Clinical Assisted Reproduction* from 2004 to 2014. He is board-certified by the American Board of Obstetrics and Gynecology and is a Fellow of the American College of Obstetricians and Gynecologists, American College of Surgeons, Royal College of Physicians (Ireland), and Society of Biology (UK). At present, Dr. Sills is Medical Director at the Center for Advanced Genetics, an in vitro fertilization program based in Carlsbad, CA.

He is registered and/or licensed for medicine in California, New York, and the United Kingdom.

Chapter 1
The Development of PGD

Joy D.A. Delhanty

Introduction

By 1987 early prenatal diagnosis of both chromosomal and single gene defects was possible via chorionic villus sampling, so why was there a perceived need to develop preimplantation genetic diagnosis (PGD)? There were two groups of patients that provided driving forces. Firstly couples known to be at high genetic risk had expressed the fervent wish to be able to start a pregnancy knowing that it would not be affected; many of these couples had experienced the trauma of repeated second trimester terminations of much wanted pregnancies. A second group, for whom PGD would obviously be of great benefit, included couples where the women had been shown from pedigree analysis to be carriers of an X-linked condition for which at the time there was no specific diagnostic test. For this group, the only option was prenatal testing of an established pregnancy to determine the sex. This then led to the termination of all male pregnancies, of which only 50 % were likely to be affected [1]. Various events around this time had made the development of PGD a possible option. In 1983 Trounson and Mohr [2] had shown that it was possible for a normal pregnancy to occur even after the destruction of blastomeres following embryo freezing. This finding indicated that it should be feasible to remove one or two cells from a cleavage stage embryo for diagnosis without compromising its further development. In the UK, Dame Mary Warnock chaired a government committee composed of people from a variety of backgrounds that considered the status of the human embryo before preimplantation with regard to the ethics of research on embryos at this stage of their development. The subsequent report was published in 1984 by the DHSS; it proposed a time limit for research of 14 days after

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fertilisation, well beyond the time when cells would be removed to allow genetic diagnosis. This paved the way for subsequent government legislation that was in line with the committee's recommendations.

Early Steps in PGD

With the aim of helping couples at risk of an X-linked disorder, the first approach to PGD was in order to sex the embryo. Handyside and colleagues at the Hammersmith Hospital in London reported in 1989 that they had been able to biopsy single cells from 30 embryos and that the expected proportion had developed to blastocysts after 6 days in culture [3]. Furthermore in all the normally fertilised embryos they were able to determine the sex by DNA amplification of a Y-chromosome-specific repetitive sequence. In 15 cases, the sex was confirmed by means of in situ hybridisation or Y chromosome fluorescence in metaphases. Shortly this was followed by the report from the same group of pregnancies from embryos sexed by Y-specific DNA amplification [4]. However, this approach proved to be error prone since crucially it relied on a negative result to identify the females. The development of the rapid and reliable technique of fluorescence in situ hybridisation (FISH) at the end of the 1980s proved a saviour and was quickly applied to biopsied cells from human embryos with excellent results [5]. The application of FISH for embryo sexing at UCL in London gave reliable diagnostic results but also gave the first indication of the frequency of aneuploidy and chromosomal mosaicism in these embryos created by in vitro fertilisation (IVF) from fertile patients [6]. Prior to this, the IVF specialists were looking forward to treating PGD patients who would be fertile, anticipating that IVF would have a much improved success rate compared with that for infertile couples. Simultaneously, FISH was being applied to biopsied cells from cleavage stage embryos by Munne's group in the USA and in 1993 they also reported the diagnosis of major aneuploidies in mosaic and full form [7]. Evidently, embryos created by IVF from couples of proven fertility were also prone to mosaic aneuploidy of an extent that was going to affect viability and implantation rates as well as the accuracy of PGD. So it was that an additional aim was added: as well as using PGD to detect heritable genetic disorders, it could be applied to help improve the success rate of IVF for infertile couples by using FISH to detect aneuploidy—this was the birth of PGS—preimplantation genetic screening.

Meanwhile, in 1992 the Hammersmith group reported the first successful PGD for a single gene disorder: the birth of a normal girl, free of cystic fibrosis, after PGD [8]. Within a few years, FISH was being applied in London to biopsied blastomeres to help couples at risk of passing on an unbalanced form of a reciprocal or Robertsonian translocation [9], and in the USA preconception diagnosis was achieved for maternal carriers by testing the first polar body alone while in Chicago it was tested in combination with karyotyping of the second polar body, also only for maternal carriers [10, 11]. While few would dispute on ethical grounds the application of PGD to avoid single gene defects that affect children, its use to avoid

passing on genes that predispose to late-onset disorders such as adult cancers provoked more controversy.

Nevertheless, the UK Human Fertilisation and Embryology Authority licensed the procedure in the case of the APC gene that causes familial adenomatous polyposis (and inevitable colorectal cancer) when mutated and the first PGD diagnosis for inherited cancer, and of this condition, was carried out in London and reported in 1998 [12].

The Development of Comprehensive Chromosomal Analysis

Initially, from 1999 onwards it was reported that the outcome for infertile couples improved significantly after PGS was applied to their embryos, compared with comparable control groups [13]. However, since FISH is perceived as an easy and reliable technique that any laboratory scientist may apply and achieve a successful outcome, it became widely used by IVF centres with no experience of genetic testing. Not surprisingly, the results for the patients were variable and doubts began to be expressed as to the benefits of PGS, mostly applied to older women with fewer embryos for testing. In 2007 a paper was published that reported on the outcome of a randomised clinical trial of PGS that showed a negative effect of screening via PGS [14]; this paper has been widely quoted but also heavily criticised on technical grounds by scientists with extensive experience of the application of FISH to human blastomeres. There are two contributory problems: one that in order to test as many chromosomes as possible, several rounds of hybridisation with FISH probes may be carried out; thus reducing the efficiency and secondly the widespread mosaicism that affects the early human embryo will clearly lead to apparent 'misdiagnoses' when testing only a single cell for aneuploidy. In the meantime, research was progressing on methods for comprehensive chromosome testing, based upon analysis of DNA extracted from a single cell. The aim was to be able to apply the technique of comparative genomic hybridisation (CGH) as used in tumour cytogenetics, where karyotyping was not possible. For this to happen, the DNA from each cell had first to be amplified in a manner compatible with analysis via CGH. Two groups from opposite sides of the world (London UK and Melbourne Australia) were successful in achieving single cell CGH analysis of blastomeres and simultaneously both groups published their work in the year 2000 [15, 16]. The results obtained confirmed the early FISH data with respect to the incidence of full and mosaic aneuploidy in apparently normally developing human embryos. These results were achieved by classical metaphase analysis after the combined hybridisation of both test and control DNAs; even with the help of computer software, that analysis required the ability to karyotype and took 72 h for the hybridisation step alone. Although both innovator groups did apply the technique clinically, these factors clearly limited full clinical application. The final step needed was the refinement of array CGH so that it could be applied to the analysis of single cells; early results from this development were described in 2009 [17]. By 2013 it was evident that

centres were seeing an improved outcome with regard to both implantation and pregnancy rates compared with those achieved previously by FISH analysis [18]. It may be concluded that the development and application of a reliable aCGH method has made a major contribution to the stated goal of 'Transferring the single euploid embryo'.

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Chapter 2
Elements of Informed Consent for Preimplantation Genetic Diagnosis

Michelle Lynne LaBonte

Introduction

The concept of informed consent exists to protect patients and research subjects from undue harm. To achieve valid informed consent, individuals should be informed of the relevant risks, comprehend the information provided, and voluntarily agree to take part in either a research study or a medical treatment [1–3]. Since PGD typically involves the biopsy of one or more cells from an in vitro fertilized early embryo followed by genetic analysis of the biopsied cells, achieving valid informed consent is especially challenging. First, the informed consent for PGD must include information about the risks associated with the three key components of the process: in vitro fertilization (IVF) using intracytoplasmic sperm injection (ICSI) to generate embryos, the embryo biopsy, and the genetic testing [4]. Furthermore, prospective parents should be aware not only of risks to themselves, but they must also be aware of risks to the resulting child and understand that they are consenting on behalf of the future child. As such, potential risks to the resulting child must be carefully outlined in the information provided to prospective parents [4, 5].

Given the complicated and multifaceted nature of PGD, it is essential that prospective parents be provided the relevant information in a manner conducive to comprehension of the associated risks. To this end, prospective parents should be provided with information in different formats and through different mechanisms and be given ample opportunities to have their questions answered [3]. There will ideally be different stages of informed consent, beginning with accurate and up-to-date educational material about risks on fertility center websites [5, 6]. Conversations about risks associated with PGD should also take place with fertility center staff and genetic counselors. It may also be wise to go over more difficult to comprehend

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aspects of the material multiple times throughout the consent process [7]. Some have suggested the use of audiovisual aids in addition to individual counseling and written documentation as mechanisms by which to inform patients prior to obtaining consent [7, 8]. Furthermore, full consent to PGD should be obtained before the in vitro fertilization (IVF) process begins, so that there are no time and financial pressures when prospective parents are making decisions.

In addition to being informed, consent must also be voluntary [3]. Fertility centers should take special care to ensure that prospective parents are not being inadvertently pressured into choosing the procedure. As such, any financial conflicts of interest or other such conflicts that might lead to undue pressure from the fertility center should be shared with prospective parents [9, 10]. It is also important that prospective parents are provided with unbiased information regarding risks so that they can carefully consider whether to initiate a PGD cycle. While there are many important elements of valid informed consent for PGD, this review will detail the risks associated specifically with the embryo biopsy and genetic testing components of PGD and provide suggestions regarding content that should be discussed with prospective parents. However, it is essential that prospective PGD users also be informed of the risks associated with IVF and ICSI, as these more widespread procedures are done before the embryo biopsy and testing components of PGD.

Categories of Consent Specific to PGD

Risks Associated with Embryo Biopsy

While informed consent procedures for PGD often cover risks to the mother, the risks to the fetus and future child are less often reported [4–6]. Some fertility center websites make reference only to studies that have found no increased risks associated with PGD. However, there are published, peer-reviewed studies that have detected subtle neurological and other differences in offspring born following embryo biopsy. While these studies are by no means definitive, they certainly warrant disclosure in the proper context to prospective parents as part of the informed consent process. This section examines the existing scientific studies of the risks to resulting fetuses and children from embryo biopsy procedures and also outlines studies indicating that preimplantation genetic screening (PGS) may decrease the chance of live birth.

Types of PGD Safety Studies

A number of mouse and human studies have addressed the issue of embryo biopsy safety in PGD/PGS, resulting in a complicated set of findings. The first complicating factor in interpreting the data is that studies have been performed in both mice and humans. Mouse studies can be quite advantageous in that they allow for large

sample sizes, more invasive and thorough analysis of offspring, and carefully controlled study design, yet embryo development in the mouse is not the same as in humans. Therefore, any interpretation of mouse studies must be made with this in mind. Even two different mouse strains can give strikingly different results [11]. Therefore, it is hard to know if findings in mice will translate to humans. However, that doesn't mean that only findings from human studies should be considered when evaluating the safety of PGD.

The second complicating factor in assessing safety studies is that study design varies markedly in published reports. A number of studies lack matched controls and very few studies report blinded analysis of outcomes. Furthermore, an important limitation of the published retrospective studies is the possibility of selection bias, as could happen if parents of children with health problems are more or less likely to enroll in a trial. Selection bias can also occur if fetuses that have been biopsied as embryos are more likely to be tested prenatally and aborted as a result of an abnormal finding.

A third complication when interpreting safety studies is that the type of biopsy used also varies when comparing studies. The three main types of preconception and embryo biopsies include polar body biopsy, day 3 cleavage-stage embryo biopsy of one or two blastomeres, and day 5 blastocyst biopsy of multiple trophectoderm cells [12, 13]. Therefore, patients should be informed not only about the results of the published safety studies but also about any important differences in embryo biopsy methodologies used by individual centers compared to those described in the published literature. A fourth complication with the existing safety studies is the limitation of time. No long-term human safety study has followed PGD offspring through adulthood, nor has any study examined the effects of PGD on the offspring of biopsied individuals. Given the potential challenges associated with assessing the safety of the embryo biopsy procedure, it is important that prospective parents are provided a balanced view of all published safety studies and made aware that no long-term safety studies have yet been completed in humans.

Results of PGD Safety Studies in Mice

While many studies have detected no increase in congenital or other abnormalities in PGD offspring [14], there is a trend in the detection of neurological abnormalities in embryo-biopsied offspring across different studies and in both mice and humans. In this section, the mixed results reported in published studies with mice are summarized.

There have been a number of studies examining the effect of embryo biopsy on fetal development, but the interpretation of these results is complicated by the different mouse strains and different developmental stages at which the biopsies occurred. For instance, a study in which one blastomere was removed at the four-cell mouse embryo stage found significant decreases in preimplantation development to the blastocyst stage and in live fetus development [11]. However, these differences were unique to the C57/BL6 strain in that no statistically significant

developmental abnormalities were seen in the B6D2F1 strain. In a different study in which one blastomere was biopsied at the eight-cell stage, hatching was premature and sometimes abnormal in biopsied mouse embryos compared to controls, yet no differences in global gene expression were found 28 h post-biopsy [15]. In a more recent study, mouse fetuses that had one cell removed at the four-cell embryo stage had significantly lower weight, lower levels of some steroid clearance enzymes in the placenta and fetal liver, and differences in steroid hormone levels in the placenta, fetal blood, and fetal liver when compared to controls [16]. Taken together, these data suggest that some but not all aspects of embryo and fetal development may be altered as a result of embryo biopsy in the mouse.

Several studies have also looked at later stages of mouse development following embryo biopsy. In one study, analysis of adult mice which underwent biopsy of a single blastomere at the eight-cell embryo stage revealed no abnormalities in blood cell counts, blood chemistry, and organ histology compared to controls [17, 18]. However, in another study, Yu and colleagues demonstrated that murine embryos which underwent biopsy at the four-cell stage performed less well than non-biopsied mice on a memory test [19]. This same study demonstrated that biopsied mice had altered expression of proteins implicated in neurodegenerative disease, suggesting the potential for long-term neurological abnormalities in biopsied mice. Furthermore, biopsied mice had altered levels of stress hormones both before and after cold stress challenge, and biopsied mice had more lipid storage in the adrenal cortex compared to controls [20]. Thus, while a number of measured outcomes have been normal in biopsied mice, the embryo biopsy procedure is associated with a variety of health problems in mice. The informed consent process for PGD should include reference to the findings from mouse studies, while at the same time making it clear that mouse outcomes may or may not translate to humans.

Results of PGD Safety Studies in Humans

Results of PGD safety studies in humans have been more promising when compared to some of the mouse studies. In an observation of the first 109 children born following polar body biopsy at the Reproductive Genetics Institute, no significant abnormalities were detected in birth weight of offspring and no increase in congenital abnormalities over the published literature for naturally conceived births was reported [21]. In another observational report of outcomes following one- or two-cell biopsy of day 3 embryos at the Centre for Medical Genetics, no significant increase in congenital abnormalities was reported [22]. However, there was a significant increase in the number of perinatal deaths and stillbirths following embryo biopsy [22]. A different observational study found that PGD offspring had low birth weight as well as decreased motor and cognitive abilities [23]. Of note, all of these studies lacked a matched control group of either ICSI and/or naturally conceived children [21–23]. While observational studies can provide important clues to issues such as the safety of embryo biopsy, it is difficult to draw clear conclusions in the absence of a matched control group.

A number of controlled studies have been carried out, however, and some of those results have been reassuring. In a controlled study comparing ICSI and naturally conceived (NC) children to PGD/PGS children who underwent one- or two-cell blastomere biopsy at the eight-cell stage, there were no statistically significant differences in mental and psychomotor development of singletons at age 2 [24]. Furthermore, no statistically significant differences were seen in language or socio-emotional development when comparing PGD/PGS, ICSI, and NC 2-year-olds [25]. A follow-up analysis of twins also revealed no statistically significant differences in mental, motor, socio-emotional, and language development in PGD/PGS offspring compared to ICSI or NC children at age 2 [26]. In addition, Desmyttere and colleagues reported no statistically significant difference in major or minor malformations in PGD/PGS offspring [27, 28]. However, BMI and arm circumference were lower in PGD/PGS offspring compared to ICSI and NC children [28]. In a matched control trial with blinded analysis, PGD offspring had significantly lower gestational age at birth and a higher number of births with low birth weight. In this same study, PGD offspring scored lower on the Locomotor subscale, yet higher on the Hearing and Language subscales of the Griffiths Scale [29]. Thus, outcomes based on these controlled trials demonstrated many similarities between biopsied offspring and controls, but a number of statistically significant differences were also observed. It is also important to keep in mind that selection bias, as might occur if fetuses with abnormalities are more often detected and aborted following embryo biopsy, can be an important limitation of such trials.

Addressing the issue of selection bias, Middelburg and colleagues reported on the results of a randomized, controlled, blinded, prospective study in which PGS offspring were compared to IVF offspring [30]. Individuals in the PGS group typically had one blastomere removed at the four-cell embryo stage, although two blastomeres were taken when necessary for analysis. Consistent with other studies, no increase in minor or major abnormalities was seen in the PGS group at birth [31]. While there were no statistically significant differences in outcomes at 18 months of age, PGS children did have an increased incidence of mild fine motor dysfunction and mildly dysfunctional posture/muscle tone. Furthermore, PGS children had more severe issues at the individual level as compared to controls [30]. At age 2, PGS and IVF offspring had similar mental, psychomotor, and behavioral scores. However, the neurologic optimality scores were statistically significantly lower in the PGS group [32]. At age 4, no differences in blood pressure or anthropometrics or received medical care were observed, yet a statistically significant increase in paramedical care (speech, physical, or occupational therapy) was seen in the PGS group [33]. Also at age 4, there were no neurological, cognitive, or behavioral differences between singleton groups. In contrast, embryo biopsy in twins was associated with “a negative effect on neuromotor condition and a positive one on sequential processing” [34]. Since some neurological deficiencies only become apparent later in life, it will be important to follow embryo-biopsied children into school age years and beyond to more carefully assess any potential adverse neurological and other outcomes [30]. These potential safety risks should be carefully weighed against the potential benefits before making a decision to move forward with the procedure [35].

Chance of Live Birth

A number of studies have examined the chance of live birth following PGD/PGS. Based on the most recent ESHRE PGD Consortium data published, the delivery rate following embryo transfer was 25 % for PGD done following testing for structural chromosomal abnormalities, 30 % for sex determination for X-linked diseases, and 25 % for evaluation of embryos for monogenic diseases [14]. These PGD data are in contrast to a 22.8 % delivery rate per embryo transfer seen following PGS [14]. However, data looking at IVF alone were not part of this collection. A meta-analysis of randomized control trials demonstrated a reduction in the chance of live birth from 26 % with IVF alone to 13–23 % with IVF and PGS [36]. Taken together, these data suggest that the chance of live birth may be reduced following PGS as compared to IVF alone or PGD. However, these data may be misleading as the indication for PGS is different than for PGD, with PGS being indicated for prospective parents who have a higher risk of pregnancy loss. In a different retrospective cohort study evaluating PGD outcomes in Sweden, it was found that the chance of pregnancy is doubled with one-cell biopsy as compared to two-cell biopsy of cleavage-stage embryos [37]. Thus, prospective parents should be informed that the chance of live birth might be reduced following PGS and that two-cell biopsies may reduce the chance of live birth as compared to one-cell biopsies.

Important Components of Embryo Biopsy Informed Consent

- Studies examining the risks of embryo biopsy to the fetus and future child have been performed in mice and humans. Some have found no risk from the procedure, while some have found neurological and other abnormalities and a higher incidence of children requiring developmental support following embryo biopsy.
- Results from mouse studies do not always translate to humans, but mouse studies can allow for more controlled study design and detailed analysis of offspring. Mouse studies should not be overlooked.
- No long-term study has been done in human children past the age of 4. Risks to older children, adults, and their offspring are unknown.
- There is some evidence that embryo biopsy may reduce the live birth rate.

Risks Associated with Genetic Testing of Biopsied Cells

Given the imperfect nature of genetic testing of embryos, there is a chance of misdiagnosis even when the testing is done by an experienced center. Prospective parents should be made aware of the need for prenatal testing if they wish to confirm the embryo testing results. Furthermore, comprehensive genetic testing, in which a wide range of genetic information will be determined, may reveal unanticipated

genetic information about the tested embryos that parents or the resulting child may not wish to know. Furthermore, selection of embryos with a decreased risk of a known disease may also inadvertently select for embryos with an increased risk of an unknown disease. Finally, genetic testing to determine the suitability of an embryo for implantation has larger societal implications.

Possibility of Misdiagnosis

Misdiagnosis can occur for a variety of reasons, and it is important that potential PGD patients be informed of this possibility. Causes of misdiagnosis include human error, PCR or FISH errors, mosaicism, unprotected sex, uniparental disomy, and many others [38]. Human error in the lab, such as tube mislabeling, is one other cause of misdiagnosis that can be reduced substantially if proper quality control measures are in place [38]. While not technically a misdiagnosis, unprotected sex can lead to natural fertilization and the subsequent development of an unselected embryo even if a selected embryo is transferred. Couples should be made aware of the risks associated with unprotected sex before beginning IVF/PGD. Another factor that can lead to transfer of an unselected embryo is mosaicism. While FISH or PCR-based analysis of the biopsied cell may in fact be accurate, mosaicism can lead to the transfer of an unselected embryo if the biopsied cell is not representative of the other cells remaining in the transferred embryo [38].

PCR-based diagnosis of biopsied cells can also result in misdiagnosis for reasons other than mosaicism. Often cited reasons for PCR-based misdiagnosis are contamination and allele dropout [38]. In an embryo reanalysis study, Dreesen and colleagues found that the initial analysis of 881/940 embryos was consistent upon reanalysis [39]. Most cases of misdiagnosis were due to mosaicism, with allele dropout and contamination cited as other reasons for misdiagnosis in their study. When the researchers further analyzed the data, they found that PCR analysis of a two-cell embryo biopsy is more accurate than analysis of a one-cell biopsy. Specifically, 3.3 % of two-cell embryo biopsies were misdiagnosed and 8.4 % of one-cell embryo biopsies were misdiagnosed by PCR [39]. Other reports of misdiagnosis, typically identified prenatally or after birth, cite lower rates of PCR-based misdiagnosis [14, 38]. Misdiagnosis rates for FISH have been cited as 0.06 and 0.07 % [14, 38], and FISH-based diagnosis has historically been considered more accurate than PCR-based diagnosis. However, a recent study found the misdiagnosis rate to be higher in FISH than in PCR [14].

Since there are risks of error associated with PGD, even when it is done properly, some lawsuits have been aimed at the lack of adequate informed consent regarding full disclosure of the risks of error that can lead to PGD misdiagnosis [40]. Surprisingly, only a minority of ESHRE Consortium members had a formal quality control program in place in 2008 to check the accuracy of PCR-based diagnosis of biopsied embryos [39]. Therefore, it is important for centers to give their own misdiagnosis rates if they have accurate ones and provide published rates as well. Given

the chance of misdiagnosis, a Practice Committee report recommends informing patients that prenatal testing can be done using amniocentesis or CVS to confirm PGD results [41]. The risks associated with these prenatal testing procedures should also be provided to prospective patients before initiating IVF/PGD.

Comprehensive Genetic Testing

Genetic testing of biopsied cells initially targeted just a single or several defined genes. However, advances in technology have made comprehensive genetic testing of biopsied cells possible. Since comprehensive genetic testing will likely reveal variants of unknown significance, information about the risk of late-onset disease, as well as nonmedical characteristics, it is important that prospective parents are aware of the risks associated with learning this type of information about their future children. A variety of suggestions have been put forth regarding how much information prospective parents should be given during the informed consent for genetic testing [42]. Ideally, informed consent would only occur after full disclosure and understanding of the details of the genetic testing. However, given the complex nature of comprehensive genetic testing, it may not be feasible to provide prospective parents with all details regarding what the test results might reveal because of concerns that comprehension may be compromised if the information provided is too complicated [42, 43].

To address the concern that consent may be inadequate if there is too much information given during the consent process, some have advocated for a generic form of informed consent for genetic information [8]. In fact, six categories of information have been proposed, including "congenital lethal disorders; early- or late-onset disorders requiring intensive medical care; early- or late-onset disorders requiring limited medical care; susceptibilities for complex disorders; conditions involving only minor health problems; and abnormal findings of which the clinical implications are unknown" [44]. However, this type of grouping can be problematic because of the different ways that doctors and parents might classify specific genetic risk information [44, 45]. Even the label of "abnormal" when applied to findings of unknown significance is potentially misleading, as many apparently healthy individuals have copy number variants and other DNA changes [46]. Furthermore, a recent study demonstrated that greater than 40 % of healthy individuals have mutations in genes that are predictive of severe early-onset disease [47]. Thus, it is not possible to predict with complete accuracy the health consequences of many genetic alterations that may be found as a result of comprehensive genetic testing [48]. Given the uncertainty regarding the predictive nature of many genetic test results, it is essential that prospective parents are aware of these limitations.

To address the limitations associated with providing only generic or specific information, Bunnik and colleagues instead offer a hybrid model in which generic consent (including categories of information as has been suggested by others) is the foundation, and then a well-organized list of specifically tested diseases is included

as well [49]. This concept is in line with the suggestion by Elias and Annas that specific consent should still be obtained for certain tests such as the genetic test for Huntington's disease [8]. Furthermore, Bunnik and colleagues suggest that consumers be required to actively select for/against different types of tests because such active decision making will aid the informed part of the consent process. While not formally part of the consent, some have also suggested that prospective parents be given the option to receive more detailed information about any of the genetic categories [49, 50]. Since specific genetic risk information will likely change over time, it will be important to constantly update this component of the consent process as new risks arise.

In addition to being informed about what the test will reveal, parents should also be involved in determining what information will be shared with them after the results have been determined [42]. In discussing prenatal genetic testing, de Jong and colleagues argue that information about late-onset disease should only be given to a woman if she plans to abort such a fetus (or in the case of PGD, not transfer an affected embryo). This thinking is in line with ethical concerns many have regarding the genetic testing of minors for late-onset disease [51]. However, because some prospective parents may not follow through with plans to avoid transfer of embryos with increased risk of late-onset disease (if testing reveals that all biopsied embryos have an increased risk of at least one late-onset disease), children could still be born with such knowledge [44]. Even if the parents don't share this information with their children, just having this knowledge may hinder the child's right to an open future [42]. Thus, it is important that prospective parents are aware of the type of information that genetic testing can uncover and that parents carefully consider what the future child might want to know about himself or herself when determining the type of genetic information that should be revealed. A delicate balance will need to be struck between a child's right to an open future and the reproductive freedoms of prospective parents, and the solution may involve limiting the type of information that is shared with parents regarding embryos that will ultimately be implanted.

Inadvertent Selection for Increased Disease Risk

It is important that prospective parents understand that by selecting against an embryo with a particular disease risk or other characteristic, they may at the same time be inadvertently selecting for an embryo with an increased risk of a different disease. This inadvertent selection could happen in the case of linked genes or as a result of heterozygote advantage [52]. For example, the disease sickle cell anemia occurs when an individual has two mutant copies of the β-globin gene [53]. However, heterozygous individuals with only one abnormal copy of the β-globin gene are less susceptible to malaria caused by the parasite *P. falciparum* [54, 55]. Therefore, while selection of embryos free of the β-globin gene mutation will virtually

eliminate the risk of sickle cell anemia in the offspring, these same offspring will also be more susceptible to malaria.

While less well characterized than the sickle cell example, many have argued that the high incidence of mutant cystic fibrosis transmembrane conductance regulator (CFTR) genes is also a result of heterozygote advantage [52]. The CFTR gene codes for a chloride channel, and individuals with two mutant CFTR genes often have cystic fibrosis. It is possible that having one mutant CFTR gene confers some protection against either diarrheal diseases or typhoid fever [52]. Given the complexity of the human genome, inadvertent selection of embryos with increased disease risk should be taken seriously, especially when prospective parents choose to select for nonmedical traits. In trying to avoid specific diseases or characteristics in their offspring, some prospective parents might be unknowingly selecting embryos that will result in future children with increased risk of unknown diseases.

Social Implications of PGD

The use of PGD and other technologies to select the characteristics of offspring has important societal implications that should be made clear to prospective parents [56]. While there is an inclination by some to assume that any deviation from "normal" is something to be avoided, many in the disability community have argued that those with disabilities can lead rich and meaningful lives and there are potential harms associated with seeking "perfection" [57]. Along those lines, in 2008 the United States passed the Prenatally and Postnatally Diagnosed Conditions Awareness Act, requiring that parents be given accurate and balanced information regarding the life experiences of someone with a particular disease so that they can make a more informed decision regarding whether to terminate a particular pregnancy or give a child up for adoption [58]. This type of awareness should be applied to the consent for embryo testing as well.

In addition to potential harms associated with selecting against future children who may deviate from what is considered "normal", nonmedical trait selection can also lead to harms at a societal level. In part due to reproductive freedoms, sex selection is permitted in the United States. However, this type of selection can lead to population-level imbalances in the sex ratio. As has been seen in countries practicing sex-based infanticide and selective abortion, the resulting skewed sex ratios have led to a host of downstream problems including female trafficking [59]. Furthermore, differences in access to PGD are likely to lead to further inequalities between people of different socioeconomic or racial groups [60]. Especially in regions where the more controversial uses of PGD are not regulated, prospective parents should be aware of these larger societal issues so that they can make their own informed choices.

Important Components of Genetic Testing Informed Consent

- There is a potential for error in the genetic testing of biopsied cells, which could lead to implantation of an embryo with the characteristic parents were trying to select against.
- Since there is this chance of misdiagnosis when biopsied cells are tested using FISH or PCR, prenatal testing may be required to confirm embryo test results. As such, the risks associated with prenatal testing should be disclosed during the consent for PGD.
- If comprehensive genetic testing is done on biopsied cells, unanticipated information regarding long-term health risks to the future child may become known. A child's right to an open future should be carefully considered when determining the type of genetic information that will be shared with parents regarding implanted embryos.
- Selection for embryos with certain genetic compositions may also inadvertently select for embryos with an increased risk of other diseases.
- There are important social implications associated with selection of future offspring based on genetic information.

Conclusion

In addition to being informed about potential risks associated with PGD, prospective parents should also be made aware of alternatives to the procedure. For instance, if prospective parents wish to select certain characteristics, they may choose to use donor gametes, adopt, or selectively terminate a pregnancy. It is especially important that prospective parents understand that in using PGD to select embryos with certain characteristics, they may in fact be harming those "preferred" embryos during the biopsy and selection process. Finally, it may be possible in the future to carry out less-invasive embryo selection using methods such as the testing of DNA in the blastocoele fluid [61–63]. Prospective parents will need to balance their wishes to have a child with certain characteristics with the possibility of directly or indirectly harming that child through the PGD procedure.

Note Added in Proof While this chapter was in production, Winter and colleagues reported no significant differences in measured cognitive and psychomotor outcomes in 5 and 6 year old Caucasian PGD singletons. In addition, Sacks and colleagues reported on neuropsychological findings of a pilot study of 4 and 5 year old PGD children [64, 65].

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Conflict of Interest The author declares no conflict of interest.

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Chapter 3
Controlled Ovarian Stimulation for Follicular Recruitment and Oocyte Recovery in IVF

Sesh K. Sunkara

Introduction

Results of in vitro fertilisation (IVF) treatment have much improved since its early days with live birth rates reaching around 33 % for women aged less than 35 years [1]. The introduction of controlled ovarian stimulation (COS) regimens has played a vital clinical milestone in improving IVF success and is mainly due to a paradigm shift from uni- or pauci-follicular natural IVF cycles to multi-follicular stimulated IVF cycles. Moreover COS allows control of the various events of follicular recruitment and oocyte maturation which are crucial for successful IVF. COS therefore remains an essential part and mainstay in IVF treatment. The aim of COS is to achieve efficacy and safety with assisted reproduction, to maximise live birth rates, to minimise side effects such as multiple pregnancy and ovarian hyperstimulation syndrome (OHSS), to maximise patient compliance and tolerability, and to minimise patient burden and costs.

Ovarian stimulation is considered an important aspect of IVF as the number of recruited follicles and oocytes retrieved is an important prognostic variable and a robust outcome for clinical success. There is a strong relationship between the number of oocytes retrieved and live birth following IVF in a fresh cycle. Analysis of over 400,000 IVF cycles has shown a steady increase in live birth rates up to 15 oocytes and a plateau between 15 and 20 oocytes followed by a decline in live birth rates beyond 20 oocytes in fresh IVF cycles [2]. This information is valuable in planning COS regimens in IVF and COS regimens should aim to optimise the number of oocytes retrieved. The ideal COS regimen obtains the best result at all stages of the in vitro fertilisation process: an optimal ovarian response (oocyte quantity and quality)

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leading to high fertilisation rates and development of good quality embryos. Availability of good quality embryos facilitates selection of the best single embryo for transfer with cryopreservation of the supernumerary embryos resulting in high success rates and at the same time reducing multiple pregnancies.

Individualisation of COS in IVF

The main objective of individualisation of treatment in IVF is to offer every single woman the best treatment tailored to her own unique characteristics, thus maximising the chances of pregnancy and eliminating the iatrogenic and avoidable risks resulting from ovarian stimulation. It is therefore important to categorise women based on their predicted response in order to individualise COS regimens. Women can be identified as having a poor response, normal response, or a hyper-response based on individual characteristics and ovarian reserve tests (ORTs). Among the various ORTs including basal follicle stimulation hormone (FSH), basal oestradiol, inhibin B, antral follicle count (AFC), and anti-Mullerian hormone (AMH), AFC and AMH have the highest accuracy for the prediction of either a poor or an excessive response following ovarian stimulation [3].

Recently published individual patient data (IPD) meta-analyses of patient characteristics and ORTs demonstrated age as being the most important among patient characteristics for the prediction of poor or excessive response and AFC or AMH as having the highest predictive accuracy among ORTs [4, 5]. The cutoff levels of AFC and AMH for prediction of poor response are an AFC of <5 to <7 and AMH of <0.5 ng/ml to <1.1 ng/ml [6]. The cutoff levels for AFC and AMH for the prediction of hyper-response are an AFC of >14 to >16 [7, 8] and AMH of 3.5–3.9 ng/ml [9, 10]. According to the European Society of Human Reproduction and Embryology (ESHRE) consensus, poor ovarian response is defined based on fulfilling two of the three criteria of (1) advanced female age ≥40 years, (2) previous poor response (≤3 oocytes) following conventional stimulation, and (3) abnormal ORT (AFC or AMH) [6]. In the absence of advanced female age or an abnormal ORT, two previous poor ovarian response cycles with maximal stimulation are sufficient to define poor ovarian response. The events involved in COS are pituitary suppression and ovarian stimulation with ovulation triggering as the penultimate step leading to oocyte maturation and retrieval. Individualisation of COS involves tailoring these events to suit each individual woman.

Pituitary Suppression Regimens in IVF

The introduction of GnRH agonists in assisted reproduction played an important role in the improvement of IVF treatment success by reducing the incidence of a premature LH surge which resulted in fewer cycle cancellations and higher

pregnancy rates [11] and allowed cycle programming. The GnRH agonists cause pituitary suppression by causing internalisation and downregulation of the pituitary receptors. GnRH antagonists, which prevent a premature LH surge by their more direct action, were subsequently introduced as an alternative to the GnRH agonists permitting a shorter duration of treatment. The GnRH antagonists competitively block the pituitary receptors and thereby cause immediate suppression of the LH [12]. The long GnRH agonist pituitary downregulation combined with exogenous gonadotrophins is the most frequently used in around 89.1 % of IVF cycles [13].

Commonly used pituitary suppression regimens in COS include the long GnRH agonist regimen, the short GnRH agonist regimen, and the GnRH antagonist regimen. With the long agonist regimen, pituitary desensitisation with the GnRH agonist is commenced in either the follicular phase or mid-luteal phase. The luteal phase regimen is more commonly used where the GnRH agonist is commenced on day 21 (in a 28-day menstrual cycle) of the previous cycle. After confirmation of ovarian quiescence approximately 2 weeks later, gonadotrophin for ovarian stimulation is commenced and continued with the GnRH agonist until ovulation triggering. In the short agonist regimen, the GnRH agonist is commenced in the early follicular phase of the cycle (day 1–3) followed by gonadotrophin (usually commenced a day later). Both the GnRH agonist and the gonadotrophin are continued until ovulation triggering. In the antagonist regimen, ovarian stimulation with gonadotrophin is commenced in the early follicular phase. The GnRH antagonist is commenced on day 6 of stimulation or when the leading follicle is ≥14 mm. Both the gonadotrophin and the GnRH antagonist are continued until the day of ovulation triggering.

GnRH agonists being small peptides are easily degradable by gastrointestinal enzymes and cannot be administered orally. They are administered parenterally, either via the intranasal route, as depot preparations, or intramuscular or subcutaneous injections. The GnRH antagonists are administered subcutaneously either as a single dose or as daily injections. Dose finding studies established that the GnRH antagonist could be administered either as 0.25 mg daily in a multiple dose protocol or as 3 mg in a single dose protocol to effectively suppress the LH surge and maintain IVF results [14] (Fig. 3.1).

Although early studies suggested the agonist regimen to be superior to antagonist regimen [15], later evidence suggested comparable pregnancy rates with the agonist and antagonist regimens [16]. The antagonist regimen is associated with a lower risk of ovarian OHSS and lower gonadotrophin consumption compared to the agonist regimen [16]. Between the long and the short GnRH agonist regimens, the long regimen has better outcomes in terms of the number of oocytes retrieved and pregnancy rates compared to the short regimen [17]. The GnRH antagonist and long GnRH agonist regimens are therefore suitable options for pituitary downregulation in unselected women.

A survey conducted in 2010 involving 196 centres from 45 countries showed a wide variation in the GnRH analogue regimens chosen for poor responders [18]. A recent randomised controlled trial comparing the long GnRH agonist regimen

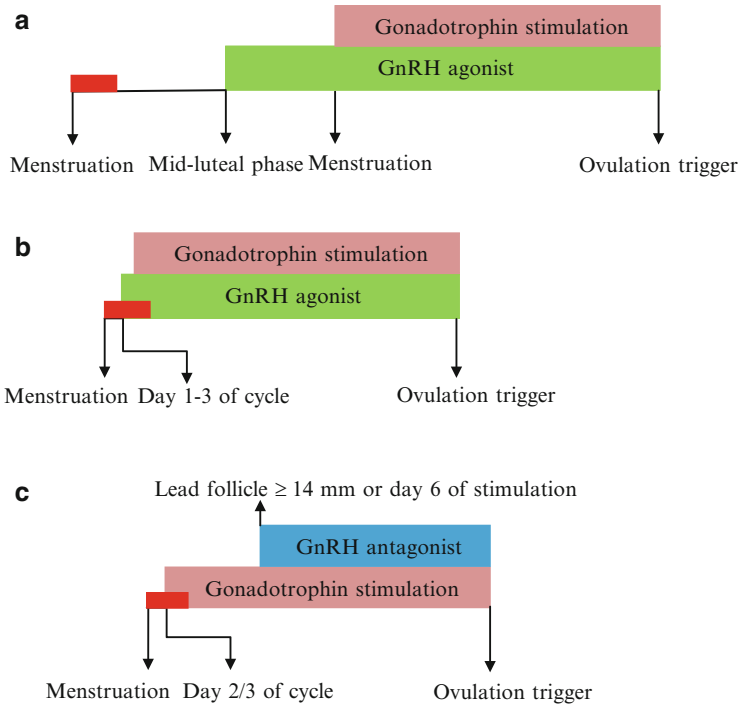

Fig. 3.1 Schematic representation of pituitary suppression regimens in IVF. (**a**) Long GnRH agonist regimen. (**b**) Short GnRH agonist regimen. (**c**) GnRH antagonist regimen

versus short GnRH agonist regimen versus GnRH antagonist regimen in women with a previous poor ovarian response demonstrated the long agonist and antagonist regimens to be suitable for these women with regard to the number of oocytes retrieved [19]. A worldwide survey in 2010 involving 179,300 IVF cycles from 262 centres in 68 countries showed the use of GnRH antagonist-based regimens in around 50 % of IVF cycles among women with polycystic ovarian syndrome (PCOS) [20]. A recent meta-analysis of studies comparing GnRH antagonist versus GnRH agonist protocols in women with PCOS involving nine RCTs from 2002 to 2013 showed comparable pregnancy rates between the two groups and a significantly lower incidence in severe OHSS in the GnRH antagonist group [21]. An added advantage with the use of GnRH antagonist-based protocols is the use of GnRH agonist trigger as a substitute for hCG in triggering of final oocyte maturation and potentially eliminating the risk of OHSS.

Ovarian Stimulation with Gonadotrophins

Gonadotrophin Dose

Exogenous gonadotrophin administration leads to supraphysiological circulating levels of FSH which facilitate recruitment of multiple follicles by exceeding the ovarian FSH sensitivity threshold [22, 23]. It is imperative to use the right gonadotrophin dose to optimise the number of oocytes retrieved and live birth rates following IVF and at the same time minimise risks such as OHSS and cycle cancellation. When exogenous gonadotrophin is administered, the number of mature follicles recruited largely depends upon the number of follicles attaining FSH sensitivity. Hence in women with a large antral follicle pool the administration of a high gonadotrophin dose may induce excessive ovarian response consequently leading to a high risk of OHSS. On the other hand, administration of an inappropriately low gonadotrophin dose may lead to the growth of a low number of follicles resulting in an 'iatrogenic' poor response.

An RCT comparing a gonadotrophin dose of 225 IU daily versus 150 IU daily in women aged 23–41 years undergoing IVF demonstrated the number of oocytes to be significantly higher with 225 IU daily compared to 150 IU daily [24]. This study excluded women with basal FSH > 10 IU/l, PCOS, previous poor response, and previous OHSS. Another RCT comparing gonadotrophin dose 225 IU daily versus 300 IU daily among women predicted as normal responders based on a total AFC of 8–21 showed no significant difference in the number of oocytes retrieved between the two doses [25]. This evidence would therefore suggest that the ideal gonadotrophin dose for women predicted as normal responders is 225 IU daily.

According to the worldwide survey on poor ovarian response, high gonadotrophin doses of >300 IU daily are used in around 50 % of IVF cycles for poor responders [18]. There is however no evidence to suggest that higher gonadotrophin doses result in a higher yield of oocytes and improve pregnancy outcome for poor responders. An RCT comparing gonadotrophin doses of 300 IU vs. 375 IU vs. 450 IU daily among women predicted as poor responders based on a total AFC of <12 showed no significant difference in the number of oocytes retrieved nor live birth rates between the three arms suggesting an unlikely benefit with gonadotrophin doses >300 IU daily [26]. The term hyper-response refers to the retrieval of >15 oocytes [27] or 20 oocytes [28] following conventional stimulation. It is vital to accurately predict women who are likely to have an excessive response and accordingly individualise the gonadotrophin stimulation dose to reduce the risk of OHSS. Women with PCOS and those predicted to have a hyper-response should be stimulated with a lower gonadotrophin dose of ≤150 IU daily as this will avoid excessive response. Excessive response (>20 oocytes) is also associated with a decrease in live birth rate in fresh IVF cycles [2] in addition to the higher incidence of OHSS with >18 oocytes [29–31].

Gonadotrophin Type

The successful therapeutic use of urinary gonadotrophins started with the first-generation product human menopausal gonadotrophin (hMG) or menotropin, which contained 75 IU of FSH and 75 IU of LH in each standard ampoule. This was followed in the early 1980s by the development of urofollitropin, the second-generation product from which the LH activity had been reduced to 0.1 IU/75 IU FSH [32]. Subsequently, the third-generation product, highly purified urofollitropin (Metrodin HP®) with practically no residual LH activity, was developed in the early 1990s. Due to its enhanced purity with very small amount of protein, Metrodin HP® could be administered subcutaneously which is an advantage over the previous generations which had to be administered intramuscularly. The more recent fourth-generation gonadotrophin is produced in vitro through recombinant deoxy ribo nucleic acid (DNA) technology, by genetically engineered Chinese hamster ovary cells. This is recombinant human FSH (r-FSH or follitropin) which is free of LH and contains less than 1 % of contaminant proteins [33]. There are two preparations of r-FSH that are commercially available for clinical use: follitropin-α and follitropin-β. There have been numerous RCTs comparing urinary gonadotrophins versus recombinant FSH for COS. Current evidence suggests that both the gonadotrophin preparations are comparable in IVF outcomes [34] (Fig. 3.2).

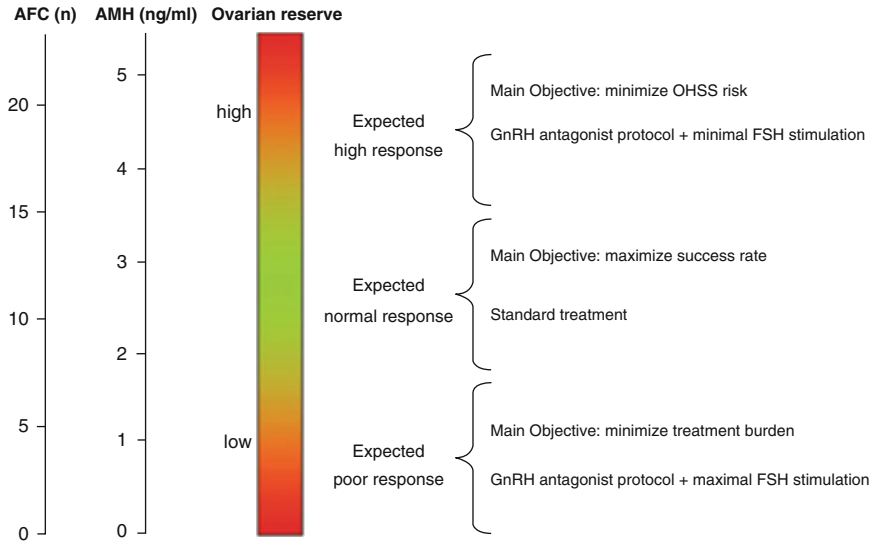

Fig. 3.2 Schematic representation of categorising women based on predicted response to individualise COS. Reproduced from La Marca & Sunkara [35]

Ovulation Trigger

Following recruitment and growth of follicles to the mature stage resulting from ovarian stimulation, the next step is maturation of oocytes facilitated by ovulation trigger in COS regimens. The LH surge that induces germinal vesicle breakdown and ovulation in a natural menstrual cycle is not reliable in stimulated multifollicular cycles necessitating artificial triggering of ovulation. hCG which is naturally produced by the human placenta and excreted in large quantities in the urine of pregnant women bears a close molecular resemblance to LH and has a similar effect on the LH receptor. hCG can be used because of its longer serum half-life (36 h) compared to the short serum half-life of LH (108–148 min) [36], thus avoiding the inconvenience of repeated administration. Administration of hCG results in luteinisation of the granulosa cells, progesterone biosynthesis, resumption of meiosis, oocyte maturation, and subsequent follicular rupture 36–40 h later. It is administered after the stimulated development of mature preovulatory follicles in order to induce maturation, but oocyte retrieval is undertaken before ovulation. The usual criteria for the administration of hCG is the presence of ≥3 follicles of ≥18 mm in diameter. The preparations of hCG that are available for clinical use are the urinary and recombinant forms and are comparable for IVF outcomes [37]. The usual dose of hCG for final ovulation triggering is between 5,000 IU and 10,000 IU as a single dose.

The GnRH agonist trigger has been proposed as an alternative to the hCG trigger by virtue of inducing an endogenous rise in LH and FSH due to its initial flare effect [38, 39]. The GnRH agonist trigger can only be used with COS regimens where prior pituitary suppression has not been achieved with a GnRH agonist, as the mechanism of action of the GnRH agonist for downregulation and desensitisation of the pituitary receptors precludes the use of the agonist trigger. Due to the specific mode of action of the antagonist by competitive blockade of the pituitary receptors and a shorter half-life, the pituitary remains responsive to the GnRH agonist, thus enabling its use for triggering ovulation. The Cochrane review comparing the GnRH agonist versus the hCG trigger in IVF demonstrated a significantly lower incidence of OHSS and a lower live birth rate with the GnRH agonist trigger [40]. It demonstrated significantly reduced live birth rates in fresh autologous cycles with the use of the GnRH agonist trigger, but there was no reduction in live birth rates in oocyte donor/recipient cycles. Following initial use of the GnRH agonist trigger, it was soon recognised of the need to modify the standard luteal support to obtain reliable reproductive outcomes [41]. Study groups have since endeavoured to fine-tune the luteal phase support in IVF cycles using the GnRH agonist trigger to optimise clinical outcomes [42, 43]. Recent suggestions and developments in overcoming the luteal insufficiency with the GnRH agonist trigger are use of (1) a “dual trigger” [44], (2) low-dose hCG supplementation [41, 43], (3) intensive luteal oestradiol and progesterone supplementation [42], (4) rec-LH supplementation [45], and (5) luteal GnRH agonist administration [46]. A recent RCT demonstrated that an individualised luteal support based on the number of follicles following the GnRH agonist trigger optimised the pregnancy rates [47]. This study proposed ovulation triggering with 0.5 mg buserelin subcutaneously followed by a bolus of 1,500 IU of hCG after

oocyte retrieval when the total number of follicles ≥11 mm was between 15 and 25 on the day of trigger and an additional 1,500 IU hCG bolus when the total number of follicles was ≤14 mm. All women received micro-ionised progesterone vaginally, 90 mg twice daily, and 4 mg of oestradiol orally commencing on the day of oocyte retrieval and continuing until 7 weeks of gestation.

Conclusion

The ultimate aim of IVF is to obtain a healthy singleton live birth with minimal adverse effects. Multiple pregnancies are recognised as a major avoidable complication of IVF. Planning of effective COS regimens is important as it leads to good quality embryos enabling selection of the best single embryo for transfer. After decades of IVF practice, it is now recognised that individualisation in IVF is the way forward. The long GnRH agonist and antagonist regimens are effective in normal responders and the ideal gonadotrophin dose is 225 IU daily. The GnRH antagonist regimen is ideal for women with PCOS and women categorised as hyper-responders. Whilst the pregnancy rates are comparable to the GnRH agonist regimen, the antagonist regimen significantly lowers the risk of OHSS in addition to enabling the use of the GnRH agonist trigger which potentially eliminates OHSS. A lower gonadotrophin dose ≤150 IU daily is recommended in these women. The long GnRH agonist and antagonist regimens are ideal for poor responders. Higher gonadotrophin doses >300 IU daily are unlikely to be beneficial in poor responders apart from higher costs and hence the maximal gonadotrophin dose should not exceed 300 IU daily.

Conflict of Interest The author declares no conflict of interest.

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Chapter 4
Biomarker-Based Flow Cytometric Semen Analysis for Male Infertility Diagnostics and Clinical Decision Making in ART

Peter Ahlering and Peter Sutovsky

Sperm Phenotype and Its Influence on Conception and Maintenance of Pregnancy After Single vs. Multiple Embryo Transfer

Paternal influence on embryo development and pregnancy establishment and maintenance can be appreciated at multiple levels. Inability of the spermatozoon to activate the oocyte after in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI) may be due to dysfunction or complete lack of the sperm-borne oocyte activating factor(s), which is most common in ICSI patients with sperm head defects such as the globozoospermia [1]. At the level of paternal genome and organelle inheritance, the contribution of the sperm-borne proximal centriole and proper reconstitution and functioning of the zygotic centrosome influence the early stages of zygotic development [2]. Structural integrity and proper packaging of the paternal chromosomes within the sperm nucleus are crucial for both early and advanced stages of embryo development [3]. Cell cycle checkpoints in the embryo assure the integrity of sperm-contributed DNA and its proper replication and apposition with the female pronucleus. Consequently, excessive, unrepairable sperm DNA damage could shut down embryo development prior to first embryo cleavage or cause embryonic fragmentation, developmental arrest, implantation failure, and pregnancy loss, often attributed to "spontaneous" miscarriage later during pre- or post-implantation development [4]. Some recent studies suggest the association of DNA fragmentation,

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measured by flow cytometric sperm chromatin structure assay (SCSA) with the sporadic and recurrent spontaneous abortion (SAB) [5, 6] and the incidence of multiple births in couples treated by assisted reproductive therapies (ART) [5]. Reliable, biomarker-based andrological evaluation of men from ART couples, in conjunction with current microscopic sperm morphological assessment techniques, might allow the attending physician to identify couples in which the sperm quality is sufficient to warrant single embryo transfer, thus reducing the incidence of unwanted multiple births after ART. The goal of this chapter is to review the emerging biomarker-based andrological approaches and more specifically automated, high-throughput semen analysis by flow cytometry (FC) that correlates with specific sperm phenotypes, clinical decision making, and treatment outcomes in ART couples.

Principles of Flow Cytometric Semen Analysis and Clinical Benefits of Biomarker-Based vs. Conventional Semen Analysis

Conventional semen evaluation by visual ejaculate assessment and light microscopic sperm motility and morphology analysis provides useful baseline information for ART clinician. However, due to its inherent subjective nature, low throughput (low number of evaluated spermatozoa per sample), and the difficulty of identifying sperm defects at subcellular or molecular level, the conventional andrological workup is not sufficiently predictive of treatment outcome. Consequently, there is a desire toward developing automated, objective high-throughput sperm quality assays better reflecting treatment strategy and outcome. Flow cytometric sperm analysis satisfies these interests as it allows for rapid, partially automated, and most importantly objective screening of a large number of spermatozoa per sample, with reasonable sample preparation time, labor efficiency, and cost. Importantly, flow cytometric approaches discussed in the following sections may reflect individuals' sperm quality and fertility more closely than conventional semen/sperm parameters [7], and fluorescent probes for FC can be combined into simultaneous or serial multiplexed assays providing a multidimensional profile of the evaluated sperm sample [8, 9]. Some flow cytometric tests can be done with minimal processing time and labor as they are applied to live spermatozoa immediately after semen collection (e.g., live/dead sperm assay, mitochondrial membrane potential, and calcium flux). Other assessments require extra processing steps and time on prepared or unprepared sperm specimens (e.g., sperm chromatin/DNA structure and TUNEL assays as well as immunocytochemical quantification of sperm proteins relevant to male fertility). As an added benefit, bacterial and other contaminant (leukocytes, immature germ cells, sloughed epithelial cells) counts can be obtained simultaneously.

In a typical FC protocol, spermatozoa are labeled with a fluorescent probe (or a combination of several fluorescent probes with well-separable excitation and emission wavelengths) and loaded into a flow cell that forces the labeled spermatozoa through a narrow nozzle one cell at a time, at a high speed. Resultant stream of cells enveloped in sheet fluid passes through one or more laser beams, exposing the fluorescently labeled cells to excitation light with probe-specific wavelength provided by a precisely

tuned laser. The resultant photon emission is captured by photomultiplier tube and processed for each flow cytometric event (cell or sample contaminant) by dedicated computer software. The output is presented in the form of a histogram depicting the relative fluorescence of a sample consisting of several thousands of cells measured in a few seconds' time (Fig. 4.1). In addition to collecting fluorescence, the visible light footprint of each cell passing through flow stream is recorded as a scattered diagram of visible light (Fig. 4.1). Even in the absence of fluorescent probe labeling, scatter plots of visible light provide useful, albeit often overlooked, information about the distribution of cell sizes within individual sample. Many of the biomarkers discussed below were validated in livestock animal species, in which fertility records from hundreds or thousands of artificial insemination services per male allow for convincing validation of correlation between biomarker and individual fertility [10, 11].

Sperm Flow Cytometry with Vital Stains and Lectins

Mitochondrial membrane potential (MMP) reflects the polarization of mitochondrial membrane and thus the metabolic state/activity of sperm mitochondria. Low MMP is indicative of elevated apoptosis or necrosis in the semen sample [12, 13]. Human sperm MMP measured by vital ratiometric dual-fluorescent probe JC-1 is correlated with sperm motility [14–16]. A recent study of normozoospermic and asthenozoospermic men revealed a correlation between sperm MMP and expression of inner mitochondrial membrane protein prohibitin/PHB [17]. Other fluorometric mitochondrial probes, such as CMX-Ros, DiOC(6)(3), rhodamine 123, and TMRE, can be used as an alternative or complement to JC-1 [18].

Sperm viability obviously has an effect on the fertilizing potential of an ejaculate specimen. Live/dead cell stains are based on differential cell membrane permeability (plasma membrane and nuclear envelope) of fluorescent DNA binding probes. The most common combination is the green fluorescent CYBR-14 probe permeant mainly to live spermatozoa and propidium iodide (PI) excluded from live spermatozoa but easily intercalated in the DNA of the dead ones [19]. While viability can be measured by PI staining alone, it is desirable to include CYBR-14 since different degrees of its exclusion differentiate not only between live and dead spermatozoa but also identify the moribund, dying spermatozoa [20], which is a characteristic similar to the sensitivity of MMP measurement.

Sperm capacitation encompasses the irreversible remodeling of sperm plasma membrane and acquisition of hyperactive motility in preparation for fertilization [21]. If induced prematurely by semen handling, storage, or cryo-damage (cryo-capacitation), capacitation may preclude successful fertilization and eventually lead to sperm death. Since capacitation is accompanied by fluxes/increases in the content of intracellular calcium [22], it can be monitored by flow cytometry with fluorescent Ca-ion reporter dyes such as Fluo-3 or Fluo-4NW [23] and used as a clinical parameter to diagnose male infertility [24]. Besides FC, capacitation status is commonly monitored by epifluorescence microcopy of fluorescent chlortetracycline labeling of spermatozoa [25], but this technique is yet to be translated into a flow cytometric assay.

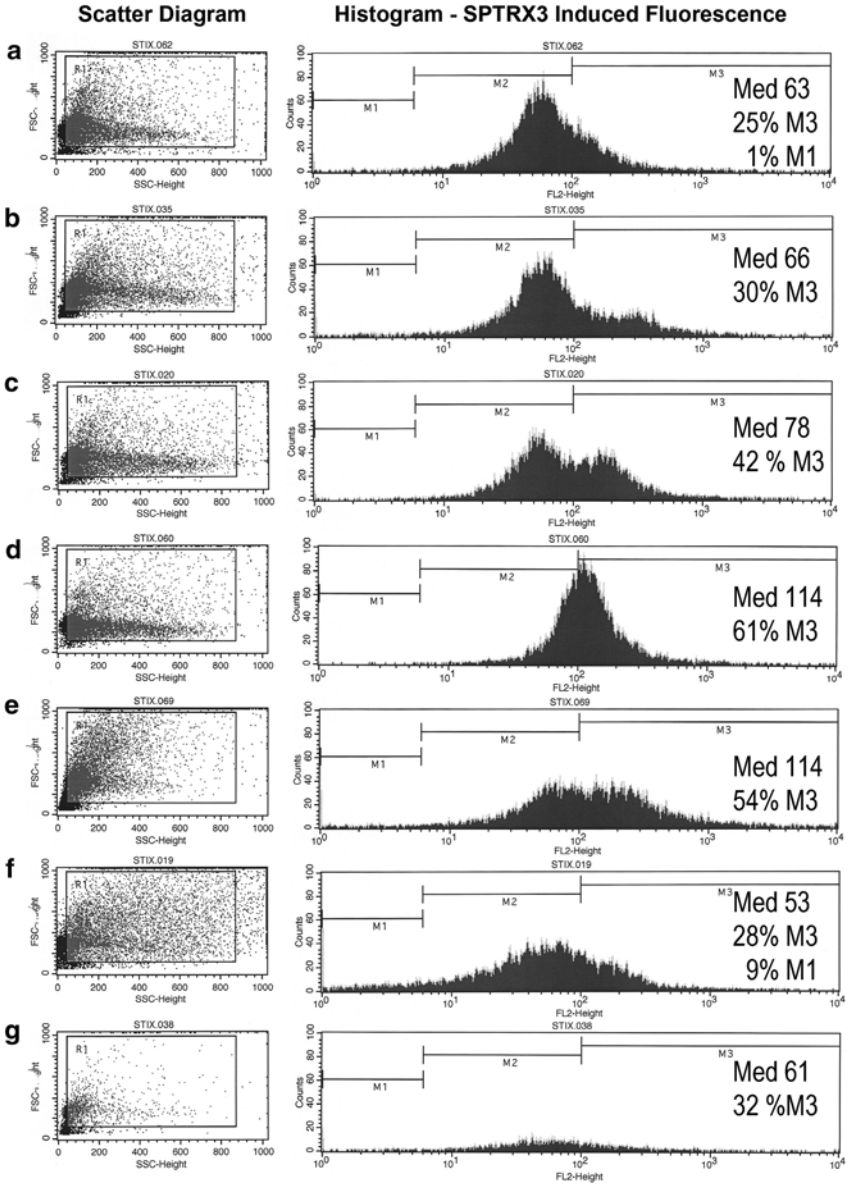

Fig. 4.1 Flow cytometric analysis of spermatid-specific thioredoxin 3 (SPTRX3/TXNDC8) in semen of ART patients. The SPTRX3 protein accumulates in the superfluous cytoplasm and nuclear vacuoles of defective spermatozoa. Scatter diagrams of visible light, reflective of the size of individual cells/flow cytometric events in each sample, are in the *left column*. Each *dot* represents one cell/event. Normal size spermatozoa cluster in the lower left corner and toward the center of diagram. Small debris is in the extreme lower left; abnormally large spermatozoa and somatic cells cluster toward the right side of the diagram. Histograms of SPTRX3-induced fluorescence are

The sperm acrosome is important for sperm interactions with the oviductal epithelia and oocyte zona pellucida. The structural and functional status of acrosomal membranes, particularly the outer acrosomal membrane, can be affected by capacitation status, acrosome reaction, mechanical damage, or cryo-injury. Sperm acrosomal integrity is evaluated by labeling of live spermatozoa with fluorescently conjugated lectins, glycan binding plant proteins with narrowly defined binding affinity to specific types of sugar residues found on sperm glycoproteins. In particular, the peanut agglutinin (PNA or *Arachis hypogea* lectin) and the green peas derived *Pisum sativum* agglutinin (PSA) display high specific affinity toward glycans of sperm acrosomal matrix and have been adapted for FC [26–29]. Thus, only spermatozoa with compromised acrosomal membranes bind PNA and PSA, which can be used in both live spermatozoa and fixed spermatozoa via a dual labeling protocol applying antibodies or DNA stains [30, 31]. Other acrosome binding lectins used for human sperm FC include wheat germ agglutinin (WGA), *Ulex europaeus* agglutinin (UEA-1, ulex, or common gorse seed lectin), and *Concanavalia ensiformis* agglutinin (Con-A or common jack bean lectin) [32, 33].

Fig. 4.1 (continued) shown on the *right*. Median (Med) is the median value of SPTRX3-induced fluorescence at which half of the events have higher and half have lower relative fluorescence (no units) of immuno-labeled SPTRX3 protein. Histograms are divided into three marker areas: M1—events representing cellular debris and sperm fragments with very low background fluorescence; M2—events representing mainly normal spermatozoa with background levels of SPTRX3 fluorescence and cells/debris of similar size free of SPTRX3; M3—events representing spermatozoa positive to SPTRX3. Marker area M3 was set differently in some of our previously published studies, resulting in higher cutoff fluorescence values for SPTRX3-positive spermatozoa. Percentages of events within each marker area are shown as %M1–M3. A total of 5,000 events were measured per sample. (**a**) Reference sample with acceptable WHO sperm parameters. Histograms show normal distribution. Med and %M3 values are low, 74 % of spermatozoa/events fall within marker area M2. (**b**) Slightly elevated Med and %M3 values are reflective of a shoulder on the right side of histogram, corresponding to the SPTRX3-positive spermatozoa. (**c**) Distinct secondary peak covers the M3 area, reflected by elevated Med and %M3 values. (**d**) Sample with normal distribution but with the histogram peak shifted to M3 area, resulting in high median and %M3 value above 50 %. In the absence of positive and negative controls, and without appropriate sample blocking prior to antibody labeling, this type of curve could also be obtained by overlabeling resulting in elevated nonspecific background fluorescence. (**e**) While the Med and %M3 values are similar to panel (**d**), the shape of histogram curve is dramatically different, essentially composed of two peaks of equal height and width. (**f**) Sample with low overall fluorescence corresponding to low Med and %M3 values similar to reference sample in panel (**a**). However, the shape of the histogram curve is unusually flat and a large number of events positioned toward the right and upper right part of the scatter diagram suggest the presence of large cells that do not express SPTRX3, such as leukocytes. While clearly a contaminant/abnormality, such cells can lower the overall reading of biomarkers associated exclusively with the defective spermatozoa. This issue can be ameliorated by dual analysis with markers of white blood cells. (**g**) Sample with only a slight increase of Med value, but a very flat histogram curve and an elevated %M3 value. This was a sample with very few spermatozoa which did not allow to measure 5,000 events, as reflected by fewer events seen in the scatter diagram. Such samples are often encountered with oligozoospermia. Due to low sperm concentration, debris and somatic cells likely make up a substantial percentage of measured events. This issue could be mitigated by double labeling with a DNA-specific probe, which would allow for gating of spermatozoa during SPTRX3 labeling

Protein Biomarkers of Sperm Quality

Normal and defective spermatozoa may accumulate certain proteins at differential levels, and they may lack certain other proteins. Whole proteome analyses comparing spermatozoa from fertile donors with male infertility patients revealed a number of such proteins [34]. Based on the observation that proteins such as ubiquitin accumulate on the surface of defective spermatozoa, we proposed the description "negative biomarkers of male fertility and semen quality" [11]. This umbrella term encompasses proteins that are increasingly or exclusively present in defective spermatozoa, often retained from the haploid phase of spermatogenesis occurring in the testis. Proteins such as thioredoxin SPTRX3, discussed below, have a function during the biogenesis of sperm accessory structures but then are degraded within the spermatid cytoplasmic lobe or jettisoned within a residual body. In defective spermatozoa that fail to complete spermatid differentiation, such proteins may be retained in structures containing residual cytosol such as nuclear vacuoles and the retained cytoplasm surrounding the sperm tail connecting piece and midpiece.

Ubiquitin and Ubiquitin-Like Protein Modifiers Ubiquitin (UBB) is a small chaperone protein that binds covalently to other proteins, most commonly in a tandem fashion giving rise to multi-ubiquitin chains making the ubiquitin-tagged substrate proteins recognizable to the 26S proteasome, which is a proteolytic holoenzyme particle responsible for regulated, substrate-specific protein recycling across the human, animal, and plant proteomes [35]. Such protein modification by this ubiquitination is reversible and has regulatory functions in addition to promoting selective proteolysis. Examination of ubiquitin as a sperm quality biomarker was initiated based on the observation that defective animal spermatozoa become surface-ubiquitinated by an apocrine secretory mechanism that assures high concentration of ubiquitin-proteasome system enzymes and non-conjugated ubiquitin in the epididymis [36, 37]. Additionally, ubiquitinated proteins from spermatid phase can be carried over in the sperm structures or sperm-borne superfluous cytoplasm. While some appear morphologically normal, most ubiquitinated spermatozoa display a variety of morphological defects and they often carry single-stranded, fragmented DNA detectable by dual TUNEL-ubiquitin FC [38]. Ubiquitin is also present in the normal spermatozoa, but the localization, ubiquitin-substrate ligation patterns, and amounts may differ from the defective spermatozoa.

In our early studies, the flow cytometric sperm ubiquitin-tag immunoassay (SUTI) for diagnosis of human male infertility correlated negatively with various conventional semen parameters, as well as with embryo cleavage rate and other embryo-development parameters after IVF and ICSI [39, 40]. Substantial proportion of men from idiopathic infertility couples tend to have elevated sperm ubiquitin content [40], hinting at the potential of SUTI assay to reveal cryptic male infertility in men with acceptable clinical semen parameters. On the opposite end of spectrum, high sperm ubiquitin levels were found in obviously infertile men with heritable stump tail syndrome/fibrous sheath dysplasia [41], in men with abnormal sperm chromatin [42], and in ART patients with self-reported occupational exposure to reprotoxic solvents [43].

In proteomic analysis, proteins related to ubiquitin-proteasome system were abnormally expressed in infertile men with high DNA fragmentation index determined by flow cytometry [34]. Contrary to elevated defective sperm surface ubiquitination, the flow cytometric measurement of the ubiquitin content intrinsic to normal spermatozoa revealed positive correlation with fertilization rate by ICSI, while no such relationship was observed for simultaneously assessed sperm protamination [44]. The sperm content of "properly" ubiquitinated normal spermatozoa can be increased by sperm gradient purification [45]. In some studies, only certain measures of sperm surface ubiquitination, such as median ubiquitin-induced fluorescence, showed negative correlation with semen parameters, while percentages of high-ubiquitin spermatozoa did not correlate with semen quality, or with various markers of apoptosis [46]. Others determined that ubiquitin was mainly associated with anuclear bodies present in semen, rather than with spermatozoa, but based on images shown, one could suspect that the immunolabeling of the examined samples was not done on properly preserved samples by strictly following published protocols. Such reports may be misleading but still put emphasis on the necessity of proper quality control of sperm immunolabeling prior to flow cytometry [47, 48]. Alternatively, these seemingly conflicting observations could simply mean that in patients with high content of semen contaminants, the measurement of surface ubiquitination unique to spermatozoa simply reveals the ratio of spermatozoa to contaminating somatic cells and residual bodies, and could in fact have a positive correlation with semen quality. Some of the issues associated with the specificity of anti-ubiquitin antibodies and other antibodies for sperm FC and the potential of immunocytochemical detection for false-negative results have been addressed by developing a very simple, single-step detection of stress-associated ubiquitinated protein aggregates, the aggresomes, using the ProteoStat aggresome detection kit originally developed for somatic cells [8].

Ubiquitin-like protein modifiers are structurally and functionally related to ubiquitin and may be involved both in selective protein recycling and in the regulation of substrate protein function. Similar to ubiquitin, the covalent ligation of these modifiers to substrate proteins requires activating and conjugating enzymes and substrate-specific protein ligases. Small ubiquitin-related modifier SUMO1 [49, 50] and its close relatives SUMO-2, 3, and 4 regulate the functions of varied substrate proteins in either reversible or irreversible manner. Similar to increased protein ubiquitination, excessive protein sumoylation by SUMO1 and SUMO2/3 was reported in infertile men and coincided with ubiquitination of several sperm proteins that appeared to be both ubiquitinated and sumoylated [51]. Sperm SUMO1 content correlated negatively with sperm motility in asthenozoospermic but not in normozoospermic men [52]. The presence of other ubiquitin-like modifiers (NEDD4/8, ISG15) in human spermatozoa is yet to be investigated.

Testis-Specific Thioredoxins Thioredoxin family proteins are involved in the regulation of cellular redox potential, thus affecting protein folding and a variety of cellular functions. There are three thioredoxins uniquely expressed in male germ line of mammals [53]. Among them, the thioredoxin domain-containing 8 (TXNDC8), commonly described as sperm/spermatid-specific thioredoxin 3 (SPTRX3), has been

found to accumulate in defective human spermatozoa. Early during spermiogenesis, SPTRX3 is detectable in the pro-acrosomic granule of round spermatids, suggesting involvement in acrosomal biogenesis [54]. While undetectable in fully differentiated normal spermatozoa of humans and other mammals [54], SPTRX3 uniquely carries over into the nuclear vacuoles and superfluous midpiece cytoplasm of defective human spermatozoa [55] (Fig. 4.2). We have found that sperm levels of SPTRX3 correlate negatively with conventional semen parameters and pregnancy outcomes of both IVF and ICSI couples [56]. Among 239 ART couples, only 9.2 % got pregnant if the male partner had >15 % SPTRX3-positive spermatozoa measured by flow cytometry, vs. 41.2 % pregnant couples in which men had less than 5 % SPTRX3-positive spermatozoa. Thus, men with >15 % of SPTRX3-positive spermatozoa had their chance of fathering children by ART reduced by nearly two-thirds [56]. Our yet to be published trials also indicate that low SPTRX3 content significantly increases the likelihood of multiple pregnancy after multi-embryo transfer.

The Post-Acrosomal WW-Domain Binding Protein PAWP (HUGO name WW-domain binding protein N-terminal like/WBP2NL) is an evolutionarily conserved, male germ line-specific signaling protein located in the post-acrosomal sheath (PAS) of mammalian spermatozoa [57, 58] (Fig. 4.2). While the downstream elements of PAWP-regulated signaling pathways in the oocyte remain to be characterized, it has been shown that the injection of PAWP cRNA or recombinant protein induces calcium oscillations identical to those observed during oocyte activation by the fertilizing spermatozoon in human and animal spermatozoa, respectively [59, 60]. Similarly, PAWP sperm phenotypes and semen content of PAWP protein determined by conventional or ImageStream flow cytometry correlate with sperm parameters and fertility in both humans and bovines [8, 61]. The FC sperm content of PAWP in men from ART couples did not correlate with conventional semen parameters or DNA-fragmentation index, but was positively associated with fertilization success and pre-implantation embryo development after ICSI [61]. Given its consistent multi-species validation, PAWP-specific probes are being developed for routine FC use in andrology laboratories. Because of distinct, easy-to-assess localization to PAS of normal spermatozoa and ectopic localization patterns in defective spermatozoa, such probes will also be suitable for light-microscopic evaluation.

The Platelet-Activating Factor Receptor (PAFR) is a G-protein-coupled receptor-like, rhodopsin-related protein receptor for the pathology-related PAF phospholipid [62]. Based on immunofluorescence and transcript profile, *Pafr* gene expression and PAFR protein distribution are altered in abnormal human spermatozoa [63, 64]. To our knowledge, PAFR FC has not been conducted in humans, while our earlier study details the relationship between PAFR and sperm quality in bulls entering artificial insemination service [65], suggesting that translation to clinical use may be possible and useful.

White Blood Cell (WBC) and Immune Response Markers The WBC frequently contaminate semen of infertile men, and sperm FC using biomarkers related to WBC surface antigens (e.g., cluster of differentiation/CD glycoproteins), immune

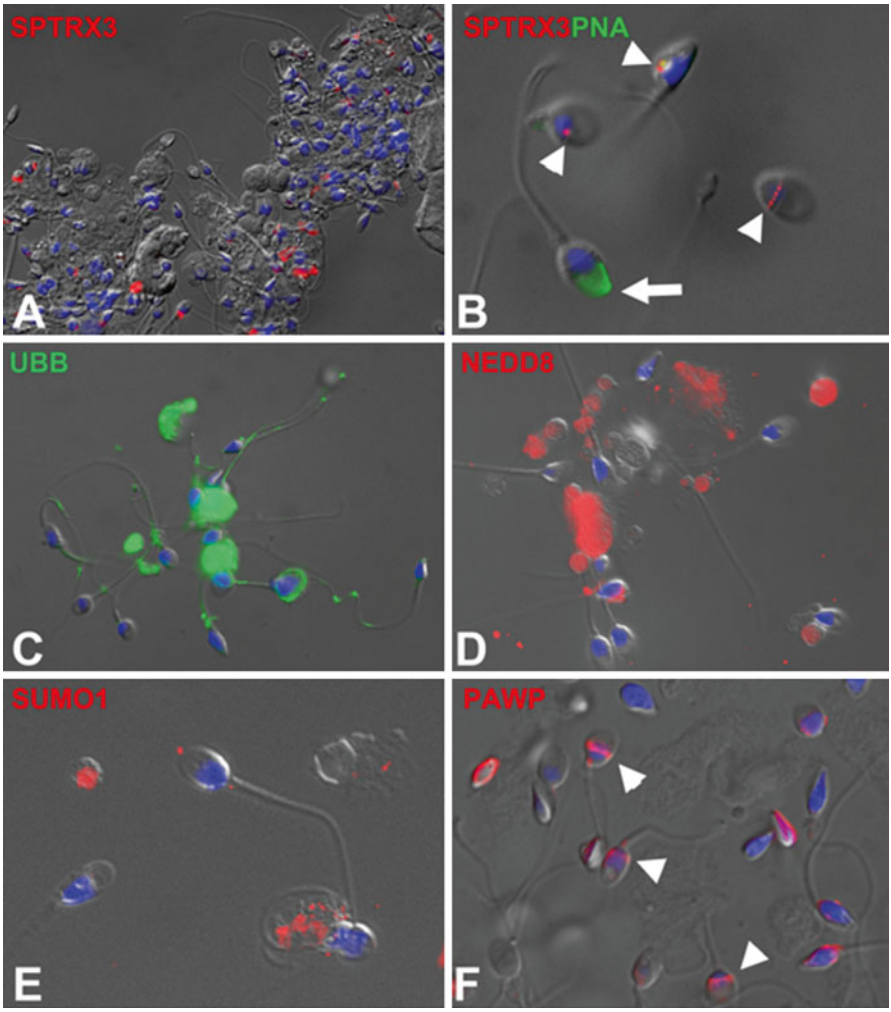

Fig. 4.2 Immunofluorescence localization of sperm quality biomarkers in the spermatozoa of male infertility patients. Sperm nuclear DNA in all panels was counterstained with DAPI (*blue*) and the epifluorescence images were superimposed over parfocal differential interference contrast (DIC) light images. (**a**) Spermatid-specific thioredoxin 3 (SPTRX3; *red*) is abundant in the redundant cytoplasm of defective human spermatozoa. (**b**) Retention of SPTRX3 (*red*) in small nuclear vacuoles of defective spermatozoa lacking acrosomes (*arrowheads*; acrosomes were counterstained green with lectin PNA—*arrow*). (**c**) Proteolysis-promoting small protein modifier ubiquitin (UBB; *green*) is found on the surface of defective spermatozoa and in the interior of anucleate residual bodies present in patients' semen. (**d**) Ubiquitin-like protein modifier NEDD8 (*red*) is localized predominantly to anucleate bodies and superfluous sperm cytoplasm. (**e**) Ubiquitin-like protein modifier SUMO1 (*red*) is associated with superfluous cytoplasm of defective spermatozoa (lower left corner). (**f**) Post-acrosomal WW-domain binding signaling protein PAWP (*red*) is found in the post-acrosomal sheaths of normal spermatozoa (*arrowheads*) but may be ectopically localized or missing from defective spermatozoa

response, and autoimmune infertility are of interest to ART practitioners. Thus, FC can be used to directly identify and quantitate WBC types in semen [66], to simultaneously assess sperm and leukocyte count and sperm apoptotic markers [67, 68], and to measure reactive oxygen species (ROS) production by WBC (main ROS source in semen) and other seminal somatic cells [69]. Exposure of normal spermatozoa to pro-inflammatory interleukins increases sperm DNA fragmentation evaluated by TUNEL-FC [70]. By FC, mast cell counts correlate positively with sperm-bound immunoglobulin IgA in ART men [71], and the CD16-positive lymphocytes and gamma delta receptor-positive T lymphocytes are elevated in autoimmune-infertile men with antisperm antibodies in semen [72, 73]. However, the influence of total semen WBC and individual WBC types on ART outcomes is unclear at present [74], partly because WBC type-specific records are commonly unavailable for ART couples and WBC are also present in the semen of fertile men. It remains to be determined if the content of any particular WBC type in semen correlates with SAB or multiple pregnancies after ART. In addition to anti-sperm antibodies on the sperm surface, immunomodulatory antigens may reflect sperm quality. We reported that the immunoregulatory human sperm glycoproteins decorated with branched, bi-antennary Lewis(x) and Lewis(y) glycans are present in normal sperm acrosome but also detected in the superfluous cytoplasm in defective spermatozoa [75]. While this study employed anti-Lewis antibodies, lectins with appropriate affinities for Lewis glycans could be adapted for sperm FC.

Sperm Protamination The protamines are sperm-specific, cysteine-rich DNA-binding proteins responsible for hyper-condensation of sperm chromatin following histone-protamine exchange during spermatid elongation in the testis. Human spermatozoa contain both known mammalian protamines, PRM1 and PRM2 [76], as well as residual somatic cell-type histones. Aberrant sperm protamination is associated with human male infertility and correlates with ART embryo development [77]. Consequently, various diagnostic assays can be used to assess human sperm protamination by the quantification of individual protamine types, or by PRM1:PRM2 or protamine:histone ratio [78, 79]. While protamination lends itself to quantification by FC (e.g., chromomycin A3 test [80]), most diagnostic approaches rely on indirect assessment of protamination via flow cytometric chromatin structure/DNA integrity tests [81], as will be discussed next.

DNA Fragmentation, Apoptosis, and Chromatin Structure-Based Tests (TUNEL, Annexin, SCSA)

Sperm DNA integrity and proper chromatin packaging have direct effect on both fertilization and post-fertilization embryo development and maintenance of pregnancy [82, 83]. Some spermatozoa with abnormal chromatin and thus enlarged macrocephalic heads may not be able to reach the oocyte or penetrate its vestments, while morphologically normal motile spermatozoa delivering fragmented DNA to

oocytes may give rise to embryos destined for apoptosis due to irreparable DNA damage within paternal genome [3]. The sperm chromatin structure assay (SCSA) is considered by some to be reflective of sperm protamination, while the most direct association may be with DNA fragmentation. To clinicians involved with fertility diagnostics and therapeutic management of couples, DNA fragmentation assessment is widely accepted as valuable. As an adjunct to traditional analyses, routine use of DNA fragmentation can streamline the evaluation process, triage to IVF/ICSI sooner in some couples, as well as diagnose "qualitative" sperm issues that otherwise are undetected by conventional semen analysis. The SCSA is based on the intercalation of metachromatic dye acridine orange with light emission wavelength shifting from green to red fluorescence when bound to single-stranded DNA [84]. As an added benefit, SCSA output can be analyzed to quantitate spermatids and various somatic cells contaminating human semen. The SCSA results are expressed as DNA fragmentation index (DFI) and high DNA stainability (HDS) value [85]. There are many convincing studies showing SCSA correlation with conventional semen parameters and embryo development after ART [3, 85–87]. Even couples with acceptable basic semen parameters may benefit from SCSA before the decision is made to treat by intrauterine insemination [88]. Importantly, several recent studies show the association of high DFI/DNA fragmentation with SAB and multiple births. Relationship between high DFI/HDS and miscarriage after ART has been recorded at varied threshold levels in ART couples [3, 89–91]. Besides significant correlation with SAB, a meta-analysis of 233 couples evaluated by SCSA reported a significantly lower average DFI in couples that had triplets after multiple embryo transfer [5]. Alternative to SCSA, the fluorescent terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) of single-stranded DNA is indicative of increased DNA damage in couples experiencing sporadic or recurrent pregnancy loss [92, 93] and can be adapted for sperm FC [38].

Various markers associated with pathways regulating programmed cell death, apoptosis, have been detected in human spermatozoa [94] and correlated with ART outcomes [95]. Among them, Annexin V is most commonly targeted for defective human sperm identification and removal from sperm samples prepared for ART [96]. The Annexin V assay adapted for FC showed relationship with human sperm mitochondrial membrane potential [97], sperm concentration and motility [98], sperm viability and DNA methylation status [99], advanced male age [100], and sperm cryo-damage [101].

Troubleshooting of Human Sperm Flow Cytometry

Contrary to most mammals, human semen contains abundant cellular debris that requires accurate gating of spermatozoa during FC. Approximate separation can be achieved by gating off the sperm-sized flow cytometric events in scatter diagram (see Fig. 4.1). Such gating will exclude large cells such as leukocytes and small FC events such as cellular debris and contaminants that may be present in reagents used

for sperm labeling. However, at the same time, abnormally large spermatozoa could be excluded along with leukocytes and sperm fragments which are also informative of sperm quality and may carry the assessed biomarker molecule. Furthermore, anucleate semen contaminants such as residual bodies could be inadvertently included in sperm analysis if they are sized similarly to spermatozoa. Consequently, a more reliable method is counterstaining of the whole sample with a fluorescent DNA probe such as propidium iodide (PI) for fixed spermatozoa or Hoechst 33342 for non-fixed samples, which reliably distinguishes between spermatozoa and somatic cells based on stoichiometry of DNA content and probe fluorescence intensity, and can be combined both with antibody/lectin labeling and with some of the vital fluorescent probes. The extrapolation between sperm phenotype and biomarker quantity is challenging in conventional FC but easily more addressed by using the ImageStream instrument which combines the high-throughput and fluorometric capabilities of a flow cytometer with multichannel imaging capability of an epifluorescence microscope. ImageStream instrument thus eliminates extrapolation between microscope and cytometer and allows for direct, simultaneous evaluation of individual spermatozoons' morphology and biomarker fluorescence intensity and localization/distribution pattern [8, 55].

Sample processing quality control is important for all fluorescent probes but particularly important for antibodies. Antibodies selected for immunolabeling of spermatozoa should be carefully validated by Western blotting (WB) for their specificity for the target protein, for their suitability for immunocytochemical procedures, and for their ability to detect the target protein in situ by epifluorescence microscopy as opposed in a denatured electrophoretically resolved sperm protein extract by WB. Batch variability should be considered, particularly for polyclonal antibodies produced by bleeding of immunized animals. Proper titers of secondary antibody conjugates should be determined to minimize background fluorescence. Inclusion of both positive and negative controls is paramount to immunolabeling accuracy. In cases when immunolabeling follows a previously validated, published protocol, care should be taken to source antibodies from the specified manufacturers and to obtain antibodies with catalog numbers identical to the ones published. In many cases, multiple manufacturers offer antibodies of varied quality and specificity. Not adhering to the validated antibody and protocol may produce conflicting results, as discussed for ubiquitin. For any fluorescent probe, quality control of every flow cytometric trial by randomly sampling and examining processed sperm batches under epifluorescence microscope prior to flow cytometric analysis is crucial.

Finally, the analysis of histograms of relative fluorescence is challenging on several levels. While the median fluorescence of the entire sample is calculated by the instrument computer, samples with greatly divergent shapes of the histogram curve may have very similar median fluorescence values (Fig. 4.1). This adds a subjective element to the analysis, while sometimes the diagnosis of male infertility can be obvious based solely on an unusual curve shape. This can be remedied in part by arbitrarily setting markers to divide the histogram into populations with low, moderate/near-median, and high relative fluoresce. Within such markers, median or mean relative fluorescence can be recorded, as well as the percentage of cells/FC events

within the marker area. The subjective, arbitrary aspect of histogram marker setting can be mitigated by cross-referencing histograms of negative control samples as the baseline for subtracting background probe fluorescence and subject cell autofluorescence from biomarker-induced specific fluorescence. Additionally, a sperm sample can be fractionated by gradient or swim-up and the histograms of the respective fractions superimposed onto the histogram of the whole source sample to establish the shape and parameters of the viable sperm subpopulation. Finally, if two fluorescent probes are simultaneously excited and acquired, their relative fluorescence of the respective biomarkers/probes can be rendered in form of a scatter plot (not to be confused with visible light scatter) that can be divided into quartiles or arbitrary fields. Altogether, analytical tools in flow cytometric software provide a variety of means for high precision analysis of FC outputs.

Clinical Summary

There is no doubt that advanced andrological testing, such as that with FC, should be incorporated into the repertoire of male evaluation. The limiting factors of more widespread clinical application of these techniques are obviously the complex nature of the laboratory techniques and the expense of FC equipment. However, commercial availability of related technologies increased over years and in-office procedures are also feasible with simplified kits that do not involve FC. As scientific research proceeds, not surprisingly, it is evident that identification of sperm factors implicating "qualitative" defects carries robust diagnostic potential even in the absence of "quantitative" abnormalities in traditional semen analysis. As such, it seems clear that advanced andrology testing will become more routine, affordable, and available to fertility centers; thus it behooves IVF programs to become familiar with these technologies.

Conclusions and Perspectives

A number of validated, accurate flow cytometric tests are available for unambiguous semen evaluation in clinical andrology laboratories. While tests such as SCSA have predictive value for spontaneous miscarriage, more effort should be invested into studies aimed at developing tests predictive of high pregnancy likelihood after single embryo transfer. In some of our studies, we already noticed that men from couples having twins or triplets after multiple embryo transfer tend to have lover sperm DNA fragmentation levels [5] and lower content of superfluous spermatid-derived carryover proteins such as SPTRX3 [56]. Adding to existing FC tool box, assays will be developed based on biomarker discovery benefiting from the differences between fertile and infertile semen samples' transcriptomes and proteomes [102–104]. At the protein level, focus will likely be on specific sperm sub-proteomes

based on posttranslational modifications of sperm proteins such as the phosphoproteome, glycome/glycoproteome, and ubiquitome [105, 106]. Progress in genomics and epigenetics will make it possible to develop protein biomarkers detecting truncated/dysfunctional protein variants arising from polymorphisms and aberrant methylation of sperm DNA and genes encoding for sperm proteins [99, 107]. Such progress will go hand in hand with the adoption of new FC instrumentation by andrology laboratories, such as dedicated sperm-specific bench-top flow cytometers [31] and flow cytometers with imaging capabilities [55]. Altogether, the improved biomarker-based andrological evaluation will facilitate the implementation of routine single embryo transfer in ART clinics and help management of paternally contributed spontaneous miscarriage.

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Chapter 5
Comparison of Methods for Assessment of Sperm DNA Damage (Fragmentation) and Implications for the Assisted Reproductive Technologies

Preben Christensen and Anders Birck

Introduction

Testing of sperm DNA damage has received an increasing amount of attention during the past couple of decades. There is a strong need for methods which can identify reduced male fertility and help to improve the treatment success rates in fertility clinics. Tests of sperm DNA damage have been developed and applied in clinical practice, and early results were promising [1–3]. However, these results were later challenged when it was observed that sperm DNA damage may be less important for assisted reproductive technologies such as IVF or ICSI [4–6]. To prevent confusion, the term "fragmentation" for this work refers to double-stranded DNA breaks which result in "fragments." All other types of changes including single-stranded breaks which make the DNA vulnerable to further damage are described as "DNA damage."

Several new methods are claimed to detect sperm DNA damage (or apoptosis) and have been made commercially available without sufficient data to demonstrate an association between the parameters measured and reproductive outcomes [7–11]. Despite its promising potential, the field of sperm DNA damage has become a somewhat controversial topic which is difficult to understand for the majority of people working with fertility treatment, and the clinical value of this technology has been questioned due to conflicting results [12–14]. The authors of this chapter are aware that the reader may not find sperm DNA damage particularly interesting or relevant in order to transfer a single embryo successfully. Few topics have been more misunderstood than that of sperm DNA damage, and it is our hope that readers will change their point of view in the course of the next few pages. However, before we can consider which test to use in the fertility clinic, we first need to "take one

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step back" and consider how DNA can become damaged and how the sperm differs from the somatic cells.

When it was discovered in the 1950s that DNA is the macromolecular carrier of essentially all genetic information, it was assumed that DNA is extremely stable. Consequently, it came as something of a surprise to learn that DNA is in fact is relatively unstable compared to most other biomolecules. When DNA damage is severe, the somatic cell is unable to replicate and will eventually die. Cellular DNA is under constant attack due to the presence of oxygen free radicals (oxidation), water (hydrolysis) and from self-generated by-products of metabolism such as the superoxide anion [15–17]. These result in apurinic sites (depurination) or in loss of amino groups at the base residues (deamination). Deamination affects cytosine mainly, creating uracil residues that result in DNA replication errors due to cytosine-to-thymine exchanges. Depurination affects the stability of the DNA backbone and leads to nicks and single-strand overhangs of the DNA fragments. Other modifications of the DNA backbone and base residues may also occur, but normally at lower rates than those of depurination and cytosine deamination. Repair of DNA is essential for the preservation and transmission of genetic information in all life-forms. Survival of the somatic cell depends on an enzymatic system for DNA repair which acts rapidly when damage occurs. In contrast to the somatic cell, the mature sperm lacks an effective means of repairing DNA damage [18–20]. Integrity of the DNA in the mature sperm depends on a tight chromatin condensation and on the formation of stabilizing disulfide cross-links when the sperm passes through the epididymis [21, 22]. The mature sperm is inactive with regard to DNA transcription and RNA translation and has lost most of the cytoplasm, including ribosomes. As a result, it is not capable of any protein synthesis.

Damage to the sperm DNA appears to occur in "two steps" [23]. In the "initial step," the sperm DNA is weakened due to different events occurring during spermatogenesis such as poor compaction of the chromatin, insertion of endogenous strand breaks ("nicks") in the sperm DNA, initiation of apoptosis which remains uncompleted in the mature sperm, or deficient disulfide cross-linking during the passage through the epididymis [24–26]. Knowledge about the causes of initial (primary) damage in sperm DNA is currently not complete, but it is likely to be multifactorial and to include several environmental and chemical factors, as well as general male health issues such as lifestyle and smoking [27–31]. The initial damage to the sperm DNA may only be a small change in structure or a few single-stranded "nicks," but this is very significant with regard to the DNA stability. The nuclear genome in mature sperm is normally quite resistant to oxidative stress, but the initial damage in the testicular sperm makes it vulnerable to secondary DNA damage when the sperm leaves the testicle [32]. As mentioned above, the mature sperm does not have the capacity to repair DNA damage and in addition lacks any antioxidant defense enzymes. The sperm DNA is therefore unprotected against spontaneous degradation [15, 16]. When the sperm becomes motile in the cauda of the epididymis, the internal oxidative stress will increase further as a result of metabolism [23]. The initial damage may only have been a few single-stranded "nicks." However, these make the DNA unstable and during the "journey" to the oocyte secondary

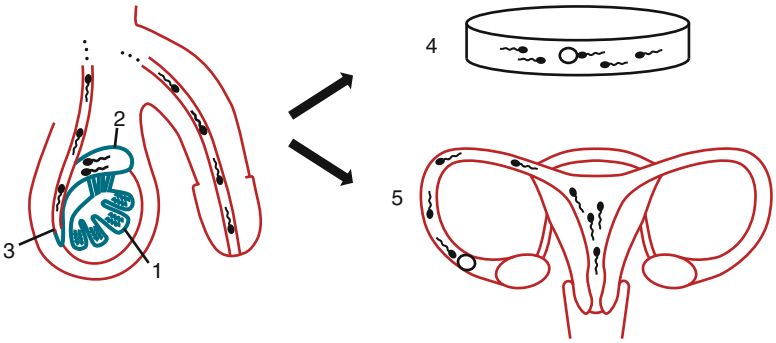

Fig. 5.1 The 'two-step' hypothesis on sperm DNA damage. Primary damage of the sperm DNA occurs in the testicle (1) as a result of uncompleted apoptosis, poor protamination, endogenous 'nicks', or by deficient disulfide cross-linking during the passage of the epididymis (2). The primary damage to the sperm DNA makes it vulnerable to secondary damage as a result of spontaneous degradation by oxygen or water, as well as oxidative stress when the sperm becomes motile (3). Secondary DNA damage may also occur during incubation in the laboratory and processing for ART (4) or during the sperm's 'journey' to the oocyte (5)

damage occurs, and is likely to result in double-stranded DNA fragmentation. Single stranded DNA damage may be repaired by the oocyte, but double-stranded DNA fragmentations are virtually irreparable and are incompatible with normal development of the embryo and fetus [33].

This "two-step" hypothesis of sperm DNA damage can explain some of the apparently conflicting results in the field: The publications by Evenson et al. [1] and Spano et al. [2] were based on results from natural intercourse and the authors assumed that the level of DNA damage would affect IUI, IVF, and ICSI treatments to the same extent [34, 35]. However, this was subsequently found to be incorrect [4, 5, 36]. The reason for this appears to be that the sperm's "journey" to the oocyte is significantly shorter in IVF. As a consequence, the DNA will be damaged less with a lower chance of having double-stranded fragmentation when fertilization is completed. With ICSI, the sperm is injected directly into the oocyte and the complete "journey," including the demanding hyperactivation and penetration of zona pellucida, is bypassed. Based on the "two-step" hypothesis for sperm DNA damage, we should expect a higher treatment success rate for ICSI in comparison to IVF, and that IVF also would be more successful than IUI treatment or natural intercourse. This is in agreement with the observations made by Bungum et al. [6]. An overview of the "two-step" hypothesis for sperm DNA damage is shown in Fig. 5.1.

Another source of confusion in this field is the publication of poor quality papers concerning the impact of sperm DNA damage on fertility or ART outcome. Several papers have been based on too few couples, bias in the selection of couples, or incorrect assumptions regarding the possible effect that sperm DNA damage might have. In comparison to animal studies, it is a much bigger challenge to obtain good fertility data in the human clinic [37]. Evaluation of fertility should only be based on the first treatment cycle to avoid bias from other potential causes of infertility in the man

or the woman. Inclusion of couples with one or several previous, unsuccessful cycles in a study will severely limit the quality of the data obtained. Furthermore, the endpoints studied should be considered carefully. It has been demonstrated that sperm DNA damage may not affect fertilization, cleavage rates, or early embryo quality [38, 39]. Sperm DNA damage may result in poor blastocyst rates, but is more likely to result in poor implantation rates or poor post-implantation development [40, 41]. Sperm DNA damage is also a frequent cause of miscarriage [42–44].

To study the relationship between fertility and sperm DNA damage, we need sensitive, precise, and accurate laboratory testing. The tests available differ with regard to sensitivity and precision, so the relationship to fertility should be evaluated separately for each test and type of fertility treatment. Tests based on microscopy of a few hundred sperm are likely to have low precision and any assessment will also be subjective. In the following pages, we will focus on the Comet, TUNEL, and SCSA tests. The advantages and drawbacks of each test will be described, including clinical studies of the relationship to fertility.

Methods

For a sperm test to be useful, a high degree of precision is necessary. Similar results should be obtained when repeated analyses of the same semen sample are performed [45]. A low degree of precision can be compared to a darts player whose darts are randomly scattered all over the dartboard (Fig. 5.2a). The first step on the road to success is the ability to place all the darts closely together on the dartboard (Fig. 5.2b). This is the equivalent of a sperm test with a high degree of precision. It is pointless to aim for the "bull's-eye" when your precision is poor, and it is equally pointless to try to predict reproductive outcome using a test with low precision. However, unlike the darts player, high precision of our test does not necessarily mean that it also is accurate and that we can hit "bull's-eye" (Fig. 5.2c). Systematic errors with the test may mean that we always are "off target" and that the

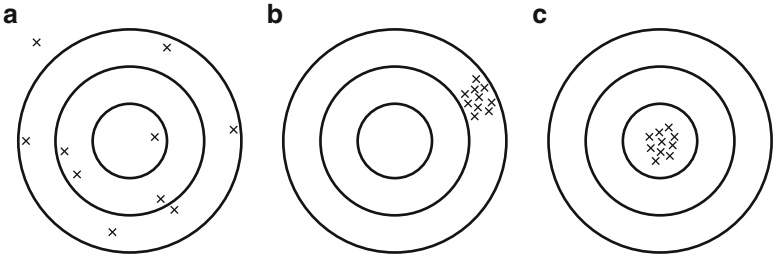

Fig. 5.2 Diagram representing the concepts of precision and accuracy: (a) represents poor precision and accuracy , (b) represents good precision, but poor accuracy, and (c) represents both good precision and accuracy

results do not correlate well with reproductive outcome. Correlation between the test and reproductive outcome will be described in the results section.

A major source of variation in most sperm tests is due to the limited number of sperm assessed [46]. Poor precision in a test is also likely to result in poor accuracy [47]. Any methods based on microscopy will generally have a low degree of precision unless several hundred sperm are analyzed per sample. In addition, microscopic tests tend to be subjective, and when assessing potential sperm DNA damage such tests are not sensitive enough to detect small degrees of change in fluorescence or color of a given dye or probe. In comparison to the electronic detection of fluorescence signals by flow cytometry, the human eye is several hundred times less sensitive. The flow cytometer, in addition to its high sensitivity, enables us to assess several thousand sperm both objectively and rapidly. Tests which do not use flow cytometry should be based on an automated assessment to ensure that a sufficient number of sperm can be analyzed objectively. Regardless of the technology used, two independent replicates should be processed separately and analyzed for each semen sample. Replication is the most essential step in the quality control of semen analysis and enables the technician to assess both errors in the sampling or processing, and technical errors such as the partial blocking of a flow cytometer. The precision of the laboratory test should always be monitored on a day-to-day basis to demonstrate that the results are trustworthy [37].

In the following sections, the protocols for SCSA, TUNEL, and Comet will be described together with the advantages and drawbacks of each method.

Comet Assay

The Comet assay or single-cell gel electrophoresis is a well-established test for genotoxicity and has been used for detection of DNA strand breaks in a broad spectrum of cells [48, 49]. Within an agarose gel, the sperm membranes are lysed and the DNA is decondensed using a high salt concentration. During electrophoresis, DNA fragments are streamed out of the "head" of intact DNA and resemble a comet tail. Before evaluation, slides are stained with a fluorescent dye that binds to the DNA. The Comet assay is known to be a sensitive test which is able to detect small amounts of DNA damage in sperm cells [50]. Another advantage of this assay is that it can be performed on semen samples containing only a few thousand cells.

One of the drawbacks of the Comet assay is that only a small number of cells per sample (100–150) can be scored with semi-automated systems. Fully automated systems allow scoring of 150–300 cells per gel and if six gels are scored per semen sample, the total number of cells may exceed 1,000 cells. The variation for repeated analyses (intra assay) for the Comet assay has been estimated at 3.7 % [51]. The Comet assay is more time-consuming to perform than both TUNEL and SCSA.

There are a variety of different protocols for the Comet assay as it has been adapted for different types of cells. The neutral version detects double-stranded DNA breaks, whereas the alkaline version detects single-stranded DNA breaks.

TUNEL Assay

The TUNEL assay relies on labeling of DNA strand breaks with fluorescent dUTP nucleotides by use of terminal deoxynucleotidyl transferase (TdT) and this method was first used for sperm by Gorczyza et al. [52]. TUNEL is a very popular assay as it targets a definitive endpoint: DNA strand breaks. However, the many different protocols for this assay have resulted in a large degree of variation in the results. The TUNEL can be performed on neat or washed sperm samples, with or without fixation, with or without detergent permeabilization, and with direct or indirect labeling [53]. The protocols usually involve several washing steps and incubation of various lengths, both of which may induce additional (secondary) DNA damage when the sperm samples are not fixed.

TUNEL can be performed using microscopy or flow cytometry. In general, microscopic assessments appear to lead to lower levels of sperm DNA damage [54–56] in comparison to results obtained by flow cytometry [57–60]. A possible explanation for this difference is the lack of sensitivity of microscopic assessments as mentioned above. To ensure accuracy, it is essential that the flow cytometric analysis of TUNEL also includes a dye which makes it possible to distinguish sperm and unstained particles. Otherwise the results of the analysis will underestimate the percentage of sperm with DNA damage [53]. It has recently been demonstrated that the probe used for TUNEL may not be able to access all parts of the sperm DNA and that this can therefore lead to an underestimation of the DNA damage [61]. TUNEL, when analyzed by flow cytometry, is a very precise assay with an intra-assay variation estimated at 3.4 % [62].

SCSA

The SCSA method was developed by Evenson et al. [63]. The principle is based on the denaturation of sperm DNA at low pH, and subsequent staining with acridine orange. Due to the metachromatic nature of this dye, denatured (single-stranded) DNA will emit a red fluorescent signal, whereas intact (double-stranded) DNA will emit a green signal. The method provides an indirect measure of DNA strand breaks since such damage is likely to occur in the areas where DNA can be denatured by low pH.

According to the protocol, analysis is performed by use of flow cytometry using 5,000 sperm per replicate [64]. The method uses neat semen samples (fresh or frozen–thawed) and the preparation is straightforward. The first step is addition of the acid solution, and after 30 s, the acridine orange staining solution is added. Analysis of the sample is performed after a staining period of 2½ min. Correct dilution of the semen sample is important as the acridine orange is an equilibrium dye. This means that binding of the dye to DNA depends on the remaining concentration of dye in the solution. All samples should therefore be diluted to approximately one million sperm/ml

prior to addition of the acid solution. A higher concentration of sperm will result in insufficient staining of the DNA and is likely to affect the outcome of the analysis. Acridine orange is a very sticky dye which adheres to the tubing and other parts of the flow cytometer. For this reason, saturation of the flow system is essential before the first analysis, and cleaning is equally important after completion of the analyses.

The protocol described by Evenson and Jost [64] is not particularly detailed with regard to the need for good quality control or the different factors which may affect the outcome of the analysis [65]. Provided good quality control is ensured, the SCSA is a very repeatable assay with an intra-assay variation below 2 % and a very high correlation between results obtained by different laboratories [66].

Results

Accuracy defines the relationship between the result of a test and the "true" value. Like the darts player, we may have a very precise test but still be "off target" due to low accuracy (Fig. 5.2b). To assess accuracy, we need to study the relationship between the results of our test and reproductive outcome. This means that a large-scale clinical study is necessary. Unfortunately, this is not an easy task when working with human fertility [37]. At first glance, a small study may appear easier to carry out, but it is also more likely to make us confused: the small number of observations will make the outcome of the study as random as "flipping a coin" [67].

In the human clinic, we usually consider couples to be either "fertile" or "infertile" and therefore regard fertility as a binomial variable. Fertility, on the contrary, is a continuous variable. In the context of increased levels of sperm DNA damage, the chances of achieving a successful pregnancy decrease and the time to pregnancy increases. A couple may manage to achieve pregnancy after several months of "trying" and will consequently be classified as fertile. To detect small differences in male fertility, the ideal fertility study should only include females with high fertility and each male should be "tested" on several females [37]. Obviously, this type of study is not possible on humans for ethical and biological reasons. Let us therefore consider a species where such a study is possible.

Boe-Hansen and coworkers have published two papers where DNA damage (assessed with the SCSA) was studied in boar semen and where the impact on fertility was assessed after insemination [68, 69]. In the study from 2005, the authors investigated the effect on sperm DNA when diluted boar semen was stored for up to 72 h at 18 °C. This kind of storage is necessary as boar sperm does not tolerate freezing and thawing at all well. Semen for all commercial insemination in pigs is therefore diluted in an extender with antioxidants and used for up to 3 days after semen collection. An interesting observation in the 2005 study was that a proportion of the stored sperm acquired DNA damage during the incubation (Fig. 5.3). This was a surprising observation as most researchers in 2005 were of the opinion that sperm DNA damage was a stable parameter. We now know that sperm DNA damage is a dynamic process and, according to the "two-step" hypothesis, the change

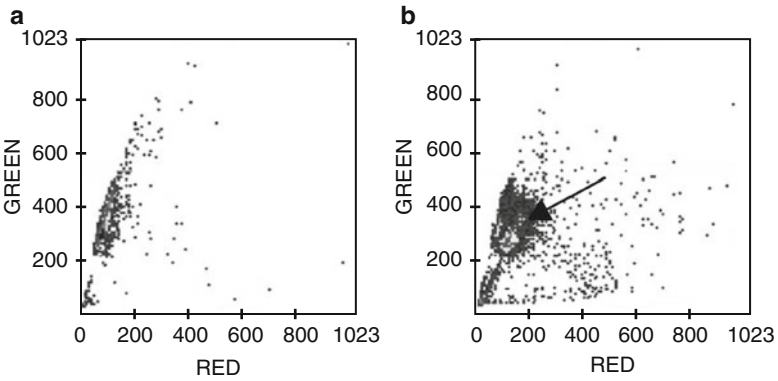

Fig. 5.3 SCSA analysis of two samples of boar semen. Increasing red signal (x-axis) indicates DNA damage and green signal (y-axis) indicates intact DNA. The two cytograms show analysis of 5000 sperm. Semen sample (a) was not stored, whereas semen sample (b) was stored for 72 h at 18˚C. In cytogram (a), 97% of the sperm display a small degree of red fluorescence indicating that the DNA is intact. Increased red fluorescence (displacement to the right) was observed for 3% of the sperm (DFI = 3%). In cytogram (b), a large proportion of the main population is displaced slightly to the right (arrow), indicating that these sperm had acquired DNA damage during incubation. DFI for this sample was 75%

observed in the boar sperm represents secondary damage caused by spontaneous DNA degradation and oxidative stress. The degree of damage acquired by the individual sperm during storage was only very minor, so the initial assumption was that this would not affect fertility. However, the authors performed a clinical study using semen from 145 boars and 3,276 experimental inseminations were performed. Results for the 2,593 litters born were published in the 2008 paper.

Sows are multiparous animals and will normally have 16–18 ovulations occurring within a few hours. When insemination is performed close to ovulation, all oocytes will typically be fertilized [70]. The average number of piglets born per litter in this study was 14.56 when semen was used without storage [69]. Boars in general have extremely good semen quality and 76.6 % of the inseminations were performed with samples where the level of DNA damage (DFI) was below 3 %. A significant effect of the DNA damage was observed for semen samples with a DFI over 3 %, as these litters on average only had 13.90 piglets in comparison to 14.91 piglets/litter when DFI was below 3 % ($P<0.01$). Litters which originated from stored semen samples with a DFI over 20 % only resulted in an average of 7.40 piglets per litter. Expressed as a percentage, the reduction in the number of piglets born was reduced by 6.8 % and 50.4 % when DFI was above 3 % and 20 %, respectively. Results from inseminations of pigs can naturally not be "translated" directly to human IVF. But just imagine how it could impact your delivery rates, if you are using sperm with a DFI of 20 % for IVF, and transferring single embryos!

It is unlikely that we will ever see a human clinical study with several thousand ART treatments, but a simple calculation of the statistical power indicates that we should be very cautious when trying to draw conclusions from a clinical study with

much fewer than 200 couples. A test with fewer than 200 couples would be equal to "flipping a coin" to decide if the sperm DNA test is useful or not. In addition to ensuring a sufficient number of couples for the study, we need to keep in mind that outcome of IVF and ICSI treatments should be assessed separately due to differing amounts of secondary DNA damage. If we want to study the outcome of both IVF and ICSI, we should enroll a minimum of 200 couples for each subgroup. Furthermore, we should only consider the first cycle of treatment to avoid bias from other factors causing reduced fertility in the man or the woman. When we study the effects of sperm DNA damage, a further essential consideration is the endpoints assessed. Several previous studies refer to "fertilization" as the most important endpoint. However, when we want to determine the possible outcome of sperm DNA damage, all the important events will occur after fertilization and will result in reduced delivery rates. Sperm DNA damage is a very likely cause of miscarriage, so this should be among our endpoints as well as an ultrasound scan at 12 weeks of pregnancy and delivery rates [44].

Some of the previous clinical studies for Comet, TUNEL, and SCSA are described below. The results are only described for studies with more than 100 couples and for studies without obvious design deficiencies, errors in the statistical analysis, or a lack of critical endpoints.

Comet

IUI: To our knowledge there are presently no clinical studies describing the relationship between sperm DNA damage as assessed by Comet and the outcome of IUI treatments.

IVF: The relationship between sperm DNA damage assessed by Comet and the outcome of 203 IVF cycles was reported by Simon et al. [51]. The live birth rate was reduced from 26.9 % to 13.1 %, when the level of sperm DNA damage exceeded 50 % ($P<0.01$).

ICSI: Simon et al. [51] also assessed the outcome of 136 ICSI cycles and observed a nonsignificant decline in live birth when the level of sperm DNA damage exceeded 50 % (30.2 % vs. 20.4 %).

TUNEL

The vast majority of studies performed with TUNEL have been based on fewer than 100 couples. Only one study used flow cytometric assessment of TUNEL and included more than 100 couples [71]. A particular problem when reviewing the literature on TUNEL is the many different protocols and different levels of sperm DNA damage (thresholds). TUNEL, as assessed by microscopy, appears to result in lower levels of sperm DNA damage than assessments by flow cytometry [59].

IUI: The relationship between microscopic TUNEL and outcome of IUI was described by Duran et al. [3] who performed a trial with 119 couples and 154 cycles. The trial concluded that no treatments with a level of sperm DNA damage above 12 % led to pregnancy (confirmed biochemically and by ultrasound).

IVF: Frydman et al. [71] assessed sperm DNA damage by TUNEL and flow cytometry in 117 couples. It was observed that more than 35 % of sperm with damaged DNA had a significantly negative effect on implantation rate and the rate of ongoing pregnancies. No effect was observed for fertilization rates, and embryo assessments.

ICSI: Benchaib and coworkers [72] is the only group who has performed a larger study of the relationship between TUNEL and ICSI outcome. TUNEL assessments were performed by microscopy on 218 ICSI cycles. Pregnancy was determined biochemically and by ultrasound after 6 weeks of pregnancy. It was observed that pregnancy was reduced (37.4 % vs. 27.8 %) when the percentage of sperm with DNA damage exceeded 15 %. This difference was only marginally significant ($P>0.05$). However, it was also the group with the highest level of sperm DNA damage that had a significantly higher miscarriage rate than where the level of sperm DNA damage was low (8.8 % vs. 37.5 %, $P<0.05$).

SCSA

The first large-scale study to demonstrate the relationship between sperm DNA damage and the outcome of natural intercourse was published by Evenson and coworkers [1]. In brief, this study showed that time to pregnancy was increased significantly if the DFI value was between 15 and 30 %, and that almost no couples achieved pregnancy with a DFI over 30 %. Additionally, Evenson and coworkers observed that the incidence of miscarriage was higher with increasing DFI. Evenson's results were confirmed by Spano et al. [2], who had followed a group of 215 "first-pregnancy planners" for a period up to 2 years or until they achieved pregnancy. Based on the studies by Evenson et al. [1] and Spano et al. [2], the assumption was made that the threshold for DFI of 30 % would also apply for IUI, IVF, and ICSI treatments. This assumption led to a great deal of controversy and was later shown to be incorrect.

IUI: The relationship between DFI and the outcome of IUI treatments was explored in a study with 387 cycles (first or second treatment cycle, [6]). Of the 66 IUI cycles performed with semen samples where the DFI was above 30 %, only two resulted in a clinical pregnancy (3 % per cycle). One pregnancy led to a miscarriage and delivery rate was therefore only 1.5 % per cycle. IUI treatments performed with semen where DFI was below 30 % resulted in an average delivery rate of 19 %. Results for IUI have since been confirmed by Yang et al. [73] who performed the SCSA test in a study with 482 first or second IUI treatments. A DFI of 25 % was used as threshold. Of the 95 IUIs performed with semen where the DFI was above

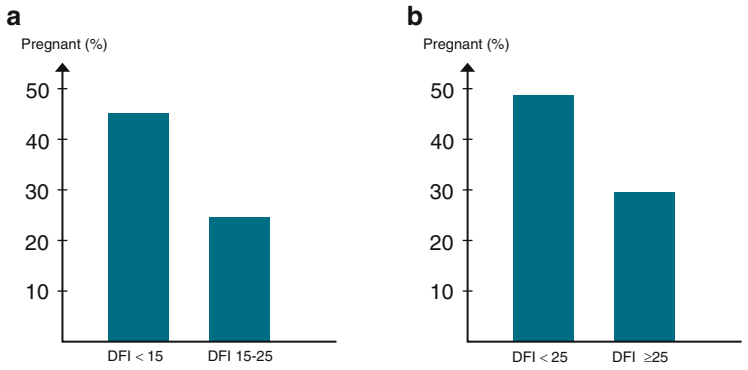

Fig. 5.4 (a) The diagram shows the percentage of ongoing pregnancies after first cycle IVF treatments for 210 couples. Pregnancy was confirmed by ultrasound at 12-week gestation. When DFI was below 15, the pregnancy rate was 45.1%. The pregnancy rate diminished to 24.6% when DFI was between 15 and 25. The odds ratio adjusted for female age, sperm concentration and motility was 2.45 (P=0.01, 95% CI 1.25 to 5.18). (b) This diagram shows the results of 196 first cycle ICSI treatments. When DFI was below 25, the pregnancy rate was 48.6%. Above this threshold, the pregnancy rate was only 29.6%.The odds ratio adjusted for female age, sperm concentration and motility was 1.97 (P<0.05, 95% CI 1.02 to 3.84)

25 %, only 5.25 % achieved a clinical pregnancy. When DFI was below 25 %, the clinical pregnancy rate was 15.25 %.

IVF and ICSI: Bungum et al. [6] also studied the impact of sperm DNA damage on the outcome of IVF ($N=388$) and ICSI treatments ($N=223$). Among IVF and ICSI couples, no statistically significant difference was observed in clinical pregnancy or delivery rates between low and high DFI groups (threshold = 30 %). When the outcome of ICSI versus IVF was compared, no significant difference was observed when DFI was below 30 %. However, if DFI was above 30 %, the results were significantly better for ICSI, with an odds ratio of 2.25 for clinical pregnancy (95 % CI 1.10–4.60), and 2.17 for delivery (95 % CI 1.04–4.51).

A retrospective analysis of the relationship between sperm DNA damage and the outcome of 210 IVF cycles was recently reported by Christensen et al. [74]. The couples were receiving their first IVF treatment and all had a DFI below 25 %. Clinical pregnancy was confirmed by ultrasound in the 12th week of gestation and the outcome was assessed for groups with DFI below or above 15 % (Fig. 5.4a). The clinical pregnancy rate was 45.1 % when DFI was below 15 % and diminished to 24.6 % when DFI was between 15 and 25 %. The odds ratio adjusted for female age, sperm motility, and concentration was 2.45 ($P=0.01$, 95 % CI 1.25–5.18). Christensen et al. [74] also reported results for 196 ICSI cycles. For ICSI cycles, the DFI varied from 2.4 % to 61.2 % and treatment outcome was assessed for groups with DFI below or above 25 %. The clinical pregnancy rate was 48.7 % when DFI was below 25 %. Above this threshold, the clinical pregnancy rate was only 29.6 % (Fig. 5.4b). Odds ratio adjusted for female age, sperm motility, and concentration was 1.97 ($P<0.05$, 95 % CI 1.02–3.84).

Discussion

The results presented above indicate that sperm DNA damage is an important parameter to assess in the fertility clinic. The most significant impact on reproductive outcome occurs after natural intercourse and IUI treatments as a result of secondary sperm DNA damage during the long "journey" to the oocyte [1–3, 6]. In IVF, the sperm suffers less secondary DNA damage as the "journey" is shorter and it is only affected by hyperactivation and penetration of the oocyte investments. However, high levels of sperm DNA damage clearly have a negative effect on the outcome of IVF treatments [51, 71, 74]. For ICSI treatments, only high levels of sperm DNA damage appear to reduce the success rate and studies do not always find any significant effects [51, 72, 74]. Although some studies may be of less significance due to differences in their design, inclusion and exclusion criteria, endpoints assessed, and especially using too few couples, the overall conclusion is that sperm DNA damage appears to be an important parameter.

At present, the literature available does not allow us to draw a conclusion as to which of the three methods: Comet, TUNEL, or SCSA, we should implement in clinics. Important factors for method selection are precision, sensitivity, and accuracy. Precision for each test can be analyzed in the laboratory and should be monitored on a day-to-day basis when the test is being carried out for diagnostic purposes. Flow cytometry is a unique technology enabling us to analyze several thousand sperm rapidly and objectively. When good quality control is ensured, this technology can give us a much higher precision than is possible with conventional methods for sperm assessment, as well as a much closer relationship to fertility [75, 76]. With good quality control, flow cytometric assessment of different sperm parameters will result in a very high degree of agreement between results obtained by different laboratories [66, 77].

Sperm DNA damage is likely to have a significant impact on fertility treatments as this type of damage appears to be a very frequent cause of reduced male fertility. Bungum et al. [6] enrolled couples randomly in the study and found that 20.1 % of the men had a DFI above 30 %. For a large proportion of these men, the classical semen parameters would be considered "normal" according to the WHO criteria [46]. This has been demonstrated by Oleszczuk et al. [78] in a study that investigated 212 randomly selected couples and identified 122 cases without apparent "male" or "female" factor. Among the 122 apparently "normal" men, 17.7 % had a DFI between 20 and 30 % and 8.4 % had a DFI above 30 %.

An important question is what strategy we should choose in the fertility clinic to minimize the likely impact of sperm DNA damage on treatment outcomes? Obviously, the first step would be to select the best embryos for transfer [79, 80]. Embryo selection may lead to higher treatment success rates, especially if we consider transferring blastocysts [38, 40]. However, despite good embryo or blastocyst development we may not be able to identify the embryos or blastocysts which later result in a miscarriage [44]. Another option could be to select the "best" sperm, for instance, by use of IMSI [81]. So far, results of this technology are still controversial

and sperm DNA damage also occurs in morphologically normal sperm [56]. Selection of non-apoptotic sperm by use of annexin V has been recommended as it appears that a relatively large proportion of the sperm may externalize phosphatidylserine, especially after cryopreservation [8, 82]. However, labeling of sperm with annexin V should not be taken as exclusive evidence of apoptosis since such sperm may also externalize phosphatidylserine as a result of capacitation [83]. Sperm with apoptotic markers, such as Fas, will probably make up only a small proportion of the sperm with DNA damage, and the apoptosis is likely to be an uncompleted process which was initiated during spermatogenesis [84, 85]. Several authors use the term "apoptosis" when referring to TUNEL, but this is misleading since different mechanisms may lead to DNA strand breaks.

A more viable approach with regard to "sperm selection" seems to be the use of testicular sperm since such sperm appears to have lower levels of DNA damage [86, 87]. Testicular sperm will not have suffered any secondary damage, but may do so if incubated after retrieval [88]. Selection of testicular sperm may be an option in some cases, but before deciding to carry out this advanced treatment, we should consider how the damaged DNA may affect the offspring and if a less invasive alternative is available. Sperm DNA damage increases as the male becomes older [89]. Increasing male age also appears to contribute to an increased risk of autism, bipolar disorder, and schizophrenia in children, as well as childhood incidences of cancer [90–92]. These diseases, as well as lower facial attractiveness, are believed to be a result of mutations [93]. Accumulation of heritable DNA damage in sperm appears to be caused by initial damage during spermiogenesis and post-testicular secondary damage [20, 23, 69]. In contrast to somatic cells such as lymphocytes or hepatocytes, which after acquiring DNA damage proceed to cell death within 2–3 h, the sperm has no transcription and is likely to remain motile for several hours or days despite DNA damage [68, 69, 94, 95]. When such sperm fertilize the oocyte, they act as a "Trojan horse" containing severely damaged DNA that subsequently may be incorrectly repaired by the oocyte. The most likely result is that the subsequent development of the embryo is compromised leading to implantation failure or miscarriage. However, in some cases the outcome will be a de novo mutation in the newborn. In this context, it appears quite interesting that a recent study has shown that 94 % of all de novo mutations seem to be of male origin [96].

It appears that sperm DNA damage not only leads to reduced success rates in the fertility clinic, but may also result in mutations in the newborn. Rather than moving to a more invasive treatment, we should investigate why the individual male has a high level of DNA damage in his sperm. It is already known that men with poor semen quality are more likely to suffer from a wide range of different diseases, including cancer in later life, and that they therefore have increased mortality rates in comparison to an age-matched control group of fertile men [97]. A recent study from Baumgartner et al. [29] has shown a clear link between DNA damage in sperm and DNA damage in somatic cells which will likely lead to cancer and/or various other diseases. One of the obvious lifestyle factors which may lead to cancer, as well as sperm DNA damage, is smoking [58]. However, smoking and other factors causing DNA damage may affect individuals differently and some male

smokers may not have sperm DNA damage [98]. A possible explanation is that epigenetics may be involved [99, 100]. Nevertheless, if a male smoker also has high level of DNA damage in his sperm, he should consider giving up smoking to improve his fertility. Another obvious cause of cancer as well as sperm DNA damage is lack of antioxidants, which can easily be identified and treated [39, 101]. Diabetes has also been shown to cause increased sperm DNA damage [102]. Several studies have shown that men with an increased BMI are more likely to have a high level of sperm DNA damage [28, 30], and a further study has shown that weight loss is likely to reduce DNA damage [103]. In conclusion, intervention with regard to lifestyle (smoking, weight loss), in combination with appropriate treatment (vitamins and antioxidants), may lead to a reduced level of sperm DNA damage. This less invasive solution may result in increased treatment success rates, reduced risk of de novo mutations in the newborn, and improved male health. At present, there is an obvious need for more research regarding the factors causing sperm DNA damage as well as randomized controlled trials to demonstrate the possible outcome of interventions.

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Conflict of Interest The authors declare no conflict.

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Chapter 6
Single Gamete Insemination Aiming at the Ideal Conceptus

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Background

Infertility is a significant global problem affecting approximately 80 million couples worldwide. As evidenced in a British study, sperm dysfunction was identified as the single most common cause of infertility [1]. Subsequent investigations have confirmed these observations [2] and highlighted dysfunctional cells in men with normal semen parameters and conversely normal sperm function in oligospermic men [3]. Because there is no drug a man can take (or add to his spermatozoa in vitro) to improve fertility, the only option to reproduce is represented by the assisted reproductive technologies (ARTs). Indeed, in a simplistic manner, this can be described by an incremental treatment approach depending on severity of the infertility (i.e., IUI for mild, IVF for moderate, and ICSI for severe sperm dysfunction). Maximizing fertilization efficiency with ICSI is always crucial, but this is especially true when the therapeutic goal is to transfer a single embryo.

Although the diagnostic and predictive value of traditional semen parameters has been debated for over 80 years, the inescapable conclusion remains that its clinical value is limited even with the latest effort of the WHO [4, 5]. Another way to look at this problem is by trying to address it—in fact spermatozoa are capable of revealing their errors/flaws externally [6], which may allow noninvasive sperm selection, e.g., indirect methods to select spermatozoa with lower chromatin damage [7, 8]. However, it remains to be confirmed whether such biomarkers do exist or if the

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heterogeneity of spermatozoa is too great for such selection methods to be applicable.

About 15 % of couples are unable to achieve a pregnancy due to male factor infertility, and ICSI is seemingly the ideal procedure to treat these couples. When a single gamete is being selected for insemination via ICSI, the importance of selecting the ideal spermatozoon is heightened as compared to other ART procedures. ICSI has provided fertilization in the most complex combination of oligo-/astheno-/teratozoospermic cases, and the procedure continues to improve its efficiency with the help of supplemental male infertility screening assays. These assays aim at shedding light on the gamete population of a given individual while tailoring to the patient's specific needs. The technology powering these assays is on the rise, and many tests are being recognized as staples in the male infertility workup. Since ICSI bypasses many of the natural barriers of fertilization, an aggregate of tests must be used in order to provide the information needed to properly treat a couple. Nevertheless, in spite of the ideal spermatozoon, this ambitious quest is particularly challenging if the goal is single embryo transfer, and only nonideal spermatozoa are available [9].

Concern over the use of "poorer-quality" sperm cells is warranted, but there is no adequate definition for what is present in a spermatozoon that might adversely impact the conceptus' development. In a normal human ejaculate, there are well over 40 million motile and presumably healthy spermatozoa, but when spermatogenesis falters, a greater percentage of spermatozoa in the ejaculate begin to show a range of abnormalities including membrane, mitochondrial, centriolar, nuclear, and chromosomal disorders [10]. Given that the object is to isolate live spermatozoa, the approaches used have been based on looking at morphology or a membrane property. A major approach in the discovery of sperm biochemical tags, independently from sperm concentration and motility in the semen, is based on the recognition of objective markers of sperm function that focus on abnormal elements occurring during the process of spermatogenesis and spermiogenesis that allow a spermatogonium to become a spermatozoon [8]. The basis of this approach relies on the concept that if an ejaculated spermatozoon has cleared spermatogenesis retaining the correct membrane properties and/or morphology, then it is most likely normal. ICSI is evolving into the most popular choice of insemination for even the simple, routine cases, and a standard semen analysis is becoming inadequate, thus welcoming the implementation of sophisticated assays (e.g., aneuploidy assessment, TUNEL, GM_1, PLCζ, centrosome, transmission electron microscopy, hyaluronan binding, MSOME) to measure the impact the spermatozoa indisputably contribute. With all of these tools and information at his/her disposal, the reproductive specialists treating patients undergoing ART procedures can then aim at providing the ideal conceptus to each couple [11].

The Spermatozoon

The sperm DNA is the instruction manual for the paternal genetic code that, when correctly fused with that of the oocytes, gives rise to the embryonic genome and makes an important contribution to proper embryo and fetal development. Once

the DNA has been introduced into the oocyte by the fertilizing spermatozoon, the highly packed sperm chromatin must be decondensed and the protamines substituted by histones. This requires the reduction of disulfide bonds by glutathione that cross-link the sperm nucleic acid to allow pronuclear formation. At that point, the spermatozoon must deploy its proximal centriole into the oocyte in order for pronuclear alignment and mitotic spindle formation to take place after fertilization [12].

In order for the embryonic genome to be read and expressed, the presence of oocyte-activating factor (OAF) is required. This essential factor is carried by the fertilizing spermatozoon that carries beneath its nuclear membrane. OAF is characterized by phospholipase C_{zeta} (PLCζ), which induces calcium release from intracellular stores. This results in activation of an inositol phosphate- and diacyl glycerol (DAG)-mediated signal transduction mechanism, followed by protein phosphorylation, expression of specific genes, and activation of the conceptus' genome capable of setting in motion the embryonic development [13].

The spermatozoon is a highly specialized cell designed to transport DNA from the male partner into the oocyte. The function of the complex self-propelling mechanism that the axoneme microtubules of the sperm flagellum represent and the biochemical drill that the sperm's acrosome constitutes have been developed and perfected through millions of years to achieve the sperm's primary function of introducing new DNA within the oocyte [10]. However, a movement of spermatozoa from the seminiferous tubule to the ampullary region of the oviduct entails a significant risk for sperm nucleic acid damage. To obviate this risk, the need for the replacement of histones with protamines extensively cross-linked with disulfide bonds provides a kind of seal to protect the sperm DNA against potential damage during its journey to the oviduct [8].

Choosing the Spermatozoon

To facilitate single embryo transfer, it is critical to optimize fertilization. In utilizing established and experimental assays to help predict and improve a given ART cycle, we can begin by evaluating the ejaculate that should present unparalleled predictive value. Although the role of spermatozoa has been described as just a "delivery device" for the male genome into the oocyte for fertilization, closer study shows that there is much more here than meets the eye.

Different mechanisms can induce abnormalities in spermatozoa either during their production or transport including apoptosis or anomalies during the process of spermatogenesis, DNA strand breaks produced during the remodeling of sperm chromatin during the process of spermiogenesis, post-testicular DNA fragmentation induced mainly by oxygen radicals during sperm transport through the seminiferous tubules and the epididymis, DNA fragmentation induced by endogenous caspases and endonucleases, DNA damage induced by radio and chemotherapy, and DNA damage induced by environmental toxins [14].

One area of sperm structure that has generated increasing interest in sperm assessment is related to sperm nuclear DNA/chromatin structure [15]. In addition to the original methodology, numerous tests have now been devised for the analysis of sperm nuclear DNA fragmentation [14]. These tests include TdT-mediated dUTP nick end labeling (TUNEL) [16], Comet [17], chromomycin A3 (CMA3) [18], in situ nick translation [19, 20], DNA breakage detection fluorescence in situ hybridization [21, 22], sperm chromatin dispersion (SCD) test [23], and sperm chromatin structure assay (SCSA) [24, 25].

The selection of the best male gamete starts with a good screening of the male partner. This begins with a good medical history to identify infertility traits in the family as well as executing a series of test that begins with a semen analysis but extends to peripheral karyotype, Y-microdeletion assay, and CF carrier status when deemed appropriate [11].

With regard to the semen analysis, although the most recent guidelines are quite stringent, our center carries out a sperm selection assay (using at least one aliquot of the specimen) to test the ability to adequately enrich the motile portion of spermatozoa. While this test measures progressive sperm motility, it also indirectly assesses membrane resilience in terms of how it will respond to the additional centrifugations and associated sperm attrition secondary to the interaction with the silica gel column when density gradients are used [26–28]. The appearance of round cells, the color of the ejaculate, and the presence of bacteria or immature germ cells can provide insights of spermiogenic dysfunction [29]. Even the presence of fructose or the level of total antioxidant capacity (TAC) can provide information on the seminal vesicles' health and potential distal obstruction [30–32].

When no spermatozoa are seen in the ejaculate, the specimen is spun to 3,000×*g* to identify cryptozoospermic cells. Should this tactic fail to yield sperm cells, then a microscopic search in microdrops under oil loaded on an ICSI dish is performed [33, 34]. The information gained will allow the reproductive physician to discuss with the male patient escalating diagnostic and therapeutic options, including a reproductive urology consultation to evaluate the condition of the genital tract and eventual surgical sampling [35, 36].

Additional sperm testing may be appropriate in certain settings, according to the specific reproductive history of the couple such as recurrent first trimester miscarriage, which entails aneuploidy assessment by FISH. This assay appears relevant also in screening infertile men with advanced age [14, 37, 38]. A history of poor embryo cleavage or unexplained habitual implantation failure with ART may also prompt the screening for sperm chromatin integrity [39, 40].

If there is a history of compromised early embryo cleavage or a mosaic karyotype of the conceptus, this invites assessment of the sperm centrosome [41, 42]. Finally, in couples with recurrent and complete absence of oocyte activation, the assessment of the PLCζ may become helpful in diagnosing these rare cases [13, 43, 44].

General sperm enrichment procedures are valuable in obtaining spermatozoa with the lowest DFI, along with the highest viability, motility, and fertilizing potential. These tests, however, are carried out on the specimen in toto and therefore can provide only extrapolated information on the characteristics of the spe-

cific cell chosen for injection. Attempts have been made to interpret morphometric measurements that range from recognition of sperm head surface irregularity [45–47], evaluation of the trajectory motion patterns achieved by computerized analysis [48], to the electrophoretic selection of spermatozoa with putatively intact chromatin [49], as well as the use of annexin V magnetic columns to separate apoptotic cells [50, 51].

The expression of hyaluronan (HA) antigens in a specially prepared ICSI dish [52] may help isolate cells that reach chromatin maturity. In addition, cell sorting of spermatozoa with putatively intact chromatin but nevertheless exposed to non-physiologic fluorochromes [53] has also been used to select sperm cells with healthy DNA. While these are obviously welcomed and laudable attempts aimed at reliably identifying surface biomarkers supposedly capable of providing indirect information on the health and function of the cell, these techniques remain unproven. In fact, these procedures merely interpret sperm head irregularities [54, 55], birefringency [56], or HA expression [57, 58]. At best, they are unproven and inconsistent in their capacity to portray sperm chromatin condition, ploidy status, or spermiogenic maturity due to the innate intra-specimen variability of the human ejaculate.

The Less Than Ideal Spermatozoon

While these highly desired and righteous attempts to select and "name" the ideal spermatozoon are creditable and fill the current literature on ART, they are being tested and debated since this attitude does not address the issue raised by situations where an extremely scarce number of spermatozoa are identifiable in the ejaculate and, most importantly, when the spermatozoa are of extremely poor morphology or even immotile.

From a purist's point of view, a commendable approach would be to suggest to the couple to avoid using the ejaculated specimen and opt for a testicular biopsy or to use donor spermatozoa or even child adoption. Acceptance of donor spermatozoa has declined as couples have become more aware of the possibilities of modern ART.

In tune with patient autonomy, it is our intent here to share our experience and illustrate some realistic expectations attainable when utilizing these less-than-desirable male gametes, whether obtained from an ejaculate or in a surgically retrieved sample.

In our daily practice, our first step would be to test a sperm for viability or to carry out a pentoxifylline assessment to see if any sign of kinetic characteristics can be elicited. If the sperm cells are abundant and there is no spermatozoon with restorable motility, the information on viability is relevant in differentiating between complete asthenospermia, e.g., as in coiled tails vs. necrozoospermia. A paradoxical situation occurs when abundant spermatozoa with vigorous motility are present in the ejaculate, but with a nucleus showing compromised morphology manifesting as

globozoospermia (genes SPATA16, DPY19L2, PICK1). Likewise, extremely amorphous heads (gene AURKc) indicate profound spermiogenic abnormalities and may require TEM or even PLCζ assessment for substantiation.

A more common and more difficult clinical circumstance is when few, motile spermatozoa with somewhat satisfactory morphology are available. At our center from 1993 to 2013, we have treated severe oligospermic men (defined as sperm concentration $\leq 1 \times 10^6$/ml) in 1,660 cycles that underwent ICSI treatment; the average concentration was $\leq 1.3 \pm 0.3 \times 10^6$/ml, with a motility of 20.6 ± 22 %, and normal morphology of only 1.0 ± 2 %. The fertilization in this group was 64.4 % (10,131/15,738) that resulted in an acceptable clinical pregnancy rate of 50.7 % (842/1,660).

When the intent of the couple to use their own gametes is resolute, then the option is to attempt the ART cycle with ejaculated spermatozoa possibly supported by some trials of specimen cryopreservation to safeguard the couple from rare but feasible occurrences of azoospermia at the time of egg retrieval. When initial specimen examination in the Makler chamber yields no spermatozoa, a high-speed centrifugation is often able to find scarce cells. In 244 cycles, after such high-speed centrifugation, samples with a mean density of $0.42 \pm 1.5 \times 10^6$/ml and motility of 35.9 ± 32 % were recovered at this institution. In this cohort, we obtained a fertilization of 60.3 % (1,500/2,488) and then replacement of an average of 2.6 embryos per patient, resulting in a satisfactory intrauterine gestation rate of 48.4 % (118/224).

After scrutinizing our overall data, we wondered just how poor can a sperm sample actually be and still provide a pregnancy. For a proxy marker for general sample quality, we decided to utilize the time spent in searching ejaculated specimens ($n = 2{,}197$) to retrieve injectable spermatozoa. In a retrospective cohort analysis, ICSI outcomes were reviewed as a function of the length of microscopic sperm search carried out in relation to increasing search time (30–60, 61–120, 121–180, and ≥181 min) and compared to control cases having abundant spermatozoa and relatively brief search intervals (<30 min).

In this analysis, we obviously had many cycles in the control group (see Table 6.1), while the study groups progressively lessened in size with longer search times. The concentration of spermatozoa in specimens allotted to the increasing search time consequently decreased; for consistency, we reported the concentration as thousand per milliliter (10^3) such that the control had 58 million, the 30–60 min had 3–4 million, the 1–2 h had 15,000 sperm cells, the 2–3 h had 6 spermatozoa, and

Table 6.1 Gamete characteristics according to length of sperm search

		Extended sperm search of ejaculates (min)			
	Control	30–60	61–120	121–180	>180
Cycles	3,559	55	27	5	2
[ ] × 10^3/ml	58,892	3,986	15.2	0.006	0.003
Sperm seen (range)	1–729	1–87	1–53	1–25	1–3
Motile sperm (range)	1–68	0–17	0–5	0–2	0–1
MII injected	31,156	719	350	62	18

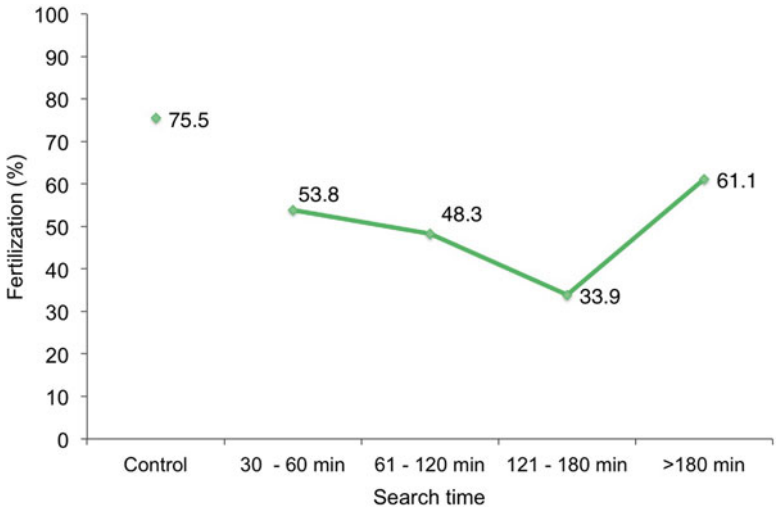

Fig. 6.1 Fertilization rate of ejaculated specimens as a function of time spent searching for an injectable spermatozoon

>3 had 3 sperm cells only. Thus, the average sperm cell count observed in this group ranged from >700 spermatozoa and went all the way down to only 3 cells. The number of motile spermatozoa also decreased in function of time. When we looked at fertilization, the control ejaculate was at 76 %, followed by 54 % for the 60 min lot, 48 for those that reached the 2 h mark, 34 by the 3 h mark, and, interestingly, 61 % for the greater than 3 h group (see Fig. 6.1). Regardless of search time, the large majority of women had embryos replaced with an average of 2.3 in the control, 2.9 in the 60 min cohort, 1.7 in the 120 min, 2 in the 180 min, and greater than 3 h study groups. To make comparisons on embryo development more consistent, since the proportion of day 5 transfer was only 9 % in the ejaculated sperm group, all embryos transferred were assessed on the afternoon of day 3. Therefore, embryo quality was defined as the average number of blastomeres on day 3 along with its fragmentation rate. For the control, the mean number of blastomeres was 7.5 with a fragmentation of 6.6 %. Of note, as the search time lengthened, the mean number of blastomeres was consistent between seven and eight blastomeres and relatively low levels of fragmentation (see Table 6.2). When clinical outcome was assessed, in the ejaculated group, the ability to achieve a clinical pregnancy was somewhat inconsistent but satisfactory particularly once the search time increased, ranging from 33 % to as high as 50 %. A similar pattern is observed with embryo implantation (see Fig. 6.2a, b).

In case of azoospermia or complete asthenospermia at the time of egg retrieval, if the patient is willing to consider the surgical risks and obvious discomfort related to surgery, then testicular sampling can be considered for retrieving spermatozoa.

Table 6.2 Embryo characteristics according to length of sperm search

	Control	Extended sperm search of ejaculates (min)			
		30–60	61–120	121–180	>180
Cycles	3,559	55	27	5	2
Replacements	3,162 (88.8)	51 (92.7)	23 (85.2)	4 (80.0)	2 (100)
Embryos transferred	7,269	51	40	8	4
Mean embryos transferred	2.3	2.9	1.7	2.0	2.0
D3 mean blastomeres	7.5±1	7.3±1	7.8±1	8.3±1	9.0±0
Fragmentation rate	6.6±3	7.9±3	6.4±3	5.2±2	3.7±3

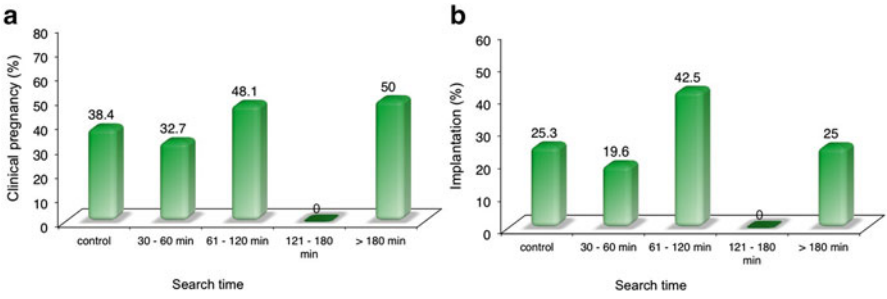

Fig. 6.2 Clinical pregnancy (**a**) and implantation (**b**) rate, according to extended sperm search time

Due to the limited number of nonideal spermatozoa identified within ejaculates, we wondered about the genomic integrity and competence of these cells. DNA damage can either be single-stranded nicks or double-stranded breaks. DNA strand breakage can occur either by free radical attack generated by the metabolic process of the spermatozoa or contaminating cells. It can also be that the spermatozoon's chromatin was not properly compacted, thus making it more susceptible to oxidative stress and enzymatic cleavage by endonucleases [59].

From this, we wondered where does the actual DNA damage occur? Was chromatin adversely affected during spermiogenesis in the seminiferous tubules, or could it be some epididymal malfunction that allowed these chromatin-damaged spermatozoa to spill into the ejaculate? Alternatively, was sperm nuclear damage caused by reactive oxygen species in the male genital tract post-spermiogenesis?

The nature of chromatin compaction in the sperm nucleus has been thoroughly described [4, 60]. In brief, sperm DNA is compacted into doughnut-shaped protamine-rich toroids that contain the DNA in a semicrystalline state [8, 10]. Interspersed between the toroids are histone-rich linker regions of DNA, required for rapid access to facilitate transcription once the spermatozoon is inside the oocyte. Sperm DNA fragmentation can be assessed by SCSA (the gold standard), sperm dispersion assay (SCD), TUNEL, and Comet.

To quickly assess if the sperm DNA fragmentation occurred at the level of sperm production on the germinal epithelium or during its station in the epididymis, we thought to assess the men's abstinence period. Careful review of records where males had abnormal DFI revealed that the longer the abstinence period, the higher the sperm DFI ($p<0.05$). Moreover, when dynamic parameters were assessed, we observed an inverse relationship between motility and chromatin fragmentation: as motility declined, DNA fragmentation progressively increased—a finding confirmed on all assays [8].

We then looked at a special group of patients where we concurrently assessed the DFI at different levels: at the ejaculate, the epididymis, and within the seminiferous tubule. In 12 men with recurrent ICSI failure (mean = 3.5 cycles/patient), their ejaculated spermatozoa yielded a fragmentation rate of 54.8 ± 29 % (up to 96 %). Here, we saw that the incidence of DNA fragmentation was remarkably higher in the ejaculate, somewhat lower in the epididymal sperm, and more drastically reduced when sperm were retrieved via testis biopsy ($p=0.007$) (see Fig. 6.3). These were non-azoospermic men with high DFI in their ejaculated sperm (ranging from 19 to 96 %); after thorough counseling, they opted to undergo testicular biopsy.

On the basis of this observation and on some published reports [61–63], we were able to compare ejaculated ($n=28$) and TESE sperm ($n=13$) cycles. While fertilization was higher with ejaculated (60.0 %) than testicular (46.9 %; $p<0.01$), the embryo cleavage rate was comparable. Following the replacement of an average of 2.8 embryos, the clinical pregnancy was 10.7 % (3/28) for the ejaculate sperm group and 30.8 % (4/13) for the TESE [61].

In a subset of individuals ($n=8$), whose semen characteristics were given in Table 6.3, a paired analysis was carried out with the ejaculated cycle closest in time to the TESE cycle. The fertilization rate was comparable between the ejaculated

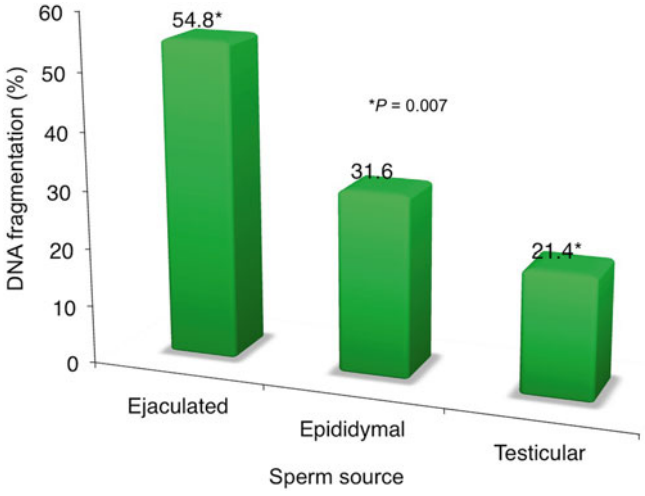

Fig. 6.3 Sperm DNA fragmentation classified by sperm source

Table 6.3 Semen characteristic of men who had DNA fragmentation assessment on both ejaculated and surgically retrieved specimens

	Ejaculated	Surgically retrieved
Men	8	
Male age	41.1 ± 5	
Concentration	12.8 ± 19	0.41 ± 0.50
Motility (%)	29.0 ± 30	4.3 ± 2

sperm cycles and the surgically retrieved sperm cycles at 55.9 % and 50 %, respectively. The embryo cleavage was lower in the ejaculate at 63.6 % resulting in a pregnancy rate of 12.5 %, while in the TESE cohort all embryos cleaved resulting in a 25.0 % clinical pregnancy rate. This study argues in favor of utilizing a testicular sperm sample when a higher DFI is measured in the ejaculated specimen.

It appears that sperm DNA fragmentation in these infertile men is most likely caused by a post-spermatogenic insult, as this is confirmed by the direct relationship between the increased DFI and the lengthening of the abstinence period. Although patients should be counseled about attendant surgical risks, potential anesthesia complications, and the possibility that even with TESE sperm may not be recovered and pregnancy may not occur, the initial results are encouraging. This is a clear indication that damage to the sperm nucleus occurs post-spermiogenesis and is obviated by utilization of sperm obtained more proximally. From these data, we can conclude that in couples with recurrent pregnancy failures with ejaculated specimen evidencing high sperm DNA fragmentation, the option to undergo testicular biopsy should be offered after appropriate counseling.

Future Approaches

A number of new technologies are undergoing refinement to allow better sperm selection to be used for standard in vitro insemination and ICSI. Epigenetic techniques (i.e., characterization of sperm DNA methylation patterns in developmental genes) have been shown to influence embryonic growth competence [64]. Interesting findings are emerging from proteomics that also show promise in assisting sperm selection [65–67]. Likewise, mRNA assessment in mature human sperm cells [68, 69] may be used as a transcriptomic screening technique particularly for idiopathic forms of male infertility [70, 71]. The application of in vitro metabolomics to identify genetically compromised cells [72] (or used to profile blood samples) could also serve as "fertility indicator" [73].

GM_1 (Monosialotetrahexosylganglioside)

At present, we are testing a method to measure the intrinsic ability of the male gamete to exert its fertilization task as a way to facilitate single embryo transfer. This approach is based on the physiologic fact that ejaculated spermatozoa are not

immediately able to fertilize an egg. Rather, they must undergo a process of functional maturation known as "capacitation." Currently, there are no sensitive and simple markers for capacitation that can be used in a clinical setting. A close approximation of such a test might be based on protein tyrosine phosphorylation events during capacitation, as this technique has been described in sperm from other species [71, 74–77]. However, the necessary polyacrylamide gel electrophoresis and immunoblotting processes may require ~48 h to complete, making it poorly suited for clinical purposes [75, 78].

Redistribution of GM_1 ganglioside in sperm induced to undergo capacitation can be used as a method for both diagnostic and predictive purposes, to assess sperm reproductive fitness. The redistribution of GM_1 during capacitation in distinct patterns has been seen in all mammalian species examined, including the bull, boar, stallion, and human [76, 79]. This assay can assess functional activity of spermatozoa before embarking on ART, to determine which insemination method is appropriate (conventional IVF vs. ICSI) [80, 81].

3D Video Imaging

As with other dynamic time-sensitive morphometric techniques to identify the ideal embryo that will most likely develop to blastocyst [82], we have adapted genetic assessment and molecular markers to screen the proportion of competent spermatozoa. Noninvasive techniques under development (i.e., 3D video imaging technology) aim to link selected bio-morphometric sperm parameters with the 360° shape of each sperm cell and its inner chromatin structure. The future holds continued application of such methods, corroborated by TEM, SEM, cytogenetic chromosomal mapping, and eventually proteomics [83].

Conclusions

Against the background of aspiring to move toward single embryo transfer (where preimplantation diagnosis mandates ICSI to avoid polyspermy), we unfortunately have no real knowledge on the long-term effect of utilizing suboptimal gametes of men with severe male factor on ICSI offspring. The contribution of the paternal genome to the development of the conceptus is definitively creating more awareness and receiving more scrutiny. As novel sperm selection methods continue to emerge, most will offer only aggregate data and do not allow selection of individual sperm cells. High microscopy magnification or disaccharide polymer markers are currently to evaluate single sperm cells, but these methods await verification. Increasing emphasis on single embryo transfer has necessarily led to a corresponding heightened interest in sperm, thus stimulating the quest for new tools to more accurately diagnose and select individual spermatozoa prior to direct injection. This will allow us to counsel and treat couples with greater confidence and efficacy and at the same

time assuage the passing onto the progeny any nonideal paternal genetic condition when treating IVF couples—one embryo at a time. What is certain is that the identification of the highest-quality sperm to be used in ART will continue to have a direct and welcome effect as a catalyst, promoting further research in this area.

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Conflict of Interest The authors disclose no conflict.

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Chapter 7
Comprehensive Chromosomal Screening from Polar Body Biopsy to Blastocyst Trophectoderm Sampling: Evidences and Considerations

Antonio Capalbo, Danilo Cimadomo, Laura Rienzi, and Filippo Maria Ubaldi

Introduction

Aneuploidies represent a major barrier for human reproduction, in particular throughout the preimplantation development window when they reach their highest incidence and can affect any chromosome of the karyotype. It is well known that, while in newborn population their incidence is relatively low (approximately 0.3 %) and mainly due to trisomies for the chromosomes 13, 18, and 21 and sex chromosomes' copy number variation, aneuploidies are responsible for more than 45 % of all spontaneous abortions [1]. When looking at the preimplantation window where no selection mechanisms against the development of chromosomally abnormal embryos are in place, the most recent evidences suggested that the incidence of aneuploidies reaches its highest values. A natural selection against aneuploid embryos from the preimplantation period onward prevents them from resulting in a live birth. These evidences highlighted how most of the couples attending an IVF treatment are subject to a significantly high risk of transferring chromosomally abnormal embryos and that aneuploidies can reasonably be considered as the single

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most important factor associated with implantation failure and miscarriage during IVF treatments. PGS theory was conceived in this scenario with the ultimate aim of selecting euploid embryos for transfer.

In this context, the main goal of an ideal PGS strategy should be to obtain the same efficacy as conventional IVF, namely the same live birth rate per cycle, while significantly increasing the overall efficiency on an IVF treatment, that is, minimizing related efforts and risks. When an effective PGS strategy is implemented in IVF programs, then many advantages can be expected, ranging from increased sustained implantation rate, because euploid embryos are supposed to implant at a higher rate compared to chromosomally abnormal embryos, and a significant decrease in abortion rate and in the occurrence of abnormal pregnancies. Importantly, the implementation of PGS might lead to adopt a single ET policy also in poor prognosis patient avoiding any kind of obstetrical and neonatal complication associated with multiple pregnancies. Furthermore, it is expected that in PGS programs a lower time to pregnancy can be obtained, since non-useful and potentially detrimental ETs will be avoided. However, it is evident that from the time PGS was theorized in the early 1990s, throughout years, several issues have arisen and have been solved in a progressive evolution of the technique. A fruitful cooperation between embryologists and molecular biologists has represented an important breakthrough, which increased our knowledge of this field of science. Across years different molecular diagnostic techniques, such as several stages of embryo preimplantation development to retrieve the cellular material to be tested, have been investigated. In order to identify a gold-standard approach, all the proposed ones have been thoroughly studied and some of their advantages and/or disadvantages have been described. In particular, the initial gold-standard protocol for PGS clinical application entailed blastomere biopsy at the cleavage stage, namely on day 3 of embryo development, and its analysis by 9-chromosome FISH.

Unfortunately, PGS failed to keep its promises by adopting this approach. In fact, Mastenbroek and colleagues [2] performed a comprehensive meta-analysis of the main nine RCTs produced in order to investigate the clinical effectiveness of PGS and demonstrated that, especially for advanced maternal age (AMA) patients, which theoretically should be the ones benefiting the most from the diagnosis, this technique actually lowered the live birth rate per stimulation cycle. This evidence held the attention on the inefficacy of such an intriguing theory. Supporters of PGS worldwide started to investigate then the causes of such a failure, and concerns were attributed mainly to technical aspects of the procedure. The potential harm to the embryo deriving from the biopsy itself, the biological and genetic features of cleavage stage embryos, and the remarkable limitations of FISH as molecular diagnostic technique especially when applied on single cells were all considered alarming issues. Thus, they started a pursuit toward different stages of preimplantation development to retrieve the biopsy material, such as the first and the second PBs from the oocyte or few cells from the TE at the blastocyst stage. Furthermore, new CCS techniques replaced the limited 9-chromosome FISH, thus extending the possibilities of diagnosis to the whole karyotype. This chapter aims at providing a comprehensive review of the literature focused on these issues, which has been produced in the last years.

Blastomere Biopsy at the Cleavage Stage

Single blastomere analysis is affected by all the concerns related with single-cell diagnostics. From a technical perspective, several artifacts compromising the reliability of the diagnosis can be introduced, thus potentially causing false-positive and false-negative results. In particular, these artifacts can turn out in erroneous copy number assessments, since few loci or whole chromosomes could be under- or overamplified [3] and can be listed as follows: (1) allele drop-out (ADO), namely random loss of alleles; (2) preferential allocation (PA), namely over-amplification of specific genomic region or even a whole chromosome; (3) allele drop-in (ADI), which is an artifact of whole genome amplification substituting an allele with another one; (4) chimerical DNA molecules formation; and (5) failure of DNA amplification occurring more often. Furthermore, none of the contemporary methods for single-cell analysis can distinguish between a cell in G1-, S-, or G2/M-phase of the cell cycle. This can inevitably determine biological false-negative/positive results, in case a cell would be at a specific point of the S-phase of the cell cycle, thus normally replicating the DNA, when it is retrieved for the analysis [4].

Another biological concern acquiring a paramount importance when conducting PGS on a single blastomere at the cleavage stage resides in the phenomenon of chromosomal mosaicism, namely the coexistence of two or more karyotypically different cell lines in the same embryo. Mitotic chromosome errors are responsible for this phenomenon and could be induced mainly by three mechanisms: anaphase lagging, non-disjunction, and structural events of DNA damage of chromatid/chromosome breakage leading to structural rearrangements (e.g., duplications, translocations) [5, 6]. An impressive influence of mosaicism up to 70 % in preimplantation embryos has been reported in some previous studies [7–10]. However, technical variation due to the reasons previously examined in this paragraph could have determined an overestimation of its real incidence. For instance, different papers showed a considerable number of false-positive results when adopting FISH to analyze single blastomere biopsy in comparison with microarray techniques [11, 12], while Mertzanidou and colleagues [13] did not report any meiotic error by analyzing all the blastomeres from 14 normally developing embryos through array Comparative Genomic Hybridization (aCGH). Such a possibility results unlikely and it supplies further evidence that single-cell analysis is not reliable enough, even though CCS by microarray techniques is adopted to perform the diagnosis. Although even if chromosomal mosaicism could potentially affect any stage of embryo preimplantation development and a proper evaluation of its incidence could not be made so far, from a biological perspective it is likely to reach its highest level at the cleavage stage.

In fact, the origin of mosaicism resides in the early mitotic divisions of cleavage stage embryos. In this time period, the cell cycle control is carried out by the maternal transcripts still present in the ooplasm, but some checkpoint mechanisms are missing a proper control until embryonic genome activation [14]. Finally, mosaic euploid embryos are also likely to self-correct by blastocyst stage [15–17], thus leading to an increased risk of false-positive diagnosis by cleavage stage PGS. Chromosome demolition, non-disjunction, or anaphase lag have been proposed as mechanisms to explain

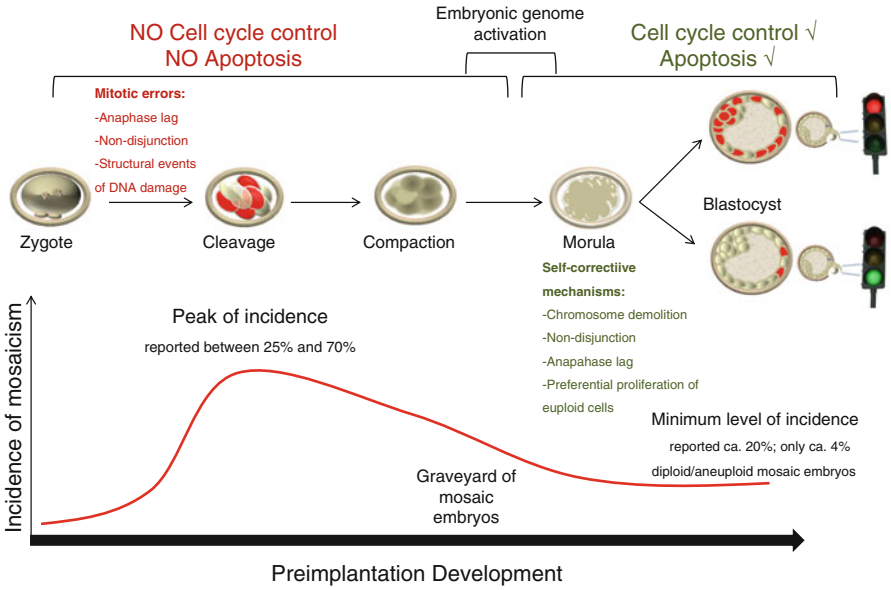


Fig. 7.1 Evolution of chromosomal mosaicism along embryo preimplantation development. Chromosomal mosaicism is likely to reach its highest level along preimplantation development at the cleavage stage. Its origin is thought to mainly reside in the chromosomal segregation errors occurring during the first mitotic divisions of the early embryo, when cell cycle is under the control of maternal transcripts still present in the ooplasm and some checkpoints are missing a proper regulation. Only at the morula stage, after zygote genome activation, these processes will be reactivated and come mosaic embryos will be prevented from reaching to the blastocyst stage. Furthermore, the embryo can undergo events leading self-correction, so that the incidence of mosaicism at the blastocyst stage has been estimated as ~21 %. However, just ~4 % of the blastocyst are subject to a risk of misdiagnosis, since this is the estimated percentage of mosaic diploid/aneuploid embryos at this stage of preimplantation development showing a mosaic error as the only aneuploidy. As reported in different papers, high-grade mosaic blastocysts, where the incidence of aneuploidies exceeds 40 % of the cells, are likely to be diagnosed as aneuploid during blastocyst stage PGS cycle, thus preventing the transfer of embryos that might have a negative clinical impact on pregnancy

this self-correction, and also a better proliferative rate of euploid cells (or apoptosis of aneuploid ones) may explain this phenomenon [3] (Fig. 7.1).

Another consideration when evaluating the effectiveness of a PGS approach is the potential for the harmful effect of the biopsy itself on embryo developmental competence. The only unbiased assessment of this aspect for cleavage stage biopsy was reported by Scott and colleagues [18] in 2013, demonstrating that even a single blastomere removal is sufficient to compromise embryo implantation potential, thus highlighting another noteworthy issue of performing PGS at the cleavage stage. In particular, they ideated an elegant prospective blinded non-selection study. Only double ETs were performed and among the two transferred embryos only one underwent blastomere biopsy before transfer. If a single embryo implanted, DNA fingerprinting was exploited to assess whether it was the biopsied one or not. When comparing the implantation rate of biopsied embryos versus control non-biopsied

ones, a significant 19 % relative decrease in implantation rate due to biopsy procedure was reported. All these evidences taken together strongly suggested that the main limitations of cleavage stage PGS could be ascribed to the technical issues of single-cell analysis and to the detrimental effect of the biopsy procedure. No RCT at the moment has been published to assess the clinical effectiveness of this method when used in conjunction with CCS methods. Thus, despite the highest worldwide experience and despite the fact that it is still the mostly used method for PGS and PGD worldwide [19], cleavage stage biopsy is going to be gradually abandoned to explore new approaches, while FISH-based screening has been already replaced by novel comprehensive methods largely more accurate.

Polar Body Approach

Failure to conceive and pregnancy loss, in both natural conception and IVF, are mainly caused by chromosome aneuploidies, whose occurrence exponentially increases with advancing female age [20]. Molecular analyses performed after natural conception or spontaneous miscarriage highlighted that trisomies arise mainly due to an impaired female meiosis, in particular the first meiotic division [21]. Thus, fertility decrease with increasing maternal age is basically ascribable to aneuploidies' increase due to an oocyte aging issue. This is mainly determined by the long-lasting arrest in the prophase of MI, which ranges from fetal life up to oocyte recruitment for final maturation, occurring between the menarche and the menopause. Unfortunately, despite the unique possibility to perform PGS without directly operating on the embryo, which makes of PB-based PGS the only practice ethically acceptable in some countries, and its compatibility with fresh ET after molecular diagnosis, this approach soon showed important limitations.

One study in particular shed light on the main drawbacks of PBs approach [17]. It was designed as a sequential biopsy associated with aCGH analysis of PBs, blastomere, and TE from the same embryo, which led to an elegant and comprehensive view on chromosomal segregation patterns from female meiosis and throughout preimplantation development up to the blastocysts stage. A unique possibility to infer the etiology of aneuploidies in AMA patient population as well as the accuracy of PB-based chromosome screening in predicting the chromosomal complement of resulting embryos was provided. The results reported in this study firstly confirm the inefficiency of PB1-only approach, because both PBs are needed since approximately half of female-derived aneuploidies in the embryos arose as a consequence of errors originating during the second meiotic division. With the inclusion of the PB2 data, more accurate information inferring oocyte chromosome copy number can be obtained. However, the inability to assess MI errors balanced at MII, the relevant proportion of meiotic errors selected against and corrected during preimplantation development, and the influence of male and mitotic-derived aneuploidies were all important source of errors described in the study and representing inescapable pitfalls of this approach which are sensibly compromising its reliability in predicting embryos' chromosomal complement.

In this scenario, these intrinsic and technical limitations of PB analysis may result in one case in discarding viable embryos, while in the other in transferring abnormal ones. It is also worth highlighting that Handyside and colleagues found a consistent proportion (21.1 %) of chromosome segregation errors detected as copy number changes in the PBs not resulting in the expected outcome in the corresponding zygote [22], which is also similar to what reported by Capalbo and colleagues in the study previously described. Moreover, also in a recent paper published by Christopikou and colleagues, 17 % of false-positive PB results were observed based on the follow-up analysis performed on the resulting embryos [23]. As previously mentioned, one of the major concerns of PB-based PGS relates to the difficulties in detecting precocious sister chromatid errors balancing in MII that was shown to be one of the major mechanisms contributing to female-derived aneuploidies in embryos [17, 22]. In the matter of this, Forman and colleagues demonstrated in a good prognosis patient population that when reciprocal aneuploidy occurs from MI premature separation of sister chromatids and compensation in MII, the resulting embryo is usually normal for that chromosome [24]. The same authors also showed in a different paper that most of these embryos could result in a chromosomally normal child after ET [25]. Thus, future studies are required to assess whether these embryos should be reanalyzed or the signal intensity of the data is reliable enough to distinguish between chromatid and whole chromosome impairments. In particular, the threshold values should be prospectively set by reanalyzing cases in which PB reciprocal aneuploidies occurred and making blinded predictions of the chromosomal status of the embryo.

At present, all the data reported so far in the scientific literature consistently demonstrate a low accuracy of PB approach in predicting the actual copy number configuration in the embryo and that reciprocal aneuploidies in PBs inevitably require a follow-up analysis in the resulting embryo. Therefore, many concerns related to whether the accuracy achievable using PB screening is good enough to improve the IVF clinical outcome still remain [17, 26, 27]. Another major problem related to PB biopsy is the paucity of material that is available. In our hands as well as in the practice of other qualified centers, around 10 % of the oocytes tested remain without a conclusive diagnosis because of amplification failure in at least one of the two PBs [28]. If PGS aims at improving IVF outcomes, it is crucial that results are obtained from all embryos tested. Economic and logistic issues should then be also considered. In particular, PBs screening results as the most time-consuming and least cost-effective among PGS approaches and it is also independent from oocyte developmental potential, since part of the analyzed oocytes/zygote will never reach to the blastocyst stage and be transferred. In synthesis, all these evidences together resulted in the breakdown of PBs approach, while some investigators proposed to move the biopsy stage forward along preimplantation development. They suggested blastocyst stage TE biopsy, arguing that several advantages could be brought from this novel intriguing approach [29]. Several efforts have been invested then in order to highlight the concrete possibilities offered by this breakthrough and to uncover its technical and clinical opportunities.

Trophectoderm Biopsy at the Blastocyst Stage

Blastocyst stage PGS on TE biopsy ensued the sharp failure of cleavage stage PGS on blastomere biopsy and the betrayed promises of PBs biopsy ones. Understandably, the same issues concerning these previous strategies were moved against this different approach. Thus, in order to prove its efficiency, a fruitful series of evidences were produced and published in literature up to date and more studies are currently in the pipeline. Hereafter, a review of the main evidences reported up to date will be provided. At first it was solved the doubt about a possible impact of the biopsy on embryo developmental potential. A particular concern dealt with the risk of decreasing the pregnancy rate per started cycle by postponing the time of the transfer beyond the cleavage stage, that is, extending embryo culture to the blastocyst stage. This issue in particular arises from the risk of embryo developmental arrest either at the time of compaction or at the time of cavitation, which, especially in poor prognosis patients, can reduce the pool of blastocysts to be screened for aneuploidies and potentially transferred. However, in the scientific literature there is absolutely no evidence that transferring embryos at the cleavage stage can result in higher pregnancy rate per stimulation cycle in poor prognosis patients compared to the use of blastocyst transfer policy and, reasonably, extended culture has not been considered to lead to embryo waste. In this regard, Guerif and colleagues [30] reported on an RCT highlighting that, in a poor prognosis patient population, the pregnancy rates per stimulation cycle was similar after both fresh ETs and frozen ETs when using a cleavage or blastocyst stage ET policy. This suggested that the extension of the culture to the latest stage of preimplantation development does not reduce the number of live births after IVF.

Blastocyst biopsy can be performed in two different ways. In the first one described by Schoolcraft and colleagues in 2010, a zona opening is made at the cleavage stage to prompt TE cells herniation on day 5 or 6 and to facilitate the biopsy procedure [31]. The main drawbacks of this method relate to the extra manipulation of embryos at the cleavage stage, especially in the current trends of contemporary IVF culture that are exploiting closed culture systems from fertilization to the blastocyst stage, and to the risk of Inner Cell Mass (ICM) herniation that may require a second hole in the zona pellucida. A different method for TE biopsy has been then recently described by our group not requiring zona breaching and avoiding any potential stress at the cleavage stage, as well as allowing a more physiological growth of embryos to the blastocyst stage [32]. As far as the impact of biopsy on embryo development is concerned, Scott and colleagues reported, in the same elegant and powerful study previously mentioned in this chapter, no significant differences in implantation rate between untested biopsied and non-biopsied blastocysts [18]. It is not clear whether this is ascribable to a smaller proportion of total cells removed from the blastocyst, to a higher stress tolerance of the blastocyst with respect to other stages of preimplantation development, or to the preservation of the ICM counterpart which originates the fetus, but still this represents a further advantage of postponing the time of the biopsy.

A crucial point of discussion is the accuracy of the analysis and the information that can be obtained from a randomly selected TE sample biopsied at the blastocyst stage. Certainly, blastocyst stage TE biopsy ensures a more accurate assessment of meiotic aneuploidies than previous strategies, since between five and ten cells are retrieved and analyzed from the embryo compared to the analysis of a single cell that is commonly performed on blastomeres and PBs. This translates in a significant reduction of the incidence of all the misdiagnosis risks derived from a single-cell analysis. In fact, all confirmation studies reported so far based on FISH reanalysis of aneuploid blastocysts following TE biopsy and CCS found between 98 and almost 100 % of correct aneuploidies prediction of meiotic errors [33–35]. Also, in a recent study from our group, we provided the first assessment of the reliability of blastocyst stage aneuploidy screening by the analysis of multiple TE biopsies from the same blastocyst with the use of different CCS methods [36]. The analysis was based on the real-time quantitative Polymerase Chain Reaction (qPCR) blinded reanalysis of 120 s biopsies of aneuploid blastocysts previously screened by TE aCGH and showed a consistent chromosome copy number diagnosis in 99.4 % (2,561/2,576; 95 % CI 99.0–99.7) of the chromosomes analyzed. The remaining 0.6 % was due to either technical variation between CCS techniques or occasionally by biological variation due to the presence of chromosomal mosaicism.

The impact of mosaicism on the reliability of the diagnosis, and especially the possibility of a nonrandom allocation of chromosomally abnormal cells exclusively to TE in case of mosaicism [37–39], is in fact another important point to be considered when blastocyst biopsy is performed on randomly selected TE cells. To this end, high concordance between ICM and TE chromosomal complement has been reported in the most recent literature [11, 35, 40], suggesting no preferential allocation of abnormal cells in a mosaic blastocyst, and that the analysis of a TE sample can be considered diagnostic of the ICM. In order to properly perform this analysis, our group conceived and published a novel method of ICM biopsy, which led to the total absence of TE cells contamination [35]. In the same paper, a preliminary aCGH analysis on a TE biopsy during blastocyst stage PGS cycle was performed, which was followed by a FISH reanalysis of three further TE fragments and of the whole ICM from those embryos diagnosed as aneuploid. The ultimate aim of this study design was to define the real influence of mosaicism on the accuracy of the diagnosis. Constitutional aneuploidies were reported in 79.1 % of cases, while mosaic in 20.9 % of cases. However, the real risk of an uncertain diagnosis due to mosaicism when testing at the blastocyst stage accounts for only 4 % of aneuploid blastocysts that were detected to be mosaic diploid/aneuploid (embryos showing a mosaic error as the only aneuploidy).

A very interesting finding of this study was that all cases of high-grade diploid/aneuploid mosaicism where abnormal cells constituted more than 40 % of the blastocysts were diagnosed as aneuploid by the original blastocyst stage aneuploidy screening cycle. This data suggested that blastocyst stage PGS performed on a randomly selected TE sample is able to avoid also the transfer of mosaic embryos with a very high prevalence of abnormal cells that might have a poor clinical outcome on pregnancies (Fig. 7.1). Indeed it is well known that, when compatible with life, mosaicism can be associated with poor fetal outcomes and neonatal morbidity.

Looking at mosaicism data in prenatal diagnosis, the overall incidence of mosaicism is very low and reported to range between 1.22 % and 1.32 % after spontaneous pregnancies and after IVF care, respectively, where true mosaicism accounted only for 0.3–0.44 % of pregnancies [41]. These data suggest that the incidence of mosaicism has been overestimated in preimplantation genetics but also that mosaic embryos can be subjected to a negative selective pressure following ET resulting in failure of implantation or early embryo loss, and at last highlight the potential benefit of blastocyst stage PGS to detect and avoid the transfer of mosaic embryos with a high prevalence of abnormal cells. However, even though all these preclinical studies were sufficient to assess no impairment of implantation potential following TE biopsy and the high diagnostic accuracy and reliability of a CCS approach for embryonic aneuploidy, they were not sufficient themselves to determine whether the test has a true clinical value.

Thus, in order to demonstrate whether a clinical benefit results from application of aneuploidy screening at the blastocyst stage and to determine the specific magnitude of this benefit, four RCTs were published up to date using different CCS methods and investigating the use of this approach in different patient populations [31, 42–44]. Taken together, all these RCTs consistently reported an increased sustained implantation and live birth rate (relative increase between 28 and 40 % with respect to the control group) following the transfer of euploid blastocyst compared to the transfer of untested embryo, suggesting that blastocyst stage aneuploidy screening can be considered today a validated technology to improve embryo selection and clinical outcome per transfer in IVF. What is still missing are RCTs evaluating live birth rate per stimulation cycle to assess whether blastocyst stage PGS can result also in similar efficacy compared to standard care according to an intention to treat analysis. A final interesting argument of discussion deals with the implementation of this PGS approach in clinical practice. Regarding this point from an economic and logistic perspective, blastocyst stage PGS on TE biopsy, conversely to previous strategies and especially to PBs biopsy one, represents the most convenient and easy to implement approach. This is mainly due to the fact that only developmentally competent embryos would reach to this stage, while incompetent ones will arrest at previous stages of development. Thus, only reproductively competent embryos will be screened for aneuploidies. This results in PGS cost reduction with the considerable advantage of being able to increase the patient population that can benefit from this technology during their IVF cycle. To summarize, blastocyst stage PGS approach has passed thorough several preclinical and clinical validation steps and fulfilled so far all the requirements that we may expect from an ideal PGS strategy.

Conclusion

Extensive progresses have been made since PGS was conceived in the early 1990s. The long and difficult pathway that was undertaken up to date conduced to the definition of a new gold-standard approach for PGS entailing CCS platforms-based analysis on TE biopsy at the blastocyst stage. The failure of PGS as it was

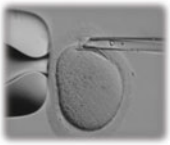
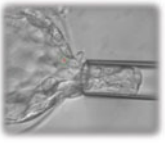
	PBs biopsy	Blastomere biopsy	TE biopsy
			
IMPACT OF THE BIOPSY ON EMBRYO DEVELOPMENT	Higher rates of cleavage arrest, fragmentation and reduction in embryo morphological quality after PB biopsy [1]	19% decrease in implantation rate after biopsy with respect to control non-biopsied embryos in a non selection RCT [2]	No significant decrease in implantation rate after biopsy versus control non-biopsied embryos in a non selection RCT [2]
RELIABILITY AND INFORMATIVITY OF DIAGNOSIS	High FP/FN [3, 4] results due to: MI errors balancing at MII, meiotic errors selected against and corrected, male- and mitotic-derived aneuploidies	Single cell analysis issues (ADO, PA, ADI, chimerical DNA molecules, amplification failure); cell cycle phase; highest impact of mosaicism	More robust genetic analysis; only 4% risk of misdiagnosis due to mosaicism [5]; high concordance ICM-TE diagnoses [5]
CLINICAL EVIDENCES OF EFFECTIVENESS	No data have been produced up to date in order to assess this aspect	**By FISH:** Meta-analysis of 9 RCTs showed detrimental effects [6] **By CCS**: No data have been published	**By CCS**: 4 RCTs produced up to date reporting ≈30% increase in implantation rate per ET versus standard IVF [6,7,8,9]
IMPLEMENTATION IN IVF	Time-consuming and poorly cost-effective	Highest worldwide experience	Least time consuming and most cost-effective

Fig. 7.2 Comparison and level of evidence of effectiveness between biopsy strategies to perform PGS. *PB* polar body,*TE* trophectoderm, *FP/FN* false-positive/false-negative, *RCT* randomized controlled trial, *ADO* allele drop-out, *PA* preferential allocation, *ADI* allele drop-in, *ICM* inner cell mass, *ET* embryo transfer, *CCS* comprehensive chromosomal screening. [1] Levin et al., Fertil 2012 [47]; (2) Scott et al., Fertil Steril 2013 [18]; (3) Capalbo et al., Hum Reprod 2013a [17]; (4) Handyside et al., Eur J Hum Genet, 2012 [22]; (5) Capalbo et al., Hum Reprod 2013b [35]; (6) Schoolcraft et al., Fertil Steril 2010 [31]; (7) Yang et al., Hum Cytogenet, 2012 [42]; (8) Schoolcraft et al., Fertil Steril 2011 [43]; (9) Forman et al., Fertil Steril 2013 [44]. The biopsy strategy to be adopted in order to perform PGS should ensure the absence of a detrimental impact on embryo development, a reliable and informative diagnosis, and clinical evidences of its effectiveness. Furthermore, an easy implementation in the IVF lab in terms of low workload and high cost-effectiveness of the procedure should also be considered. Currently, according to the data reported in literature up to date, TE biopsy is the only approach fulfilling these criteria and thus it should be considered as a gold-standard approach to perform preimplantation aneuploidy screening

performed at first did not weaken the conviction of its value. In fact, all the different levels of evidence reported in literature and reviewed here proved the efficiency of this last approach against all the issues causing the failure of previous strategies (Fig. 7.2). Nowadays, we can confidently sustain that CCS-based PGS on TE biopsy is the closest approach to an ideal PGS strategy that is currently available. In the last years, the implementation of this strategy in IVF labs was delayed by the low worldwide experience in blastocyst culture and vitrification protocols. Nowadays instead, a strong impulse to increase the IVF laboratories' experience with blastocyst

culture, handling, and cryopreservation will derive from the application of the freeze-all approach and of the cycle segmentation theory [45], which are increasingly being recognized as effective approaches in IVF. In particular, cycle segmentation theory entails a GnRH agonist triggering in a GnRH antagonist cycle as stimulation protocol, which was reported as free from the risk of ovarian hyperstimulation syndrome (OHSS), associated with oocyte and/or blastocyst vitrification in order to perform ET on a receptive endometrium in a natural cycle, thus also reducing the risk of extrauterine pregnancies and improving obstetrical and neonatal outcomes of IVF-derived pregnancies [46]. Conducting CCS on TE biopsy entails the further advantage to be totally integrated in this protocol. In fact, vitrified euploid elective single embryo transfer (eSET) could be performed following this approach, consequently escaping the risk of multiple pregnancies, while increasing implantation rate and decreasing abortion rate per ET. Next progresses in PGS will be mainly technical advances dealing with a reduction of costs and a parallel increase of throughput. In this regard, NGS-based PGD and PGS represent the most promising tools to be implemented in this field. However, even though PGS conducted through NGS can in prospect become accessible to every couple approaching an IVF cycle and with suitable indications to it, we should pay attention not to be excessively optimistic because several technical and ethical considerations should be made about this technique. In particular, an exhaustive genetic counseling will be required in order to thoroughly describe all the possible advantages and limitations of this novel and potentially higher throughput platforms, especially at the beginning of its implementation in preimplantation genetics. Now that the optimal stage of biopsy to accurately detect chromosomally abnormal embryos has been identified, the future challenges will deal with the implementation of embryo evaluation methods beyond aneuploidy screening to further enhance selection among euploid blastocysts. Noticeably, neither blastocyst morphological grade nor embryo developmental rate to the blastocyst stage do significantly correlate with implantation potential of euploid embryos [32], suggesting that the commonly used parameters of blastocyst evaluation are not good indicators to achieve this aim. Thus, future researches are required to identify noninvasive biomarkers of reproductive potential and to further enhance selection beyond euploidy assessment.

Several studies are currently in the pipeline aiming at investigating the correlation between implantation and the -omic sciences world. Genomic, transcriptomic, exomic, methylomic, miRNomic, proteomic, metabolomic, and spent culture media analysis studies represent an unexplored source of knowledge that can help us filling the gap between failure and success of a PGS cycle. If we expect mainly a decrease in costs and an increase in throughput from new advances in the field of CCS-based PGS, the prospect to further increase the outcomes of a PGS cycle resides in these new tools of analysis. The future in blastocyst developmental competence assessment is yet to come and we expect it to be fruitful.

Conflict of Interest The authors declare no conflict.

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Chapter 8
Polar Body Diagnosis (PBD): An Alternative and Supplement to Preimplantation Diagnosis for Single Embryo Transfer

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Introduction

Polar body biopsy (PBB) and diagnosis (PBD), the earliest form of preimplantation genetic diagnosis (PGD) and prenatal diagnosis (PND), was developed in response to two main factors: (1) for countries with legal restrictions against embryo biopsy and/or testing and (2) for couples with ethical concerns against the genetic testing of cleaved embryos.

Interest in PBD grew dramatically after prospectively randomized studies reported no benefit in analysing a limited number of chromosomes in day 3 embryos with fluorescent in situ hybridization (FISH) for aneuploidy screening [1, 2]. In contrast to blastomeres, polar bodies (PBs) do not exhibit mosaic problems and are not necessary for the fertilization process and normal embryo development. Thus, they can be removed and tested. Human PBD was described for the first time as early as 1990 by Verlinsky et al. [3]. However, it took some time to develop this method of testing, also known as preconception genetic diagnosis.

Whether PBD provides more accurate genetic information than PGD derived from cleaved eight-cell-stage embryos or blastocysts (due to lack of mosaicism in PBs) remains under debate [4]. What is undisputed, however, is that PBD offers limited information, i.e. information on the maternal genome only. In addition, both

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the workload and the cost of PBD are much higher than for PGD of cleaved embryos or the trophectoderm of blastocysts, as only a fraction of mature oocytes develop to embryos and blastocysts that are worth testing.

Indications and Genetic Counselling

PBD provides genetic information from the maternal side only. Nevertheless, maternal meiosis is the major contributor to embryonic aneuploidy [5]. In addition, PB analysis circumvents possible diagnostic errors arising from chromosomal mosaics caused by post-zygotic chromosome instability—a common event which occurs early during human embryogenesis [6]. In vitro fertilization (IVF) and preimplantation genetic diagnosis (PGD)/preimplantation genetic screening (PGS) are complex and extensive processes which are best managed using a multidisciplinary approach. If a couple is considering PGD/PGS, they need to receive expert consultation and should receive detailed information to help them understand these demanding procedures [7]. The European Society for Human Reproduction and Embryology (ESHRE) Preimplantation Genetic Diagnosis Consortium stated that, in order to offer a good clinical service, centres providing PGD should involve the medical expertise of both assisted reproduction and clinical genetics departments [8]. Those offering genetic counselling should be appropriately qualified, e.g. be a medically qualified clinical geneticist or genetic counsellor. The counselling must be non-directive and address family history, reasons for requesting PGD/PGS, a review of the diagnosis, help with understanding the risks, a review of reproductive options, an explanation of the treatment, and a discussion of the advantages and disadvantages related to the various techniques, as well as the respective legal situation, procedure costs, and how much of the cost is likely to be covered by health insurance.

Indications for PGD by PBD

PGD using PBs is indicated for couples at risk of transmitting a known maternal genetic abnormality to their offspring. In order to reduce the risk of inheriting the maternal genetic condition and/or a late pregnancy termination (following prenatal testing), solely non-carrier zygotes are considered for transfer after PGD tests. Primary candidates for PGD by PBD are:

- Women who are carriers of an X-linked disorder
- Women who are carriers of chromosome translocations. Translocations are common causes of implantation failure, recurrent pregnancy loss, and of mental/physical features in offspring [9]
- Women who are carriers of recessive or dominant diseases, including cancer predisposition disorders and mitochondrial disorders [10, 11].

Indications for Preimplantation Genetic Screening by PBD

Chromosome aneuploidies, which occur most often during female gametogenesis, are the major cause of pregnancy failure and loss and of abnormal live births following both natural conception and IVF [12, 13]. The aim of PGS is to allow the transfer of embryos without aneuploidy, thereby increasing implantation and clinical pregnancy rates, reducing the incidence of miscarriage, avoiding abnormal pregnancy, and increasing the live birth rate. Primary candidates for PGS are:

- Women of advanced age
- Couples with a history of unexplained recurrent miscarriage
- Couples with repeated implantation failure

Polar Body Diagnosis: Description of Technique

Information and Treatment in the Fertility Centre

Couples are referred to (or themselves contact) a fertility centre with the ability to test polar bodies for PGD, if the female partner is a known carrier of a serious inherited disease or if they are already parents of an affected offspring with a genetic disease of maternal origin [14]. PGS for aneuploidy testing upon assisted infertility treatment is offered largely due to advanced maternal age (>37 years) or due to a history of unexplained recurrent miscarriage. The idea is to select euploid oocytes in situations with an increased incidence of aneuploidy, thus improving pregnancy and delivery chances. Initially promising results were reported with the established, but not very precise, FISH analysis of five chromosomes—13, 16, 18, 21, and 22—at this centre [15] and elsewhere [16]. However, this genetic test method was widely abandoned after the publication of two prospectively randomized studies with disappointing findings [1, 2]. This precaution for PBD was taken despite the fact that the investigations were accomplished not with PBs but with blastomeres of cleaved eight-cell-stage embryos. A revival of PGS with the comprehensive testing of all available PB chromosomes using array comparative genomic hybridization (array-CGH) is possible and plausible. PBD with array-CGH has also led to pregnancies and deliveries in our group (personal communication). However, the results of the ESTEEM PB study (ESHRE Study into The Evaluation of oocyte Euploidy by Microarray analysis) must be awaited before array-CGH-PGS can again be routinely recommended for PB aneuploidy screening.

Before embarking on any intervention, couples are informed by an infertility specialist about the techniques of intracytoplasmic sperm injection (ICSI) and PBD. The procedure involves opening of the zona pellucida and is always combined with ICSI to avoid polyspermia. This includes discussion of the procedure; chances of success; limitations of the procedure, including the weaknesses of the

genetic testing; costs; and potential alternatives. This is crucial as many patients do not consider the possibility of misdiagnosis and estimate the chances of success for a pregnancy with an unaffected child as unrealistically high. The cost issue is a difficult matter, especially in Switzerland. Here the costs of neither ICSI nor biopsy and genetic analysis are included in the catalogue of services of the public health system (or any private insurance). It is not a rare occurrence for young couples with limited financial resources to have to withdraw their interest in the costly PBD procedure, especially when they realize that prenatal testing and an abortion (if required) are fully reimbursed.

If patients agree to PBD, they are referred to a geneticist for thorough genetic counselling and preparation for the genetic analysis of the planned PBD. Following clearance from the genetic institute, controlled ovarian hyperstimulation (COH) is started as described earlier [17] with the aim of harvesting at least 10 eggs. Increasingly, the antagonist protocol as proposed by Griesinger et al. [18] is used. This protocol almost completely prevents the development of severe ovarian hyperstimulation syndrome—the most dangerous complication of a COH, which occurs more often as more follicles grow and oocytes mature.

In the early years of PBD, immediate analysis of the PBs with a reliable genetic result within a few hours was necessary to replace an unaffected embryo in the fresh cycle. This very short time was required as the legal situation in countries such as Switzerland allows no more than three pronuclear stage eggs (PN) cultured to cleaved embryos, embryo selection practices are not permitted, and no cleavage stage (2–3 day) embryos can be frozen, thus requiring all embryos to be transferred. Hence, the results of the FISH analysis had to be available before the onset of syngamy of the pronuclei. To enlarge this time window by freezing of the fertilized oocyte was not an option, as cryopreservation of biopsied PNs with the slow-freezing protocol was less than optimal [19]. However, the introduction of vitrification—a novel ultra-rapid freezing technology—resulted in sustainable relief [20]. With this technique, the PBs may be biopsied and the corresponding PNs are subsequently vitrified and stored until the result of the genetic investigation is available. If the number of testable oocytes is too small due to either a poor stimulation response or a low fertilization rate, a second round of hormonal stimulation and oocyte pickup can be discussed before the genetic analysis.

After obtaining the genetic data, the results are reviewed with the couple. It is the couple (and not the geneticist nor the infertility specialist) who finally decides about the future of the tested oocytes.

In our unit, the embryo transfer takes place in context of an artificial thawing cycle. After endometrial preparation with estradiol (Estrofem® N 3×2 mg/day orally; start at cycle day 1) and the additional PBD application of micronized progesterone (Utrogestan® 1,000 mg/day vaginally) when the endometrium reaches at least 8 mm thickness, one or two PNs are warmed and 1–2 days later are transferred as two- to eight-cell-stage embryos. The implantation rate is comparable to the implantation rate in the conventional thawing cycle without PBD, which is 25.1 % in our unit (Limoni C, FIVNAT-CH, Swiss National IVF Registry, 10.3.2014).

Due to the low number of centres performing PBD, only anecdotal reports exist regarding the incidence of misdiagnosis using this technique [21]. However, all patients must be informed before PBD and at the time of very early pregnancy about the possibility of prenatal diagnosis to confirm the PBD result.

Polar Body Biopsy Technique

Obtaining an intact PB is the most demanding part of the overall PB testing procedure. In fact, technical problems may arise during any phase of the procedure, resulting in the loss or lysis of PBs, or even oocyte injury. This chapter provides an overview of the most technically difficult aspects with the aim of providing the reader with a complete theoretical knowledge about the technique of the PB biopsy procedure.

Chronology

In contrast to blastomere- or trophectoderm-based PGD where timing of biopsy is extendable, the timing of PB biopsy is defined precisely and depends mainly on the method of genetic testing being performed. For FISH-based testing, for instance, the most optimal time to accomplish the simultaneous biopsy of both PBs seems to be at the time of fertilization check, or about 16–18 h after ICSI [22]. However, one should keep in mind that the DNA of the first polar body (PB1) will degrade gradually after fertilization, so postponing the biopsy for 16–18 h following insemination could sometimes make proper interpretation of FISH results difficult. Conversely, fragmentation (an additional consequence of PB1 ageing) is less problematic because any dilemma in distinguishing PB1 from PB2 morphologically can be resolved easily later with FISH; PB1 chromosomes are represented by paired chromatids, each of which produces a hybridization signal; PB2 gives only a single signal for each chromatid [22].

On the other hand, the introduction of array-CGH into clinical practice has initiated a different timing of PB biopsy which actually fits exactly with the timing of biopsy for PGD of single gene (Mendelian) disorders [23]. Thus, it becomes more advisable to remove PB1 separately from PB2, because potential mistakes in prediction of the origin of PBs might lead to the misdiagnosis of genetic disorders. Accordingly, the removal of PB1 in such cases is scheduled a minimum of 1 h before or, at the latest, 1 h after ICSI. Timing of the PB2 biopsy is very challenging; this needs to take place no earlier than 9 h after ICSI, since an earlier time of biopsy, at, e.g., 4–6 h after ICSI, might lead to problems with amplification—a procedure that provides an amount of DNA sufficient for successful genetic diagnosis [24].

Breaching the Zona Pellucida

The simplest way to give the biopsy micropipette free access to the PB through the zona pellucida (ZP) is to use laser-assisted technology. After placing the PB in the focal plane of an inverted microscope, an opening in the ZP can be created with a few laser pulses delivered near the PB. Care should be taken not to create too large an opening, which could facilitate leakage of cytoplasm from the ZP and consequently cause the arrest of zygote development. Too small an opening, however, may arrest blastocyst growth by entrapping the embryonic cells that must hatch through the hole in the ZP during preimplantation development. Thus, the ideal size of opening should be confined to between 15 and 17 μm. The same opening must be used for the removal of both PBs, irrespective of whether simultaneous or sequential biopsy is intended. Of note, we reported recently that the opening in the ZP made by the laser might affect the viability of embryos developed from biopsied oocytes. Although the underlying reason for the decrease in blastocyst viability in this latter study remains unknown, blastocysts developed from the laser-treated group of oocytes were noted to have initiated hatching much earlier than the control group; this was thought highly likely to be linked to decreased viability [20].

Another effective but less invasive and more economical approach to ZP opening was developed in Chicago by Verlinsky et al. [25]. Their three-dimensional method of dissection, known as partial zona dissection (PZD), is well described and illustrated in the original publication by Verlinsky and Kuliev [25]. Thus, what follows here is a brief description of only the most important parts of the procedure:

1. The oocyte is affixed on the holding pipette (Fig. 8.1a) in such a manner that PB1 is kept out of the focal plane just above the 6 o'clock position (Fig. 8.1b).
2. Using a very sharp microneedle, the ZP is penetrated tangentially at 4–5 o'clock, passing through the perivitelline space, and out at 7–8 o'clock (Fig. 8.1c).
3. The opening in the ZP is made by gently rubbing the microneedle attached to part of the ZP against the holding pipette until a cut of about 20–40 μm long and 1–2 μm wide is created (Fig. 8.1d).
4. The oocyte is further oriented with the help of both the holding pipette and the microneedle so that PB1 is positioned in the same focal plane as the small cut in the ZP made earlier (Fig. 8.1e).
5. The biopsy micropipette, which has an inner diameter of 15–20 μm and is primed with 10 % PVP solution, is then inserted through the hole into the perivitelline space, and, by applying negative pressure, PB1 is separated gently from the oocyte and drawn into the micropipette (Fig. 8.1f–i). The same access and the same aspiration procedure should be also used for the removal of PB2.

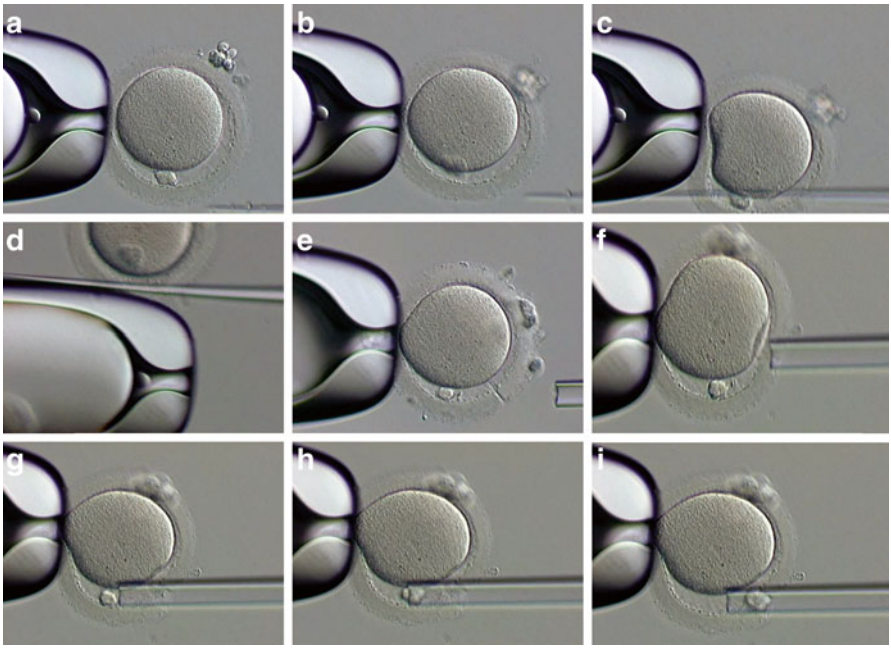

Fig. 8.1 Six principal steps in biopsy of the first polar body (PB1) using the partial zona dissection method: (**a**) Fixation of the oocyte on the holding pipette. (**b**) Positioning of PB1 out of the focal plane. (**c**) Penetration of zona pellucida (ZP) with a sharp microneedle. (**d**) Rubbing of the microneedle against the holding pipette in order to open the ZP. (**e**) Positioning of PB1 in the same focal plane with a small cut in the ZP. (**f**) Insertion of non-bevelled biopsy micropipette through the ZP into the perivitelline space. (**g**–**i**) Aspiration of PB1 into the non-bevelled micropipette

Bevelled vs. Non-bevelled Biopsy Micropipettes

There are no universal rules governing the correct micropipette shape for PB biopsy. Under optimal biopsy conditions, for instance when both PBs are mature and loosely connected within the oolemma, it is advisable to use a non-bevelled micropipette, as this enables a very smooth mode of PB aspiration (Fig. 8.1). Difficulties during biopsy usually arise when one PB (usually PB2) is still firmly held to the oolemma; in such cases, the use of a bevelled micropipette is technically more practical as it is easier to dislodge the attachment. If removal of the PB still proves technically difficult, a higher pressure combined with some rigorous back and forth movements of the micropipette might help break the tight connection between PB and cytoplasm. However, anomalies at fertilization, such as the appearance of oocytes with a single pronucleus (1*pn*) or three pronuclei (3*pn*), are often seen after this exceptional extirpation of PB1 or PB2 from the cytoplasm of the oocyte.

Alternative solutions, such as prolongation of oocyte incubation for a couple of hours, as suggested by Montag et al. [24], can sometimes be very helpful in facilitating the biopsy procedure by allowing completion of the meiotic cell cycle leading to complete PB1 or PB2 extrusion.

PB Transfer to Reaction Tube

Transfer of PBs from culture medium to reaction tubes following biopsy is another delicate step of this procedure. This "tubing" step requires a witness to ensure that patient name, PB number, and negative control are all correct. Tubing should in all cases be done in a running laminar flow under strict hygienic conditions to reduce DNA cross-contamination. Under a high-contrast stereomicroscope installed under laminar flow, the PB is washed at least twice in washing buffer droplets (e.g. PBS). Next, the PBs are transferred into a PCR-grade plastic capillary tube (120–170 μm inner diameter). Tube composition is critical to prevent possible PB loss if a glass capillary tube were to shatter or fracture. The volume of carry-over medium should be as small as possible.

Cryopreservation/Vitrification After Biopsy

Immediately after the introduction of PBD, it became clear that cryotechnology could play an important role in preservation of the developmental potential of biopsied oocytes. Nevertheless, the past period of almost two decades since the first use of PBD is notable for the almost total absence of published reports regarding cryopreservation of biopsied oocytes. It is also somewhat astonishing that not even the most experienced PBD teams, such as the Chicago group, released any report on cryopreservation of biopsied oocytes or embryos following biopsy during this period. The lack of literature data on cryopreservation after PBD can be interpreted in several ways, but the presence of a low number of genetically normal oocytes in general is definitely one of the important factors explaining why cryopreservation of biopsied oocytes has not been offered routinely to PGD couples. Indeed, apart from cases leading to a few genetically normal oocytes, which were kept in culture for fresh embryo transfer, PBD often terminated in producing a cohort of affected oocytes. In addition, the interpretation of genetic results was found not uncommonly to be impossible because of amplification and other problems. So, these two groups of PBD oocytes had to be excluded from further culture. An even stronger argument for the frequent rejection of freezing after biopsy was that the conventional slow-freezing method used did not offer optimal conditions for the cryopreservation of biopsied oocytes, and the survival rate of biopsied oocytes after slow freezing was so low that the number of successfully established pregnancies was practically negligible [26, 27].

One of the greatest technological breakthroughs in human embryology was the introduction of vitrification into routine clinical practice. Ever since Kuwayama and co-workers published their data on the successful use of the novel vitrification protocol, interest in cryopreservation of biopsied oocytes has increased dramatically [28, 29]. After thorough investigation of this new method of cryopreservation, our group was also able to demonstrate that vitrification is a very effective procedure for freezing mouse and human biopsied oocytes [20, 30]. We have since developed a strategy for vitrifying all oocytes immediately after sequential PB1 and PB2 biopsy. We named it "comprehensive cryopreservation" since it includes vitrification of all oocytes displaying two PNs following PB biopsy [20]. With embryo transfer in such instances postponed until the next cycle, this comprehensive vitrification approach may improve the overall success of PBD procedures in several ways. First, it could lead to an increase in overall diagnostic accuracy because it allows sufficient time for complex genetic tests. Second, the legal problems provoked by embryo freezing due to an unplanned prolongation of PBD and genetic analysis can be simply avoided with the inception of the ultra-rapid vitrification protocol. Finally, PBD combined with vitrification may facilitate the implementation of an elective single embryo transfer protocol for a selected group of young patients in order to avoid complications due to multiple-gestation pregnancies without adversely affecting pregnancy rates.

In the meantime, this comprehensive vitrification approach has been confirmed clinically at our institution with several pregnancies and live births having been achieved in a relatively short period of time following PBD. However, for full clinical validation, this protocol still awaits the results of prospective randomized studies, and also the final report of the ESTEEM polar body study, which was initiated after a successful multicentre pilot study organized a few years ago by the ESHRE PGS Task Force [31, 32].

FISH/Array-CGH

Fluorescence In Situ Hybridization

FISH on fixed nuclei of biopsied cells with target-specific DNA probes allows detection of chromosome imbalances associated with chromosome rearrangements. FISH has also been used to screen embryos for sporadic chromosome aneuploidy in PGS. However, using FISH in PGS on PB has several disadvantages. First, only a limited number of chromosomes can be analysed. Second, the interpretation is very challenging since the DNA is frequently of very low quality and degraded. Last but not least, it is technically very demanding to process a single cell for FISH. Accordingly, several clinical trials have failed to show any improvement in delivery rates either for poor prognosis [2, 33–35] or for good prognosis patients [36–39].

The principle of FISH is to use target-specific DNA probes labelled with fluorochromes or haptens to detect the copy number of specific loci. The biopsied cell has to be spread within a predefined area on the slide in order to facilitate its localization following hybridization; extreme care needs to be taken in ensuring that the cell is lysed, that the cytoplasm is dispersed, and that the nucleus is visible and intact. Stringent rules and protocol/interpretation guidelines should be applied [40]. Best practice guidelines for FISH technique have been summarized by Harton et al. [41].

How to Perform FISH [42]

Solutions

- 0.01 N HCl
- Pepsin 10 mg/ml
- 1 % Paraformaldehyde/PBS
- 20×SSC: 3 M sodium chloride, 0.3 M tri sodium citrate, pH 7.2
- 60 % formamide/2×SSC
- 20 % Tween 20
- 4×SSC/0.05 % Tween
- 1 ml Vectarshield + 6 µl DAPI

Pretreatment

1. Pre-wash fix nuclei on slides using PBS for 5 min at room temperature
2. Add 0.5 ml pepsin to preheated (37 °C) 0.01 N HCl. Incubate the slides in this solution for 20 min at 37 °C
3. Rinse the slides briefly in H_2O and PBS
4. Fix the slides using 1 % paraformaldehyde/PBS for 10 min at 4 °C
5. Rinse slides briefly in PBS followed by H_2O
6. Dehydrate the slides (3 min each in 70 % EtOH, 90 % EtOH, 100 % EtOH) and air dry
7. Add the FISH probe to the slide, add coverslip, and seal the coverslip with rubber cement
8. Denature slides for 3 min at 75 °C, followed by incubation at 37 °C for a minimum of 2 h (best results with overnight incubation).

Post-treatment (in the dark)

1. Remove coverslip and incubate the slides at 42 °C 5 min in 60 % formamide/2×SSC.
2. Wash at 42 °C for 5 min in 2×SSC
3. Wash at room temperature for 5 min in 4×SSC/0.05 % Tween 20.
4. Dehydrate the slides (3 min each in 70, 90, and 100 % EtOH) and let the slides air dry
5. Mount the slides in Vectarshield
6. Store the slides in the dark at 4 °C until ready for analysis.
7. Score signal by direct visualization using a fluorescence microscope.

Array-CGH

Array-CGH circumvents the limitations of the FISH technique by allowing the parallel analysis of all chromosomes rather than just a limited number. The ESHRE PGS Task Force conducted a pilot study in which both PBs were biopsied simultaneously following ICSI and analysed by array-CGH in order to predict aneuploidy status in the corresponding embryo [31]. The protocol can be completed within 12 h. The study demonstrated that the euploid/aneuploid status of the PBs was highly concordant (94 %) with the status of the corresponding zygotes. Analysis of the pattern of segregation errors of the chromosomes in the two PBs and corresponding zygote demonstrated that almost all errors in the first meiotic division (MI) are caused by inappropriate early division of sister chromatids rather than the non-disjunction of whole chromosomes and that many oocytes have multiple meiotic errors [43].

Since aneuploidy correction might occur due to premature division of sister chromatids for one of the homologous chromosomes followed by random segregation of the chromatid in meiosis II (M II), both PBs from an oocyte should be tested. If PB1 shows a chromosome aneuploidy and PB2 a reciprocal loss or gain of the same chromosome (Fig. 8.2), then the corresponding oocyte (and thus the resulting zygote) might be euploid [44]. In cases where there are other oocytes with normal PB1 and PB2, these would be selected for IVF. However, this concept is important in cases where none of the tested oocytes has normal PBs.

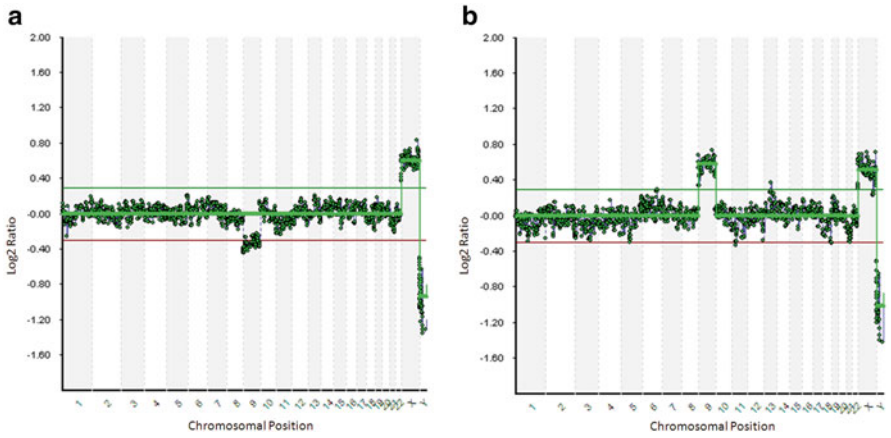

Fig. 8.2 Array comparative genomic hybridization (array-CGH) profiles of an example of a reciprocal chromosomal aneuploidy correction (Array-CGH: 24sure V3; Illumina, San Diego, CA, USA; image analysis: BlueFuse Multi, Illumina, San Diego, CA, USA). There is one error in meiosis I in PB1: a chromatid loss for chromosome 9 (**a**), which is balanced by a chromatid gain in PB2 (**b**). All other autosomes are within the bounds of normality. *Green* and *red horizontal lines* represent the 95 % confidence interval for normal copy number

How to Perform Array-CGH

The principle of array-CGH includes the following steps:

1. Whole genome amplification of the sample DNA. The cell is lysed and the DNA is fragmented and amplified by polymerase chain reaction (PCR) using universal primers.
2. Label amplified sample and control DNA with Cy3 and Cy5 fluorescent dyes, respectively. The master mix contains the reaction buffer, primer solution, dCTP-labelling mix, and the appropriate fluorescent dye. The labelling reaction is performed at 37 °C for 2–16 h. Labelled sample and control DNA are then combined and COT DNA is added to block repetitive sequences of the genome, followed by a co-precipitation step.
3. Hybridization of combined labelled sample and control DNA on microarray slides, after a washing step with EtOH. The clean pellet is then re-suspended in pre-warmed hybridization buffer and denatured at 75 °C. Denatured DNA is thereafter applied to a microarray slide and hybridized at 47 °C for 4–16 h.
4. Microarray slide washing.
5. Scanning of arrays slides using a two-channel scanner.
6. Analysis of the results and interpretation.

PGD for Single-Gene (Mendelian) Disorders by Molecular Genetic Analyses of Polar Bodies

Genetic analysis of PB1 and PB2 can be used for the diagnosis of various single-gene disorders with a maternal contribution (dominant or recessive mutation). The genetic testing procedure in PGD comprises a personal consultation with the couple and an initial genetic examination of both partners and additional family members, followed by analysis of PBs. PGD is considered only if the DNA of an index patient is available. Moreover, additional family members are included for segregation analysis and the identification of informative flanking markers. The couple is provided with the following information: an explanation of the genetic origin of the disease and its mode of inheritance, risk assessment and recurrence risk, principles of the molecular genetic testing procedure, reliability of the test and possible misdiagnosis, alternatives to PGD, time frame, and costs [7].

Linkage Analysis

In addition to confirmation of the disease-causing mutation in the family, the PGD procedure involves linkage analysis with mutation-flanking polymorphic markers to increase reliability and to reduce misdiagnosis due to allele dropout (ADO).

For this purpose, DNA from the couple and additional family members is analysed with highly polymorphic intra- or extra-genic variants to determine which marker alleles co-segregate with the disease-causing mutation. This haplotype analysis requires at least two generations and an index patient in the family. Two types of markers are commonly used in PGD: microsatellites (short tandem repeats or STRs) and single nucleotide polymorphisms (SNPs).

DNA Amplification from a Single Genome

A major challenge in genetic testing is the limited amount of genetic material available, as this leads to a number of problems compared to routine diagnostic PCR where the amount of DNA is not limited. For efficient and reliable DNA amplification, different techniques have been developed. Two frequently used methods are (1) multiplex PCR and (2) whole genome amplification (WGA).

1. Multiplex PCR involves the combination of multiple primer pairs in one PCR reaction to amplify multiple loci simultaneously [45]. For detection of the amplified alleles, conventional gel electrophoresis, capillary electrophoresis of fluorescently labelled PCR products, or Sanger DNA sequencing can be used [45]. The advantage of this technique is the increased specificity and sensitivity afforded by amplifying unique sequences; however, the method requires a family-specific design, which is time-consuming and labour-intensive [46].
2. WGA, which amplifies the entire genome, is an alternative approach that provides a sufficient amount of DNA for downstream applications [47].

   Currently, the most frequently applied WGA approach in PGD is multiple displacement amplification (MDA). The average product length of MDA is >10 kb and the error rate of the polymerase is 1 in 10^6–10^7 nucleotides [47, 48]. Several investigators have established MDA protocols for PGD and have successfully identified polymorphic STR markers [49, 50]. However, the relatively high allele dropout (ADO) rate of STR genotyping by PCR is still the major drawback and increases the risk of misdiagnoses [51, 52].

Mutation Detection Strategies

Different mutation detection strategies exist to determine the mutation directly or confirm the presence of the mutation-containing allele. Respective guidelines and recommendations have been issued by ESHRE [53]. To reduce the risk of misdiagnosis, a combination of direct mutation detection with at least two flanking markers at each site is strongly recommended. If one marker fails, the remaining marker together with the mutation is still informative.

Lysis and DNA Release from Single Cells and Polar Bodies

Lysis of the biopsied single cell is the most critical step in PGD as it can significantly influence the amplification efficacy and thus the efficiency and reliability of this procedure. There is no consensus regarding which lysis buffer and procedure should be applied. Two methods are commonly used for single-cell analysis in PGD: proteinase K/SDS (PKS) and alkaline lysis (AL). PKS treatment can efficiently lyse the cell and inactivate RNAses and DNAses in the presence of SDS [54]. The AL protocol disrupts membranes of the cell and nucleus, denatures nucleases, and dissolves the DNA during incubation at alkaline pH [55].

Pitfalls in PGD by PCR

Amplification Failure

Amplification failure (AF) is the absence of amplification in single-cell PCR. The major consequence of this phenomenon is a reduction in the number of oocytes available for transfer. Amplification failure affects approximately 5–10 % of oocytes or single cells and is presumably caused by loss of the isolated cell during transfer to the PCR tube, transfer of an enucleated or degraded cell, or the failure of cell lysis [56, 57].

Allele Dropout

One of the leading causes of misdiagnosis is ADO, a phenomenon whereby one of the two parental alleles in a heterozygous genome fails to amplify by PCR [58, 59]. In the case of PBD, the sequential genetic analysis of PB1 and PB2 can be an efficient approach to identify ADO (Fig. 8.3). Three different scenarios should be considered:

1. A homozygous mutant PB1 and hemizygous normal PB2 would indicate a normal oocyte. However, if ADO of the normal allele has occurred in PB1, this conclusion would be incorrect and the oocyte would be transferred, even though it carries the mutant allele.
2. A homozygous normal PB1 and a hemizygous mutant PB2 would indicate a mutant oocyte. However, if ADO of the mutant allele has occurred in PB1, the oocyte would be excluded from transfer, even though it carries the normal allele.
3. Heterozygosity of PB2 is a clear indication of contamination, or error in meiosis II whether PB1 is homozygous mutant or normal. In this case, no reliable prediction about the allele that is retained in the oocyte is possible.

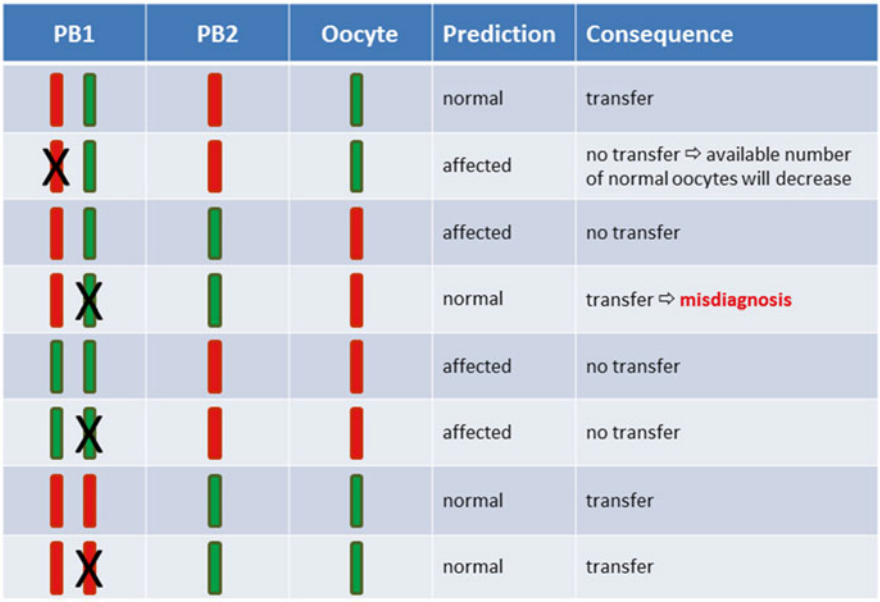

Fig. 8.3 Genetic analysis of polar bodies 1 and 2 (PB1 and PB2). Normal and mutant alleles are represented by *green* and *red bars*, respectively. PB1 contains two DNA molecules; PB2 and the oocyte contain only one. Allele dropout (ADO) in PB1 is indicated by an X. The most significant consequence of ADO is a loss or amplification failure of the normal allele in PB1. This leads to a misdiagnosis of the oocyte. In contrast, if the mutant allele in PB1 is affected by ADO and PB2 shows the mutant allele, the corresponding oocytes are not implanted although they contain the normal allele. This reduces the number of oocytes available for transfer

Hence, oocytes with a heterozygous (mutant and normal) PB1 and a hemizygous mutant PB2 always have the highest priority for transfer to the uterus. Using one, two, or three polymorphic markers closely linked to the mutation can reduce or even eliminate the risk of potentially affected pregnancy caused by ADO. In order to avoid misdiagnosis, the sequential genetic analysis of PB1 and PB2 is essential.

Contamination

Contamination of the single-cell PCR reaction is the other key contributor to misdiagnosis. To avoid maternal contamination, the cumulus cells have to be removed completely before biopsy, and the polar bodies have to be washed in PBS or medium. Similarly, paternal contamination can be prevented, if exclusively ICSI is used. So-called carry-over contamination, caused by the presence of previous PCR products, is probably the most significant source of contamination in single-cell PCR [45]. To prevent this, stringent conditions need to be applied, including a separate

PCR preparation room, working in laminar flow hoods, regularly cleaning and decontamination of work surfaces and equipment using DNA degrading detergents and ultraviolet light, aliquoting PCR reagents, and using dedicated laboratory coats, hair-caps, masks, gloves, and overshoes. In addition, numerous negative controls and blanks should be included and monitored during the whole process [45, 60, 61]. More details are available in the ESHRE best practice guidelines for PGD [7].

Conclusions

PBD is currently the only preconception genetic diagnostic method available in oocytes today, and this technique can play a meaningful role in selecting single embryos for transfer. The relevance and reliability of PBD is high, but workload and costs are also high. In addition, biopsy and genetic testing of PBs require a high degree of skill from embryologists, geneticists, and clinicians. The future of PBD is dependent on three main issues: (1) legal issues, (2) PBD results, mainly from the ESTEEM polar body study, and (3) results of preimplantation testing with cleaved embryos and blastocysts. The combination of PBD with trophectoderm preimplantation tests might increase the accuracy of both methods and become the diagnostic technique of choice.

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Conflict of Interest The authors declare no conflicts of interest.

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Chapter 9
Efficiency of Polar Body Biopsy on Aneuploidy Screening by DNA Microarray for Single Euploid Embryo Transfer

Shutao Qi, Ghassan Haddad, Craig Witz, and Weihua Wang

Introduction

It has been more than 50 years since the first identification of the relationship between trisomy 21 and Down syndrome [1], bringing to light the clinical importance of aneuploidy. Aneuploidy is a condition in which there is not an exact multiple of the haploid number of chromosomes. Aneuploidy is the most common chromosome abnormality in humans, and it is estimated that more than 5 % of all clinically recognized pregnancies are aneuploid, including trisomy, monosomy, and mosaicism [2]. Almost all autosomal monosomies and most trisomies are nonviable and spontaneously terminate during early pregnancy, which makes aneuploidy a leading cause of miscarriage. Some trisomies (such as trisomy 21) can survive and develop to term, but will lead to severe mental retardation and congenital birth defects. Recently, great progress has been achieved in the investigation of the prevalence and origin of aneuploidy in humans. It has been reported that embryonic aneuploidy increases dramatically with advanced maternal age and is one of the most important factors causing implantation failure of embryos produced by assisted reproduction technology (ART) [3–5].

To increase the implantation rate of human embryos produced by ART, several strategies have been carried out to select euploid embryos for transfer. Preimplantation

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genetic screening (PGS) is a technology used to screen for abnormal chromosome numbers in oocytes, cleavage embryos (typically day 3 embryos), and blastocysts. Such technologies have been used in human ART for many years. Conventional PGS uses fluorescence in situ hybridization (FISH) of 5–12 specific chromosome probes to examine chromosome integrity [6]. However, it has been found that conventional PGS procedures by FISH do not increase implantation rates and may even have negative effects on embryo implantation [7–9]. This is because FISH-based PGS only examines 5–12 chromosomes, but anomalies in human embryos can occur in any chromosome [10]. Hence, the information obtained by FISH-based PGS does not accurately represent the complete chromosome complement.

The technical limitation of FISH-based PGS can be overcome by comprehensive DNA analysis techniques, such as array-based comparative genomic hybridization (aCGH). All 23 pairs of chromosomes can be simultaneously examined with aCGH. It has been demonstrated that transfer of euploid blastocysts screened by aCGH can significantly increase implantation rates of embryos [10–12]. Currently, aCGH can be applied to oocytes before and after fertilization (polar body samples) or to embryos from the cleavage stage and the blastocyst stage. A comparative study of these three biopsies was reported previously and blastocysts were considered to be the optimal embryo stage to perform biopsies as multiple trophectoderm (TE) cells can be used for the test [13]. However, several studies have reported that the rates of mosaicism in human embryos at cleavage stage (Day 3) and blastocyst stage are extremely high [10, 14–16]. Thus microarray of biopsied blastomeres from day 3 cleavage embryos or TE cells from blastocysts may not accurately predict the chromosome complement in some embryos [10]. Furthermore, biopsies of both cleavage embryos and blastocysts are invasive procedures, and the risk of biopsy to embryo development should not be ignored. It then follows that in this situation, biopsy of polar bodies may be an alternative choice since mosaicism is not present in the oocytes or zygotes, and polar body biopsy is technically noninvasive.

Origin of Aneuploidy in Human Embryos

Theoretically, there are three possible origins of aneuploidy in human embryos: the oocyte (maternal meiosis), the sperm (paternal meiosis), and the zygote (early mitosis). Indeed, these three types of aneuploidy have all been found in aneuploid embryos. However, it is estimated that most cases of aneuploidy observed in human embryos are from maternal meiosis [17]. This is mainly due to the unique features of meiosis in human oocytes. Human oocytes have a prolonged arrest at the dictyotene stage in a process that begins during fetal life and completes after ovulation and fertilization. The first phase (meiosis I) consists of DNA replication followed by two rounds of DNA reduction, which is initiated during fetal development; meiosis resumption will not occur until after puberty, meaning that the oocytes will be arrested at prophase of meiosis I for 10–50 years.

The complexities of chromosome segregation and the long time of meiosis arrest provide ample opportunities for errors to arise. The second division (meiosis II) is

triggered by fertilization and is a relatively brief period compared to meiosis I. The timing of meiosis II appears to be critical. Long delays are poorly tolerated. Oocytes at this stage must be fertilized in a short time window. Missing this window may also cause abnormal meiosis. Thus, this short period of meiosis II arrest also provides opportunities for errors to arise.

According to a study by Kuliev et al., of over 20,000 oocytes from 2,830 in vitro fertilization (IVF) patients, 31.1 % of aneuploidies originated from meiosis I, 33.7 % originated from meiosis II, and the remaining aneuploidies resulted from both meiosis I and II [18]. This suggests that aneuploidies originate equally from both meiosis I and meiosis II. Furthermore, based on analysis of natural conception and spontaneous miscarriage, it has been suggested that aneuploidy originates mainly from female meiosis I [17].

On the other hand, Handyside et al. analyzed aneuploidy in patients of advanced maternal age undergoing IVF using aCGH and found that over half of aneuploidies resulted from errors in meiosis II, and a significant proportion of meiosis I errors did not result in aneuploidy in the zygotes [19]. Moreover, the incidence of aneuploidy varies among different chromosomes. For example, trisomies 21 and 16 are predominately from meiosis I errors, while trisomy 18 is typically from meiosis II errors [18, 20]. These results demonstrate that aneuploidy in human oocytes randomly occurs in either meiosis I or meiosis II, or both stages.

The origin of aneuploidy in meiosis I was assumed to arise mainly from the failure of segregation of homologous chromosomes at anaphase I (non-disjunction). However, Angell et al. observed that oocytes contained additional or missing chromatids rather than whole chromosomes during the analysis of oocytes from an IVF program [5, 21]. It was then proposed that precocious separation of chromatids prior to anaphase I is the main factor causing aneuploidy in women. In fact, both types of chromosome error can be detected during female meiosis, but the frequency may be different based on patient age [22, 23].

According to Handyside's analysis of the pattern of segregation errors of the chromosomes in the two polar bodies and corresponding zygotes, almost all errors in meiosis I are caused by premature segregation of sister chromatids rather than non-disjunction of whole chromosomes [19]. For women of advanced maternal age, the premature separation of sister chromatids may be quite common [22–24]. Several studies have demonstrated that the cohesin complex which maintains the tight association of homologous chromosomes and sister chromatids is severely reduced in oocytes from old mice, which may cause weaker centromere cohesion and premature segregation of sister chromatids [25–27].

Polar Body Biopsy for Aneuploidy Screening by DNA Microarray

During oocyte meiosis, there are two rounds of chromosome reduction needed to yield a haploid egg. Meanwhile, two by-products, the first polar body (PB1) and the second polar body (PB2), are produced from meiosis I and meiosis II, respectively.

The polar body contains genetic material complementary to the corresponding oocytes. If the polar bodies contain abnormal (additional or reduced chromosomes/chromatids) chromosomes, then the corresponding oocytes should also have the incorrect chromosome complement. Therefore, aneuploid information in oocytes and embryos can be predicted by detecting the chromosomes/chromatids of PB1 and PB2. Polar body analysis was started 20 years ago and was mainly used for FISH-based genetic diagnosis [28]. After aCGH introduction to human ART, PGS with polar body biopsy by aCGH has been used to screen for aneuploidy in human oocytes and its clinical values have been evaluated [29–32].

Efficiency of Polar Body Biopsy for Aneuploidy Screening by DNA Microarray

There are many advantages of polar body biopsy for aneuploidy screening. Firstly, as polar bodies are by-products of meiotic divisions, they are extracellular material with no biological role after extrusion. Thus, polar body biopsy is noninvasive and should not interfere with fertilization and embryo development. In contrast, the biopsy of day 3 blastomeres can reduce the implantation potential of the biopsied embryos [33]. Secondly, as polar bodies are extruded during meiosis, they can be biopsied and analyzed before and after the oocytes are fertilized. Thus, the information can be obtained during early embryo culture. This is very important as the conventional aneuploidy screening processes are time-consuming, requiring at least 12 h to obtain the chromosome information [31, 34]. Biopsy of embryos, especially at the blastocyst stage, may require that the embryos be cryopreserved and subsequently transferred after the biopsy results are available. Although cryopreservation technology is advanced now, there are still risks that cryopreservation and thawing may affect the quality and developmental potential of embryos. However, for polar body biopsy and testing, the aneuploidy information could be obtained before the blastocysts are formed, so patients can still have fresh embryo transfers. Moreover, the origin of chromosome errors of the embryos could be determined from polar body testing while it could not be obtained from blastomere or TE testing.

Several studies have reported that the mosaic rates in human embryos at cleavage stage (day 3) and blastocysts stage are extremely high [10, 14–16]; thus PGS of samples biopsied from both day 3 cleavage embryos or TE cells may not accurately predict the chromosome status if the embryos are mosaic. By analyzing polar bodies, errors related to embryo mosaicism can be avoided since mosaicism is not present at oocyte or zygote stages.

Although polar body biopsy has many advantages compared to biopsy of blastomeres or TE cells, there are several limitations. First, the polar body analysis can only detect chromosome anomalies from maternal meiosis, so aneuploidies from sperm or early mitosis cannot be detected. Second, aneuploidies can occur equally from either meiosis I or meiosis II, so it is necessary to analyze both PB1 and PB2 [35]; then, two biopsies and examinations are required, which will increase the

cost of PGS. Third, polar body biopsy and DNA amplification are technically much more difficult, as both PB1 and PB2 are tiny in size and the amount of DNA is limited. In some cases, the first polar bodies may degenerate rapidly if not biopsied in time [36]; thus incorrect information may be obtained after array analysis [36].

Accuracy of PB Biopsy as Compared with Day 3 and Blastocyst Biopsy

The most important criteria to assess polar body biopsy is its accuracy in predicting the aneuploidy of embryos and the improvement of implantation and pregnancy rates. A study was undertaken by the European Society for Human Reproduction and Embryology (ESHRE) PGS Task Force to investigate the reliability of PGS by polar body biopsy with whole genome amplification and aCGH analysis. In this study, both PB1 and PB2 were biopsied from a total of 226 zygotes in 41 couples with an average maternal age of 40 years. The corresponding zygotes were then processed for aCGH analysis if aneuploidies were found in either or both of the polar bodies. According to this study, the concordance rate between the PBs and the corresponding zygote was 94 %, which showed high reliability of aCGH analysis of polar bodies [31].

This work was reinforced by the study of Christopikou et al. in which 93 % of the aneuploidies in the cleavage stage embryos were associated with copy number changes in the polar bodies, 98.5 % of samples had been predicted to be aneuploid, and 100 % cleavage stage embryos predicted to be aneuploid by polar body aCGH analysis were confirmed to be aneuploid [30]. However, Scriven et al. reanalyzed the published data of the ESHRE pilot study with a theoretical model and found that the accuracy of polar body testing may be high, but the predictive value of an abnormal test result was <100 %, especially when the overall aneuploidy rate was low, indicating that some (>10 %) normal zygotes may therefore be excluded incorrectly [37]. Furthermore, Capalbo et al. compared complete comprehensive chromosomal screening data including PB1, PB2, corresponding blastomeres of day 3 embryos, and TE samples of blastocysts from women over 40 years old and all samples were analyzed by aCGH. They found that only 79.5 % (62/78) of the meiotic errors in either one or both PBs were consistent with the aneuploidies observed in their resulting embryos, and as high as 20 % of female-derived aneuploidies detected on polar bodies and confirmed on Day 3 were corrected at the blastocyst stage [38]. They suggested that the accuracy of the polar body biopsy approach was significantly lower than the blastomere and TE cell analysis. Therefore the time of biopsy should be postponed to the blastocyst stage in order to obtain the most reliable results. Also, it would appear that there is controversy about the real accuracy of polar body analysis. This may be due to the differences in the population of samples used by different studies or the presence of mosaicism in the cleavage embryos and blastocysts.

Table 9.1 Comparison of clinical outcomes by the transfer of embryos biopsied at various stages and screened with aCGH

Biopsy	Clinical pregnancy rate per transfer	Ongoing pregnancy rate per transfer	Implantation rate per transfer	References
Polar body[a]	33 % (8/24)	29 % (7/24)	26 % (10/39)	[31]
	35.2 % (38/108)	27.7 % (31/108)	27.7 % (31/112)	[29]
	43 % (3/7)	43 % (3/7)	40 % (4/10)	[30]
Day 3 blastomere	Unknown	54 %	39 %	[40]
	81.8 % (27/33)	72.7 % (24/33)	52.63 % (30/57)	[11]
	60.3 % (123/204)	53.9 % (110/204)	53.5 % (161/301)	[39]
TE from blastocyst	70.9 % (39/55)	69.1 % (38/55)	70.9 % (39/55)	[41]
	70.2 % (26/37)	70.2 % (26/37)	63.5 % (33/52)	[10]
	58.8 % (20/34)	Unknown	54.3 % (25/46)	[12]
	Unknown	67 %	61 %	[40]

aCGH array-based comparative genomic hybridization, *TE* trophectoderm
[a]Both polar bodies I and II were biopsied

As for the clinical outcomes for polar body aCGH, as shown in Table 9.1, the clinical pregnancy rates after polar body biopsy and aCGH were 19–25.3 % per cycle and 33–43 % per transfer, and the ongoing pregnancy rates were 13–20.7 % per cycle and 27.7–43 % per transfer. The implantation rates were 26–40 % per embryo transferred [29–31]. In contrast, the reported clinical pregnancy rates of day 3 blastomere testing were 38.4–69.23 % per cycle and 60.3–81.8 % per transfer, and the ongoing pregnancy rates were 34.4–61.54 % per cycle and 53.9–72.7 % per transfer. The implantation rates were 39–53.5 % per embryo transferred [11, 39, 40]. The reported clinical pregnancy rates of TE cell testing were 27.8–70.9 % per cycle and 58.8–70.9 % per transfer, and the ongoing pregnancy rates were 50–69.1 % per cycle and 67–70.2 % per transfer. The implantation rates were 54.3–70.9 % per embryo transferred [10, 12, 40, 41]. Therefore, the overall clinical outcomes of polar body approach were not as good as the results of day 3 blastomeres and TE cell analysis. However, as polar body aCGH is not widely used, the sample size of these pilot studies are small and the results may not provide sufficient statistical power to make a final conclusion. Obviously, a randomized clinical trial with a larger sample is needed to validate the clinical outcomes of polar body aCGH as compared to day 3 blastomere and TE cell approaches.

Single Embryo Transfer After Biopsy and aCGH

Limited data are available on the clinical outcome with single embryo transfer after polar body biopsy and DNA microarray analysis. After polar body biopsy and DNA microarray analysis, the resulting euploid embryos may be transferred fresh on day

3 at cleavage stage or days 5 and 6 at blastocyst stage. They could also be frozen for a later frozen embryo transfer. So a comparison of efficacy is more difficult due to these numerous factors. Based on our experience with a limited number of cases ($n=55$) of frozen single blastocyst transfer in which the embryos were biopsied and tested with DNA microarray at the blastocyst stage, the clinical pregnancy rate was as high as 63.6 % in women of advanced ages (37–42 years old) during 2012. The rate is comparable to the rates with fresh single blastocyst transfer (19/38, 50 %) and frozen–thawed single blastocyst transfer (122/222, 55 %) in young patients (<35 years old) without aneuploidy screening. These results were also comparable to multiple embryo transfer in our clinic. These results indicate that aneuploidy screening by aCGH is helpful for patients of advanced maternal age to obtain high pregnancy rates and single euploid blastocyst transfer is recommended.

As polar body biopsy is a noninvasive procedure and theoretically and practically it is safer than blastocyst biopsy, it would be possible to get the same pregnancy rate if the biopsy is properly done in the oocyte and zygote stage, and the resulting embryos were transferred at later stages. However, because blastocyst development rates of human eggs obtained from patients undergoing ART are 50–60 %, it is suggested that single embryo transfer should be performed after the embryos develop to blastocyst stage (i.e., biopsy should not be performed on day 3 cleavage stage or earlier stages). The single embryo transfer can be done with either fresh or frozen blastocysts. Similar pregnancy and implantation rates should be obtained with fresh and frozen blastocyst transfer as blastocyst cryopreservation by vitrification does not affect the embryo survival with the optimized protocol.

Conclusions

DNA microarray technology has revolutionized life science research and medical diagnosis. Its application in PGS has brought about great improvement to the success of human ART. However, due to the complexity of various aneuploidies and chromosome mosacism in cleavage embryos and blastocysts, it is difficult to examine all chromosome abnormalities by a single PGS approach. For different patients and different situations, different PGS procedures should be applied in order to get the best clinical results. Although the blastocyst stage is now considered to be the optimal embryo stage to perform biopsies for PGS, polar body biopsy technology, for its noninvasive nature (and earlier PGS result availability), may be worthy of consideration in patients with few eggs and further embryo culture and/or blastocyst transfer is not necessary. Further randomized clinical trials are required to confirm the clinical value of polar body analysis for the improvement of clinical outcomes.

Conflict of Interest The authors declare no conflict of interest.

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Chapter 10
Prediction of Embryo Viability by Morphokinetic Evaluation to Facilitate Single Transfer

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Introduction

Optimal culture conditions and reliable embryo selection constitute two major challenges for successful IVF treatment. Embryo quality is typically assessed by the use of grading systems based on morphological evaluation under a microscope at certain, distinct time points. This methodology has several limitations. The inability to accurately assess embryo quality constitutes a hindrance for evaluating the impact of culture conditions and for estimating the reproductive potential of an embryo. The recent development of clinical time-lapse instruments has enabled continuous monitoring of human embryos, hereafter referred to as time-lapse imaging (TLI). TLI, where consecutive images are obtained during embryo culture by using a microscope and a camera, allows for a refined evaluation of known morphological parameters and represents a new method of evaluating embryo viability. Several retrospective studies have demonstrated a correlation between timing of key events and developmental or implantation potential, which suggests time-lapse imaging as a promising method for a more reliable embryo selection than morphology alone. However, as we expand our knowledge of pre-implantation embryo development, it becomes increasingly clear that timing is influenced by several patient- and treatment-related factors. This may complicate the establishment of a prediction

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model for optimal embryo development that may be applied under a variety of conditions across heterogeneous patient groups. This chapter addresses the use of TLI in the evaluation of pre-implantation embryo development and pregnancy potential in an effort to provide an overview of the feasibility and potential use of TLI in IVF treatment.

Scoring of Static vs. Dynamic Parameters

Traditionally, the quality and viability of pre-implantation embryos are evaluated by a microscopic inspection at a few, well-defined discrete time points. There is a well-documented close correlation between morphological appearance and developmental stage of the embryo at given time points and developmental competence (as reviewed by ALPHA and ESHRE [1]). Due to the simplicity and cost-effectiveness of static morphological grading and lack of documentation for existing alternative methods, traditional morphological evaluation therefore remains the choice method for embryo evaluation. Nevertheless, this approach has several recognized limitations. Firstly, the information obtained with a few, discrete time point provides an incomplete picture of the inherent dynamic process of embryo development, as illustrated by the observation that embryo score may change markedly within a few hours [2]. This limitation is obviously overcome with continuous monitoring. Furthermore, morphological scoring of embryos has shown substantial inter- as well as intra-observer variation, which in turn has implications for the decision to transfer, cryopreserve, or discard the embryos [3–5]. A probable cause for this variation is that assessment in categories tends to be rather imprecise. In contrast, the assessment of time-lapse parameters appears to have a high degree of intra- and interobserver agreement [6]. Theoretically, this agreement will depend on the instrument used, in particular the resolution, the number of focal planes, and the intervals between the photographic recordings. Any variation in clinical decision-making remains to be assessed, as no model has presently been prospectively validated, as discussed in detail below. TLI necessitates periodical light exposure, use of moving devices, and magnetic fields that constitute potential risks to the embryos. The safety of TLI for IVF has been documented in two trials conducted with the same instrument. Embryo development was the primary endpoint in both trials [7, 8]. As for any new method introduced in the ART laboratory, a sufficiently powered study using pregnancy rate or live birth rate with pediatric follow-up would be preferable before any definitive conclusions are drawn. Likewise, it must be noted that both trials were conducted using the same TLI instrument and that the conclusions may not necessarily extend to include other systems.

Introduction to Time-Lapse Parameters

While time-lapse monitoring is a rather novel method in the ART lab, the method has been used for nearly a century to study embryo development for research purposes [9]. Prior to the introduction of clinical instruments, research was conducted

on embryos from various animal species or more seldom, surplus human embryos. Initially, the studies were aimed at describing the process of development, but as IVF was introduced, the attention was directed toward the potential use of time-lapse imaging to characterize division patterns and dynamic parameters that potentially would identify embryos that are viable beyond the time of observation. The following section describes typical in vitro development of a pre-implantation human embryo and the events that are visible and thus recordable in a time-lapse analysis.

Development of a human embryo begins with fertilization. The spermatozoon penetrates the extracellular multilayer glycoprotein coat, zona pellucida (ZP) [10], and the spermatozoon membrane fuses with the oocyte membrane [10]. The associated formation of the male pronucleus can be visualized with time-lapse monitoring. During normal fertilization the fusion of the two membranes initiates oocyte activation, leading to the completion of the second meiotic division of the oocyte. This stage is visualized by the extrusion of the second polar body 3–7 h after fertilization [11] followed by the visible formation of the male and female pronuclei. The male and female pronuclei (*pn*) start replicating their DNA as they migrate toward each other in the zygote. This process can be visualized morphologically as syngamy/abuttal of *pn* [12]. After DNA replication, the two nuclear envelopes break down, and the 2*pn* are no longer visible. The zygote subsequently enters the first mitotic division and cleaves and two embryonic cells, or blastomeres, are formed. The process from formation of the cleavage furrow until complete separation of the two daughter cells is denoted first cytokinesis [13, 14]. The first cleavage cycle is completed with the first division early on "day 2," 24–29 h after fertilization [15–17]. The two embryonic cells divide during the second cleavage cycle, forming a 4-cell embryo on day 2.

The third cleavage cycle results in the formation of an 8-cell embryo on day 3, followed by a final round of cell divisions, before compaction occurs, visualized as obscured intercellular boundaries, and the embryo develops into a morula on day 4. Shortly after the morula stage a fluid-filled cavity develops. This appearance of this cavity, the blastocoel, defines the beginning of the early blastocyst stage [10]. This cavity expands until it fills most of the embryo (full blastocyst stage). Continued expansion leads to a progressive thinning and, eventually, focal rupture of the surrounding zona pellucida (ZP). Escape of the mammalian embryo from the ZP, referred to as hatching, is initiated on day 5–6 in vitro.

Deviations from the above description of a normal in vitro development are often observed and are of particular interest as they presumably represent underlying abnormalities. An extreme short duration of the first division cycle (the 2-cell stage), referred to as a direct cleavage from one to three cells, is often observed in tri-pronuclear embryos presumably as a result of an excess centriole [18]. Direct cleavage from one to three cells is however also observed in embryos with a presumed normal chromosomal content where the deviation is associated with a significantly lower implantation rate compared to embryos with a normal cleavage pattern [19, 20]. Likewise, an aberrant first cytokinesis has been correlated to decreased developmental potential [14]. These studies illustrate the potential benefits of characterizing not only optimal division patterns but also deviations from the normal pattern as a single embryo is selected for transfer.

Predictive Algorithms

First to demonstrate the potential of morphokinetic-based predictive models, Wong and colleagues [14] predicted the developmental fate of 4-cell embryos with exceptional sensitivity (93 %) and specificity (94 %). In this model, a combination of three morphokinetic parameters (duration of first cytokinesis, interval between first and second mitosis, and interval between second and third mitosis) was used successfully to predict blastocyst formation or developmental arrest. More recently, Conaghan and colleagues chose to reevaluate solely these parameters during development of a morphokinetic model for prediction of usable blastocysts (blastocysts selected for transfer or frozen storage on day 5) [13]. In this large study, morphokinetic data from five clinical sites were collected from embryos cultured to blastocyst. No patient and treatment selection criteria were used or restrictions to culture conditions enforced [13]. Notably, the resulting predictive algorithm did not achieve the same sensitivity as Wong et al., but when validated on a large independent dataset it was much better at identifying embryos that were less capable of developing to usable blastocysts than those that did (specificity of 84.7 %, sensitivity of 38.0 %, PPV 54.7 %, and NPV 73.7 %).

Although blastocyst formation and quality has been used as a measure of embryo viability in a number of morphokinetic studies and confers a number of practical advantages when researching and validating new technologies [21, 22], the information generated only becomes useful when translated into pregnancy and live birth outcome. Only a few studies have investigated the compatibility between morphokinetic prediction of blastocyst formation and quality and prediction of pregnancy outcome and these studies have demonstrated conflicting results. The aforementioned blastocyst prediction model [13] was subsequently tested on a large combined set of transferred embryos with known clinical outcome [23]. This study demonstrated that the model was somewhat effective with a relative increase of 30 % for implantation in the model-selected group of embryos, but it fell short, as there was a concomitant large rejection of embryos from the test cohort, which actually resulted in pregnancy. This highlights the limitations of predicting blastulation only.

Hlinka et al. [24] showed that only 26.4 % of timely blastocysts resulted in a successful implantation, not surpassing current IVF success rates [24]. Moreover, both Kirkegaard et al. [25] and Chamoyou et al. [26] identified several morphokinetic parameters as significant predictors of high-quality blastocyst development, but these same parameters were unable to discriminate between implanted and unimplanted embryos [25, 26]. In dramatic contrast, Dal Canto et al. [16] showed that significantly shorter cleavage times from the 2-cell to 8-cell stage were predictive of embryos that develop to blastocysts, expand, and implant [16]. In another study, optimal cleavage stage timings proposed for implantation success have also been successfully shown to identify a large proportion of embryos that develop to blastocysts with good morphology [15]. It would seem that further studies are needed to elucidate the interpretation of these discrepancies and determine if predictive algorithms trained to predict blastocyst development could be used to predict implantation.

The first group to construct a morphokinetic-based model to predict implantation potential developed a hierarchical model that uses both morphological observations and kinetic timings to rank embryos in 10 different categories of descending implantation potential [17]. First, embryos are discarded by a set of exclusion criteria including poor morphology, direct cleavage from 1 to 3 cells, uneven blastomere size at 2-cell stage, and multinucleation at 4-cell stage. Then timings of three morphokinetic parameters were ordered according to predictive strength: time to 5-cell stage, time interval between second and third mitosis, and time interval between first and second mitosis are used to characterize embryos depending on timings lying in or out of acceptable ranges. These optimal time ranges were defined by the timings of 247 implanting and non-implanting embryos that were first subdivided into quartiles and the two consecutive quartiles with the highest number of implanting embryos were then selected as in-range values. Embryos that did not develop within these time intervals were considered out of range. This group suggested that categorization of embryos from high to low implantation potential according to this model was improved when compared to using morphology alone (AUC 0.72 vs. 0.64). Nevertheless no statistical difference in implantation rate was found between embryos in the highest scoring category compared to embryos of highest morphological grade [17]. Subsequently, the same group tested the application of this model to data collected from 10 clinical sites in a larger retrospective study and suggested that a relative improvement to the clinical pregnancy rate of 20.1 % per embryo transfer could be achieved compared to a control group of embryos cultured in conventional incubators and selected solely by static morphological grade [27]. However, this study was not randomized and the improved clinical pregnancy rate could also be explained by better culture conditions supplied in a time-lapse incubator compared with the traditional incubator or selection bias. So far no prospective controlled trial has been published to determine if embryo selection using this time-lapse model can improve IVF success rates. The IVI group has recently completed a randomized study. Yet unpublished results report significantly improved ongoing pregnancy rate (51.4 % vs. 41.7 %; $p=0.01$) and implantation rate (44.9 % vs. 37.1 %; $p=0.02$) for embryos selected using time-lapse criteria compared with selection by standard morphological criteria (Rubio et al. [20]). It has been demonstrated, though, that the tested selection model was not transferable from one clinical setting to another without modifications [28], thus underlining the difficulties in determining universal criteria for optimal division patterns.

Since these studies were published, similar hierarchical models to predict implantation have been described and again quartiles yielding highest number of implanting embryos were used to define optimal time ranges and embryos developing in range have been shown to have higher implantation rates than those embryos developing out of range [29, 30]. Additionally, several investigators have confirmed that shorter durations of cell cycles and synchronous divisions of sister blastomeres are strongly predictive of implantation and that prolonged durations in one or more cell cleavage cycles and aberrant cleavage behavior are characteristics of non-implanting embryos [16, 20, 24, 30]. Most strikingly, abrupt cleavage from one to three cells, defined by a short 2-cell duration of <5 h, has been shown in a number

of studies to be a strong negative marker of implantation [17, 20, 25]. This abnormal cleavage pattern has largely been unnoticed in static routine observations before the introduction of TLI monitoring. It may be argued that the superior ability of morphokinetic models to identify less viable embryos rather than identify embryos of highest reproductive potential may create the basis for a strategy of time-lapse based embryo selection that will translate into improved clinical outcome. Such an approach will have particular relevance in the setting of single embryo transfer.

Recently, the correlation between timing of kinetic parameters and embryonic aneuploidy, has been the focus of several morphokinetic studies [19, 29, 31–33]. In the past, morphology and sequential embryo scoring systems have had limited success at identifying aneuploid embryos [34–37] and static observation of multinucleation on days 2 and 3 has been shown to have a positive association with aneuploidy and used routinely to deselect embryos [38, 39]. However, a number of preliminary studies suggest that morphokinetic behavior can be used to increase the probability of selecting euploid embryos without invasive genetic screening. A number of small studies report possible correlations between timings of early mitotic divisions and embryonic aneuploidy [33, 40–42]. One of these studies suggests that delayed first and second cleavage divisions and a prolonged transition from the 2- to 4-cell stage were significantly correlated to aneuploidy, in particular multiple aneuploidies [40]. This study also confirmed that embryos undergoing abrupt cleavage from 1- to 3-cells and 2- to 5-cells are predominately aneuploid. Chavez et al. [33] observed cell cycle parameters for 45 embryos up to the 4-cell stage and found that euploid embryos displayed tightly clustered timings when compared to aneuploid embryos, which had more widely distributed comparative timings. In this study, only 30 % of aneuploid embryos displayed normal timings and these normal timings were determined to predict embryonic euploidy with 100 % sensitivity and 66 % specificity [33]. Most recently, a much larger study analyzing the chromosomal content of 504 embryos by blastomere biopsy on day 3 and array CGH created a hierarchical model to subdivide embryos into four categories (A–D) according to expected risk of aneuploidy [29]. The two morphokinetic variables used in this algorithm included time interval between 2 and 5 cells (>20.5 h) and duration of the third cleavage cycle (t5–t3) (11–18 h). Embryos categorized according to in- or out of range timings suggested by this model showed a significant decrease in the percentage of normal embryos for each decreasing category (A, 35.9 %, B, 26.4 %, C, 12.1 %, and D 9.8 %; $p < 0.001$). Interestingly, this algorithm was better at predicting blastocyst formation, which was interpreted by the authors as strengthening their findings. The area under the curve was 0.634.

A similar number of time-lapse studies have not identified an association between early cleavage timings and blastocyst aneuploidy as determined by trophectoderm biopsy and 24-chromosome analysis [19, 43–45]. In contrast, one of these studies suggested a simple classification model using timing of initiation of blastulation and timing of full blastulation to classify embryos into high-, medium-, or low-risk categories, with an area under the curve of 0.72 [19]. An assumption that TLI parameters correlate with aneuploidy is hardly justified if the same parameters are not

predictive to implantation potential. When this model was tested on a group of transferred blastocysts ($n=88$) from un-selected non-PGS IVF patients and related to implantation and live birth outcome, the risk classification was shown to correlate to clinical outcome. Interestingly, the relation was consistent, even when accounting for an important confounding parameter, such as age [31, 32]. The other significant variable identified to differ, between embryos with multiple aneuploidies only and euploid embryos in the Campbell study, was the time to the start of compaction (tSC) [19]. Several other small studies considering ploidy and morphokinetics have reported peri-compaction and cavitation delays in aneuploid embryos diagnosed by comprehensive chromosome screening methods of trophectoderm biopsies. Montgomery et al. reported that where the duration of compaction was <22 h, fragmented embryos were significantly more likely to produce a euploid blastocyst ($p=0.009$) compared with embryos with longer compaction periods [46]. Melzer also reported longer duration of compaction in aneuploidy blastocysts compared with euploid, using TLI and blastocyst biopsy techniques ($p<0.004$) [47]. Delays in later developmental stages were also described by Hong et al. [48]. This group reported longer duration to the start of cavitation in aneuploid embryos. The two significant variables providing some discrimination of aneuploidy risk were the time from first cytokinesis ($p=0.02$) or from the 5-cell stage ($p=0.01$) to the onset of cavitation ($p=0.01$)—when the data were considered in quartiles. Ultimately, morphokinetic-based embryo selection models should focus on healthy euploid live birth as the outcome measure. A promising study of over 200 embryos with known implantation outcome data, which did this, presented an early cleavage algorithm with an area under the curve of 0.8 [49].

Limitations for Model Building: Sensitivity, Specificity, and Confounders

In summary, a large number of publications confirm that timing of development does indeed differ between viable and nonviable embryos. The challenge is that most studies show divergent results and that no consensus therefore exists on which parameters are the most predictive. Only a few publications have offered clinically applicable models of embryo selection [13, 17, 31] and these models remain to be validated in randomized trials.

Developing valid time-lapse models applicable to heterogeneous patient populations and in different clinical settings is difficult, as multivariate hierarchical selection models [17] have been shown not to be transferable from one clinical setting to another without modifications [28]. Similarly, in a hypothetical experiment, where a blastocyst prediction model [13] was applied retrospectively on a large set of transferred embryos, a theoretical increase of 30.0 % in implantation rate for embryos grouped as usable compared with the entire test cohort was demonstrated. Notably, 50.6 % of embryos that were categorized as having a low chance of forming usable blastocyst nevertheless resulted in fetal heart beat [23]. While a part of

the explanation may be found in heterogeneous patient populations and different clinical settings, it also emphasizes one of the crucial dilemmas in developing diagnostic tests in general—the balance between sensitivity and specificity. The study very nicely illustrates the risks of defining too narrow time intervals for optimal division in order to achieve a high specificity at the expense of a low sensitivity. It thus underlines the importance of carefully considering that a model must not only provide a substantial increase in implantation, but equally important, that a low rejection rate of viable embryos is secured.

Other plausible explanations for the diverging conclusions on which parameters are most predictive are most likely to be found in the distinct differences in the population of embryos studied, the parameters evaluated, the endpoints chosen, and the differences in treatment-related factors and culture conditions between the studies.

Embryos from fertile oocyte donors have been shown to proceed faster through the first cellular divisions compared to embryos from infertile patients [50]. However, it remains unanswered whether the significant differences in age may explain that particular finding. In mice, culture in 20 % oxygen significantly delays all stages of embryonic development compared to culture in 5 % oxygen [51] as well as influences the embryonic metabolism [52]. In humans, culture in high oxygen appears to delay pre-compaction development [53]. ICSI-fertilized embryos have been reported to display an earlier first cleavage than IVF-fertilized embryos [16, 54–56]. The observed difference most likely originates from a difference in the starting time of registration or oocyte activation [57], and can therefore be overcome by normalization to an early event or durations of events. The difference does, however, complicate the comparison of absolute time points between IVF and ICSI populations which is overcome by using intervals between events.

The choice of medium has been shown to influence the cleavage rates for human embryos [58–62]. Surprisingly, a recent study did not report any correlation [63], which indicates that the impact may depend on the type of medium and perhaps a combination with other factors as well. Factors relating to the infertility treatment, such as gonadotrophin doses, have been reported to affect timing, with embryos from oocyte donors receiving higher doses of gonadotrophin reaching advanced developmental stages later than those receiving lower doses [64]. Since gonadotrophin doses were presumably administered according to the treatment response of the patient, it could be argued that the finding might be correlated with patient-related factors, such as age and ovarian response which are both interrelated—and correlated to prevalence of aneuploidy—rather than the differences in stimulation per se. Finally, studies on time lapse have been conducted using different time-lapse systems. A difference in technology could potentially influence the assessment of the embryo and limit the comparability between studies.

These confounding factors become even more important if the time intervals of optimal division are defined too narrowly, as small displacement in timing may result in viable embryos being declared nonviable. Arguably, low sensitivity may be attributed to the influence of many of these confounding factors demonstrated to affect embryo kinetics and viability, in particular maternal age, oxygen tension, fertilization method, and culture media, which are rarely considered in the predictive models.

Albeit even controlling for these confounders during collection of morphokinetic data, moderate predictive values were still attained by some predictive models [25].

Future Directions

An increasing number of studies demonstrate that timing of development differs between viable and nonviable embryos, evaluated by blastocyst development, clinical pregnancy, or euploidy. The future challenge is to translate this knowledge into clinically useful models that will improve pregnancy rates after single embryo transfer.

A major challenge is that timing not only reflects viability but is also influenced by patient and treatment-related characteristics, which must be considered when predictive models are developed. How much of the variation in timing that is explained by viability and culture conditions remains to be clarified. The difficulties in transferring a model from one clinic to another may, however, indicate that one model does not fit all. Furthermore, concern must be taken for both sensitivity and specificity.

Several studies have reported that shorter durations of cell cycles and synchronous divisions of sister blastomeres are strongly predictive of implantation while prolonged durations in one or more cell cleavage cycles and aberrant cleavage behavior are characteristic of non-implanting embryos. The superior ability of morphokinetic models to identify less viable embryos by deviations from the normal division pattern, rather than to identify embryos of highest reproductive potential by defining optimal division patterns, may indicate that the true potential for TLI lies in de-selection of embryos. The association between poor viability, aneuploidy, and certain TLI patterns supports this. Likewise, attention could profitably be directed toward identifying normal and abnormal patterns in embryos from the individual patient combined with a traditional evaluation of morphology, rather than focusing exclusively on defining time points for optimal division, which most likely vary depending on external factors. This might be combined with broad intervals for timing with sufficient respect for sensitivity and specificity, in particular perhaps at the later stages of development.

Conflict of Interest The authors declare no conflict of interest.

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Chapter 11
Utilisation of Transcriptome-Based Biomarkers for Single Embryo Transfer

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New Approaches for Embryo Selection

The only currently available method for non-invasive assessment of oocytes and embryos is based on different morphological parameters. These are assessment of the polar body, meiotic spindle, zona pellucida, and cytoplasm in oocytes and pronuclear oocyte morphology, the time to the entry into the first mitotic division, fragmentation rate, blastomere number, and morphology in embryos [1]. However, there is growing evidence that the subjective morphological assessment alone does not accurately predict oocyte's developmental potential and embryos with the highest chance of implantation, as even embryos considered to be morphologically perfect do not always implant in the uterus [2, 3]. For this reason, non-invasive, objective, and reliable markers that could identify the best quality oocytes and embryos with the highest implantation potential without compromising the success of IVF procedures in single embryo transfer (SET) are needed [4].

There have been several methods for embryo quality assessment proposed in recent years. These are measurement of amino acid turnover in embryo culture media [5], proteomic analysis of embryos [6], follicular fluid content analysis [7], embryonic metabolism analysis [8], apoptosis of granulosa (GC) and cumulus cells (CC) [9, 10], and the morphokinetic assessment of embryo viability using time-lapse technology [11].

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In the past decade, intensive research has been made in the area of gene expression activity in oocytes, GC and CC, and embryos, believing that gene expression profile could provide an insight into oocyte competence and embryo viability [12].

Transcriptomics in Reproductive Medicine

The use of transcriptomic analysis in reproductive medicine could improve understanding of important physiological processes [13] or for discovery of genetic biomarkers of oocyte and embryo quality and endometrial receptivity [14–16]. The most common approach for the investigation of gene expression involves a combination of microarray analysis followed by quantitative real-time PCR (qPCR). DNA microarray technology is a tool for the determination of genome-wide gene expression at the level of messenger RNA (mRNA) [17, 18] and usually provides us with a long list of potentially differentially expressed genes. It is essential to confirm the validity of these data by using qPCR, which gives a more reliable quantification of mRNA levels for the selected, potentially important genes [19].

Transcriptomic Analysis of Oocytes

It is well known that the presence of a mature oocyte is essential for its fertilisation and early embryo development [20]. Oocytes of several organisms show large transcriptional activity that reflects the importance of maternal RNA and proteins not only during oogenesis but also during early embryo development, until the activation of embryonic genome [21, 22]. The precise control of complicated transcriptional mechanisms during oogenesis is required for the oocyte's final maturation and competence acquisition. However, these mechanisms are still largely unknown and transcriptomic analysis enables the improvement of understanding of oocyte maturation and competence acquirement.

Assou et al. [23] established that there are more than 400 genes overexpressed (and over 800 genes underexpressed) in mature human metaphase II (MII) oocytes when compared to immature oocytes [germinal vesicle (GV), metaphase I (MI)]. Among the overexpressed genes there are those involved in meiosis process (*CDC2/CDK1*, *CCNB1*, *CCNB2*), spindle checkpoint components (*BUB1*, *BUBR1*, *MAD2L1/MAD2*), as well as several oocyte-specific genes, such as the Zona Pellucida genes (*ZP 1*, *ZP 2*, *ZP 3*, *ZP 4*).

Furthermore, there was a progressive decrease of the number of genes expressed during oocyte nuclear maturation with the lowest number of genes expressed in MII oocytes compared to GV and MI. Similarly, analysis of global gene expression profiles of mouse GV and oocytes revealed substantial differences, with over 1,600 genes overexpressed in GV and over 2,000 in MII oocytes [24]. Comparison of gene expression profile between sheep oocytes and GC also revealed overexpression of genes involved in meiosis in these oocytes [25].

Reproductive capacity declines with female age due to depletion of number of oocytes as well as decline in their quality. Several studies have found that the global gene expression profile in oocytes is related to female age [26–28]. The expression of oocyte genes related to cell cycle regulation, cytoskeletal structure, energy pathways, transcription control, and stress responses [26] as well as those related to the control of spindle organisation, protein metabolism, DNA repair, and meiosis [27] are all dependent on female age. Gene expression profiling of young and aged mouse oocytes revealed there were 530 genes significantly differentially expressed; 449 showed decreased expression, and 81 showed increased expression with maternal ageing [28]. Moreover, a group of genes providing protection against stress responses, cellular damage, and apoptosis showed decreased expression in aged oocytes. All of these findings unravel the possible involvement of different genes and biological pathways associated with oocyte senescence and decline of quality.

Of note, mature MII oocytes derived from women with polycystic ovary syndrome (PCOS) are morphologically indistinguishable from oocytes of women with normal ovaries. However, a comparison of gene expression profiles of normal and PCOS oocytes showed these oocytes have quite dissimilar gene expression profiles [29]. Annotation of the most differentially expressed genes revealed two distinct groups: maternal-effect genes and genes involved in the meiotic/mitotic cell cycle. Maternal-effect genes produce mRNA and/or protein during oogenesis required for early embryo development, before the activation of zygotic genome [30]. These results suggest that there are abnormalities in PCOS oocytes at the molecular level that could be responsible for reduced quality of PCOS oocytes and reduced fertility of women with PCOS.

A comparison of transcriptomes of in vivo and in vitro matured, MII, oocytes revealed important differences in gene expression [31]. The process of in vitro oocyte maturation could be useful in women with PCOS who have an increased risk for ovarian hyperstimulation syndrome (OHSS) after the use of gonadotropins for controlled ovarian hyperstimulation and in cases where immature oocytes are obtained after ovarian puncture in IVF procedures that might otherwise be discarded. Wells and Patrizio have established that the expression of genes for nuclear maturity is similar in in vitro and in vivo matured oocytes. The expression of genes for cytoplasmic maturity in in vitro matured oocytes, however, was more similar to expression in GV [32]. Disturbances of cytoplasmic maturity of in vitro matured oocytes could contribute to the development of poor-quality embryos and early pregnancy loss [33].

Despite the careful description of complex gene expression profiles in human oocytes, the connections among expressed genes and the fertilisation process, embryo development, and conception remain poorly understood.

Transcriptomic Analysis of Granulosa and Cumulus Cells

Oocyte maturation and competence are acquired during follicular development where GC and CC play an essential role [34]. The oocyte plays a dominant role in regulating GC and CC functions during folliculogenesis via secretion of paracrine factors that maintain an appropriate microenvironment for acquisition of its competence [35, 36]. Growth differentiation factor 9 (GDF9) and Bone morphogenetic

protein 15 (BMP15) are mitogens that are secreted by the oocyte and they induce follicle growth, cumulus expansion, and signalling pathways in GC and CC. Accordingly, it is believed that functions of GC and CC indirectly reflect oocyte competence [37]. Cell functions and active cell processes are regulated through gene expression. Gene expression analysis in GC and/or CC could therefore provide a non-invasive method for identification of the most competent oocytes and embryos. Furthermore, these cells are easily accessible and discarded during IVF procedures and can be sampled without compromising the oocyte.

The concept of finding biomarker(s) of oocyte and/or embryo quality in GC and/or CC that would objectively and reliably predict successful embryo implantation is highly desirable. By finding such biomarker(s), single embryo transfers could be used in IVF procedures without compromising the clinical success rate. One of the first studies in this area investigated the correlation between CC gene expression and in vitro embryo development [38]. These investigators compared the expression of *HAS2*, *PTGS2,* and *GREM1* in CC between high- and low-grade embryos by using qPCR. The expression of these genes (each of which are activated by *GDF9* and contribute to CC expansion during folliculogenesis [34, 39, 40]) was higher in high-quality embryos. Another study compared gene expression in CC from unfertilised oocytes and those that developed to an 8-cell embryo on day 3 of IVF procedure [41]. Microarray analysis revealed there were 160 genes differentially expressed. Subsequent qPCR analysis confirmed that higher expression of *PTX3* is associated with oocyte development. However, in another study there was no difference in CC *PTX3* expression between high-quality embryos on day 3 and unfertilised oocytes or poor-quality embryos [42] (Table 11.1).

One of the first studies where biomarkers of successful embryo implantation were sought by using CC gene expression analysis was that of Assou et al. [14]. The results of this study revealed higher expression of *BCL2L11* (involved in apoptotic pathways) and *PCK1* (involved in regulation of gluconeogenesis), but lower expression of transcription factor *NFIB* in CC whose embryos resulted in pregnancy after transfer. However, these gene expression profiles need further validation as they were discovered in a study where double embryo transfer was performed. Elective SET was performed in a study where real-time PCR was used to analyse the expression of 13 genes in CC [43]. They have analysed the expression of genes involved in the regulation of metabolism (*ALDOA*, *LDHA*, *PFKP*, *PKM2*), extracellular matrix formation (*HAS2*, *PTX3*, *TNFAIP6*, *VCAN*), and signalling (*AHR*, *GREM1*, *PTGS2*, *STS*) in order to find genes whose expression differentiated between embryos that led or did not lead to pregnancy. The expression of *VCAN* and *PTGS2* was significantly higher ($p < 0.02$) and the expression of *PTX3* tended to be higher ($p = 0.066$) in CC whose oocytes led to pregnancy. An interesting finding of this study was that no genes correlated with clinical embryo morphology scores. This observation implies that there is no relationship between the CC gene expression profile and the embryo morphological assessment.

In 2012, Wathlet et al. [44] analysed the expression of 11 genes in CC in relation to day 3 and 5 embryo morphology and pregnancy by using qPCR. The selection of genes was based on their unpublished microarray data and they were involved in

Table 11.1 The representation of some studies identifying biomarkers in cumulus cells for predicting oocyte, embryo, pregnancy and clinical outcome

Study	Methodological approach	Samples	Observed outcome	No. of patients included	Proposed biomarkers
McKenzie et al. [38]	QPCR	Whole cumulus complex	Oocyte, embryo	8 patients	*GREM1, HAS2, PTGS2, PTX3, TNFAIP6*
Zhang et al. [41]	Microarray and QPCR	Cumulus cells	Embryo, pregnancy	20 patients for array and 16 patients for QPCR	*PTX3*
Cillo et al. [42]	semi-QPCR	Cumulus cells	Oocyte, embryo	45 patients	*GREM1, HAS2, PTX3*
Feuerstein et al. [84]	QPCR	Cumulus cells	Oocyte, embryo	47 patients	*STAR, COX2, AREG, SCD1, SCD5, Cx43*
Hamel et al. [15]	Microarray and QPCR	Granulosa cells and cumulus cells	Embryo, pregnancy	40 patients	*CYP19A1, CDC42, PYSL3, HSD3B1, EREG, SERPINE2, SERPINA3, TNFAIP6, SCARB1, INHA, SPRY2, FDX1, RGS2, NRP1, EGR1, PGK1, BACH2, IL6ST*
Van Montfoort et al. [85]	Microarray and QPCR	Cumulus cells	Oocyte	6 patients for array and 12 patients for QPCR	*CBL, CCND2, CTNND1, CXCR4, DHCR7, DVL3, GPC4, GPX3, GUK1, HSPB1, HTRA1, ITPR1, RAB6IP2, TRIM28, VEGFC*
Assou et al. [14]	Microarray and QPCR	Cumulus cells	Pregnancy	30 patients	*BCL2L11, PCK1, NFIB*
Anderson et al. [86]	QPCR	Cumulus cells	Pregnancy	75 patients	*PTGS2, BDNF, GREM1*
Gebhardt et al. [43])	QPCR	Cumulus cells	Embryo, clinical outcome	38 patients	*VCAN, PTGS2, GREM1, PFKP*
Wathlet et al. [44]	QPCR	Cumulus cells	Embryo, clinical outcome	33 patients	*TRPM7, ITPKA, STC2, CYP11A1, HSD3B1, EFNB2, CAMK1D, STC1, STC2*
Fragouli et al. [53]	Microarray and QPCR	Cumulus cells	Oocyte, clinical outcome	28 patients	*SPSB2*
Iager et al. [45]	Microarray and QPCR	Cumulus cells	Clinical outcome	58 patients	*SCL2A9, NR2F6, ARID1B, FAM36A, GPR137B, ZNF132, DNAJC15, RHBDL2, MTUS1, NUP133, ZNF93*

key cellular processes (*TRPM7*, *ITPKA*, *VCAN*, *SDC4*, *CAMK1D*, *STC1*, *STC2*, *EFNB2*, *PTHLH*, *CYP11A1*, *HSD3B1*). For embryo morphology prediction, *TRPM7*, *ITPKA*, *STC2*, *CYP11A1*, and *HSD3B1* were the most informative genes. Expressions of *ITPKA* and *EFNB2* were statistically higher in the CC of oocytes giving pregnancy, and *CAMK1D* showed the same trend. This investigation emphasised that gene expression-based analysis of embryo quality is independent of morphology. Another study tried to identify biomarkers for pregnancy prediction by using microarrays followed by qPCR validation on CC derived from patients from three different clinics [45]. They reported on a novel set of 12 genes that were included in a prediction model which had a 78 % accuracy. Seven genes (*FGF12*, *GPR137B*, *SLC2A9*, *ARID1B*, *NR2F6*, *ZNF132*, *FAM36A*) were upregulated in pregnancy samples compared with non-pregnancy samples, and five genes (*ZNF93*, *RHBDL2*, *DNAJC15*, *MTUS1*, *NUP133*) were downregulated in pregnancy samples compared with non-pregnancy samples.

Besides biomarker(s) search, transcriptomic analyses of GC and CC have been performed to better understand folliculogenesis [13, 46] and the impact of controlled ovarian hyperstimulation (COH) and patient characteristics on CC gene expression [47–52] and to examine the follicular environment of aneuploid oocytes [53].

It has been established that global gene expression profile of human GC and CC significantly differs [13, 46]. Gene ontology analysis revealed that differentially expressed genes belong to pathways of immune response, organism injury, protein degradation [13] and steroidogenesis, cell-to-cell communication, and extracellular matrix formation [46]. These studies have helped towards better understanding of fundamental aspects of folliculogenesis; better understanding of folliculogenesis could help improve protocols for oocyte in vitro maturation procedures and improve COH protocols.

It has been speculated that COH used during IVF procedures affects oocyte and consequently embryo quality [54]. The influence of COH on CC gene expression was assessed in a study where gene expression in CC surrounding mature oocytes derived from unstimulated and stimulated IVF cycles was compared [47]. There were 66 genes significantly differentially expressed; a gene ontology analysis revealed oxidation–reduction processes were significantly enriched in CC derived from unstimulated IVF cycles, implying pronounced reactive-oxygen species production might be one of the reasons for lower success rates of unstimulated IVF cycles. In a study by Devjak et al. [48], gene expression patterns were assessed in CC after ovarian stimulation protocols incorporating GnRH agonist or GnRH antagonist and transcriptomic analysis revealed no differences. This finding supports clinical data considering pregnancy and delivery rates, where slight (and statistically non-significant) differences have been reported which favour GnRH agonists in IVF [55].

On the other hand, comparison of transcriptomic profiles of GC after COH with recombinant follicle-stimulating hormone (rFSH) or urinary human menopausal gonadotropin (hMG) showed significant differences in gene expression [49, 50]. Differentially expressed genes were involved in signal transduction and transcriptional regulation, signalling pathways, oocyte maturation, and metabolic pathways [49].

Also, expression levels of luteinising hormone/human chorionic gonadotropin (LH/hCG) receptor gene and genes involved in biosynthesis of cholesterol and steroids were lower and anti-apoptosis genes were expressed at higher levels in hMG protocols than in rFSH [50]. Differential gene expression in GC implies that gonadotropin stimulation protocols for IVF could have an impact on oocyte's functional status and quality. Another study compared transcriptomic profiles of CC between rFSH and highly purified hMG (hMG) and found 94 genes were significantly differentially expressed [51]. In CC after treatment with HP-hMG, there was overexpression of genes involved in lipid metabolism and intercellular signalling, whereas in CC following rFSH treatment overexpressed genes were involved in cellular assembly and organisation—crucial functions in oocyte maturation and competence acquirement [56]. Interestingly, *STC2* and *PTX3* were related to in vitro embryo quality in both gonadotropin treatments, and it was postulated that these may serve as informative biomarkers regarding embryo quality.

Regarding patient characteristics, it has been shown that age, BMI, and FSH concentration at the end of COH correlate to CC gene expression [52]. Comparison of CC gene expression associated with chromosomally normal and abnormal oocytes revealed that aneuploid oocytes have reduced mRNA levels, indicative of impaired transcriptional activity [53]. Furthermore, signalling, metabolism, apoptosis, and transport pathways were all adversely affected in CC from aneuploid oocytes. This finding implies that aneuploid oocytes tend to be surrounded by dysfunctional or damaged CC. qPCR validation of microarray data confirmed statistically significant overexpression of *SPSB2* and *TP53I3* in CC of euploid oocytes. The CC expression of *SPSB2* and *TP53I3* was further quantified using qPCR in 38 IVF cycles; embryos were transferred according to the morphological assessment and gene expression were analysed retrospectively. Both genes tended to be overexpressed in CC whose oocytes led to live birth, indicating that *SPSB2* and *TP53I3* could serve as potential non-invasive biomarkers of pregnancy in IVF procedures.

Transcriptomic Analysis of the Endometrium

The endometrium is a dynamic tissue that changes under the influence of hormones in order to create optimal conditions for embryo implantation. To better understand the molecular mechanisms of endometrial receptivity, several research groups have performed transcriptomic analysis of the endometrium of mice [57, 58], rats [59], and rhesus monkeys [60]. The human endometrium has been studied in pathological conditions (such as endometrial cancer) to better characterise the molecular pathways involved in pathogenesis [61] and throughout the normal menstrual cycle [62]. The latter study showed that the endometrium may be 'dated' to specific phase of menstrual cycle based on its transcriptional profile. Moreover, specific gene clusters characteristic of the different phases of the menstrual cycle have been described [62]. In IVF procedures, one of the major challenges has been to identify the endometrial window of receptivity and several groups have tried to find it by using

transcriptomic analysis [63–66]. These investigators have generated extensive lists of genes proposed as markers of endometrial receptivity; however, only one gene—osteopontin—appears on all rosters. Osteopontin is involved in cell adhesion, but its role in human embryo implantation remains poorly understood [67].

Comparison of endometrial gene expression in unstimulated IVF cycles, stimulated IVF cycles, and immediately after removal of IUD showed there were 25 genes expressed during the window of implantation (WOI) in common for all three conditions [68]. Interestingly, these genes seemed to be regulated in one way in unstimulated cycles but in the opposite way in both stimulated and IUD cycles. In other words, if a gene was overexpressed in unstimulated cycles, it was downregulated in other two conditions and vice versa. Recently, the group of Simón [16] introduced an endometrial receptivity array (ERA) containing 238 genes, related to endometrial receptivity. By using ERA, we could determine an individual WOI for women with repeated implantation failure and thus perform the embryo transfer on an optimal day of IVF cycle.

Transcriptomic Analysis of Embryos

To better understand the molecular mechanisms during preimplantation development, several studies have analysed global gene expression profile of embryos in humans [69–71] and mice [72, 73]. Precise control of gene expression during the preimplantation embryonic development is of particular significance. The first cellular differentiation occurs at this time and the embryo transfers from a reliance on maternal RNA derived from the oocyte to expression of its own genome. Wells et al. [70] examined the expression of nine known genes implicated in important cellular processes such as cell cycle regulation, DNA repair, apoptosis, maintenance of accurate chromosomal segregation, and construction of the cytoskeleton throughout the preimplantation phase of embryo development by using qPCR. The genes tested were *BRCA1*, *BRCA2*, *ATM*, *TP53*, *RB1*, *MAD2*, *BUB1*, *APC*, and *β-actin*. They established that the expression levels of all nine genes decreased dramatically after fertilisation and then recovered between the 4- and 8-cell embryo stages. Further increase of gene expression (or in some cases a slight reduction) was seen at the morula stage before gene expression jumped significantly at the blastocyst stage. Of note, global transcriptomic analysis of mouse embryos has revealed a requirement for maternal RNA depletion before embryonic genome activation. This process happens in two stages: zygotic genome activation and mid-preimplantation gene activation [72]. After zygotic genome activation de novo gene transcription begins, it is needed for morula to undergo morphological and functional changes and develop to blastocyst.

Many morphologically normal embryos do not achieve pregnancy after embryo transfer. It has been postulated that many failed IVF cycles occur because of chromosomally abnormal oocytes or embryos. For this reason, several groups have tried to screen oocytes and embryos for aneuploidies by using transcriptomics to identify

euploid and viable oocytes and embryos with greatest chances for implantation [6, 53, 74]. Wells and Delhanty [75] introduced a molecular cytogenetic method allowing the simultaneous enumeration of all of the chromosomes in a single cell called comparative genomic hybridisation (CGH). They report an improvement in embryo implantation and pregnancy rates with the proportion of CGH screened embryos resulting in live birth was 80 % as compared to 60 % for patients without CGH screening [76, 77]. The major pitfalls of using CGH were the long time required for the method (approximately 4 days), which was incompatible with a fresh transfer timeframe during IVF.

Translation of Discovered Biomarkers into Clinical Practice

With developing technology of transcriptomics, proteomics, and metabolomics, new biomarker identification has greatly accelerated. With that has come an intense discussion on how best to measure newly discovered biomarkers. Understandably, there is great interest of implementing discovered biomarkers into clinical practice. For example, the American Society of Clinical Oncology has presented a paper where it is estimated that routinely testing people with colon cancer would save at least US $600 million a year [78].

In the past decade, we have witnessed increased numbers of biomarker publications, but most of them do not have sufficient sensitivity and/or specificity to be clinically useful. This weakness is reflected in the relatively low number of patent applications and the even lower number of successful market applications [79] for such discoveries. The major pitfalls in the translation from biomarker discovery to clinical utility are:

- Lack of making different selections before initiating discovery phase
- Lack in biomarker characterisation/validation strategies
- Robustness of analysis techniques used in clinical trials [79]

In order to overcome these limitations, certain authorities [e.g. American Society of Clinical Oncology, U.S. Food and Drug Administration (FDA), European Medicines agency (EMA), European Association for Predictive, Preventive and Personalized Medicine (EPMA), National Institutes of Health (NIH)] have developed guidelines on validation process for studies of biomarker discovery. For the purpose of this chapter, these recommendations can be extracted by terms analytical validity, clinical validity, and clinical utility [80].

Biomarkers can be classified into the following categories: pharmacodynamic, prognostic, or predictive [81].

1. Pharmacodynamic biomarkers indicate the outcome of the interaction between a drug and a target, including both therapeutic and adverse affects.
2. Prognostic biomarkers were originally defined as markers that indicate the likely course of a disease in a person who is not treated, although they also include markers that suggest the likely outcome of a disease irrespective of treatment.

3. Predictive biomarkers suggest the population of patients who are likely to respond to a particular treatment.

As a rule it can be considered that ideal biomarkers for use in diagnostics and prognostics, as well as for drug developing and targeting, should be highly specific and sensitive [82]. But in reality, only rare biomarkers have high sensitivity and specificity. According to Issaq et al. [82], the following factors attribute to this:

1. Small number of samples are analysed
2. Lack of information on the history of the samples
3. Case–control and control specimens are not matched with age and sex
4. Limited metabolomic and proteomic coverage
5. The need to follow clear standard operating procedures for sample selection, collection, storage, handling, analysis, and data interpretation.

Status on Validation Process of Transcriptomic Biomarkers for SET

As described previously, many biomarkers have been proposed for various endpoints in IVF cycle (oocyte maturity, oocyte fertilisation, embryo quality, pregnancy). Biomarkers for pregnancy seem to be most appropriate for SET in clinical practice. But a major drawback of all biomarkers CC and GC thus far discovered remains the lack of validation. Only a few of all proposed biomarkers have been validated by any statistical method.

In the study of McKenzie et al., *HAS2*, *PTGS2*, and *GREM1* were validated by a logistic regression model for oocyte maturity, oocyte fertilisation, and embryo quality. Regression models for embryo quality yielded an AUC 0.76, 0.76, and 0.81 for *HAS2*, *PTGS2*, and *GREM1*, respectively. Combining *PTGS2* and *GREM1* improved the predictive power only slightly (AUC 0.82 vs. 0.81) [38].

Whatlet et al. investigated *PTGS2*, *SDC4*, *VCAN*, *GREM1*, *ITPKA*, *CALM2*, and *TRPM7* and used a multivariate regression model for embryo quality and pregnancy. Better cleavage-stage embryo prediction relied on *TRPM7* and *ITPKA* expression, and the pregnancy prediction relied on *SDC4* and *VCAN* expression. The developed multivariate regression models for prediction of pregnancy had a sensitivity of 0.70 and a specificity of 0.90 in the analysed dataset [44].

Another prognostic model for pregnancy was published by Iager et al. [45] where 12 genes (*FGF12*, *GPR137B*, *SLC2A9*, *ARID1B*, *NR2F6*, *ZNF132*, *FAM36A*, *ZNF93*, *RHBDL2*, *DNAJC15*, *MTUS1*, *NUP133*) previously recognised by microarray were tested by qPCR for their predictive power. They used a "signal-to-noise" ratio to assess the predictive value of a gene using weighted voting. The AUC value for pregnancy prediction was 0.76 ± 0.08.

Even though certain results seem promising, the general lack of overlap among genes identified as potentially useful biomarkers is evident, as noted by Fragouli et al. [83]. This suggests that the transcriptome of follicular cells could be affected

by multiple intrinsic factors, having to do with the patient and possibly the aetiology of infertility, as well as extrinsic factors, such as hormonal stimulation.

Conclusion

Although all of these studies have revealed some promising biomarker genes for use in reproductive medicine, further well-designed validational studies are necessary in order to reach consensus and find biomarker(s) with high sensitivity and specificity. In order to overcome the challenges of translating discovered biomarkers into clinical utility of SET, validational studies will have to be large, and, if possible, multi-centre. Moreover, clear standard operating procedure for sample selection, storage, handling, analysis, and data interpretation will be critical. If these biomarker(s) meet requirements in validational process, then they would be expected to improve oocyte and make embryo selection more informative, thus leading to an increased use of elective SET without lowering success rates of IVF. It is likely that a combinatorial evaluation of different parameters will be needed for the development of universally applicable biomarker(s) to facilitate SET.

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Chapter 12
Array CGH and Partial Genome Sequencing for Rapidly Karyotyping IVF Blastocysts Before Single Transfer

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Introduction

Assisted reproduction treatment employing in vitro fertilisation (IVF) often results in a surplus of embryos potentially suitable for transfer. The transfer of several embryos at once can enhance immediate pregnancy rates, but it also increases the chance of multiple pregnancy, with its risks of serious complications during pregnancy and the perinatal period [1–3]. This led to a trend of electively transferring a single embryo at a time [3–6]. It has been revealed recently that a significant percentage of early embryos, however, harbour substantial chromosomal anomalies which may be incompatible with implantation (or with establishment of normal gestation), so, in the absence of effective screening, elective single embryo transfer can appear to reduce the immediate, first-transfer IVF pregnancy rate [1–3]. Traditionally, a hierarchy of best embryos (or best remaining embryo for transfer after cryostorage) has been inferred from a combination of developmental and morphological features based on cell number, cleavage rate, blastomere fragmentation fraction, presence of intracellular vacuoles, and, most recently, the ability to form blastocysts suitable for transfer on day 5 or 6. With the exception of cleavage rate

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and blastulation, these factors are subjective [7, 8] and are ultimately inadequate for choosing the embryo with the best potential for initiating a normal, singleton gestation; a morphologically normal embryo can fail to implant, or might initiate a pregnancy only to miscarry later, because of chromosomal aneuploidy.

As many as 65 % of clinical miscarriages in the first trimester have a major abnormality of chromosomal copy number identifiable with classical low-resolution karyotyping on products of conception (POC) [9–11]. While aneuploidy may involve any (or several) of the 24 chromosomes, some typically larger chromosomes appear to be so crucial that their aneuploidies are lethal during preimplantational development; they are almost never observed among tested clinical POC. Recent reports show that more than 50 % of oocytes from women in their mid-30s can be aneuploid [12]. Given similar aneuploidy rates identified in embryos, even among relatively young IVF patients [13], identifying such embryos and excluding them from transfer logically offers the possibility to increase implantation rates substantially using those that test normal and should also decrease the risk of miscarriage among any pregnancies that follow. While such preimplantation screening of embryos with partial characterization of chromosomes using fluorescent in situ hybridization (FISH) has been performed for nearly 20 years, no objective demonstrable improvement in IVF outcomes was reported, irrespective of whether biopsies were performed on day 3, where embryos are essentially 8 identical cells, or on morphologically normal day 5 and day 6 blastocysts, when there are typically more than 100 cells and differentiation has occurred of outer trophectoderm (TE, the future placenta) and the inner cell mass (ICM, or embryo proper). If any incomplete analysis of potential chromosomes involved in aneuploidy at either stage or, in the case of blastocysts, any mosaic observation that then rules out the transfer of that embryo, then such preimplantation genetic screening potentially disadvantages live birth rates per embryo transfer event compared with standard IVF practices [14].

The debate on what constitutes effective screening for aneuploidy has been protracted, but it is clear that FISH, while very convenient, falls far short. The particular blastomere or cells removed and tested from an embryo may be euploid and considered normal. In contrast, if the blastomere were aneuploid, this could be representing a true meiotic non-disjunction or may be a mosaic in the embryo arising from anaphase lag, chromosome gain, or mitotic non-disjunction followed by trisomy and monosomy mixtures among clonally surviving daughter cells. Such mosaic aneuploidy has been attributed to loose cell cycle controls during rapid cell mitosis in the early embryo [15–17] and is paralleled by confined placental mosaicism observations in otherwise healthy pregnancy outcomes. Depending on the ‘dosage’ and level of survival disadvantage of the mitotically derived aneuploid cell line for the embryo, partial or complete resolution can take place naturally [18]; given the chance, this will lead to a normal gestational outcome in at least some cases. Accordingly, the clinical significance of such occurrences at the embryonic stage is unknown. But secondly, and perhaps most importantly, many instances of meiotically founded aneuploidy are missed through the limited number of chromosomes able to be examined with FISH [19].

A method of total chromosome screening employing comparative genomic hybridization (CGH) of metaphases at single-cell level following DNA amplification by degenerate oligonucleotide-primed PCR was developed and reported by Wells and colleagues as long ago as 1999 and applied to human preimplantation blastomeres from three embryos of normal appearance a year later [20, 21]. The following year the technique was also employed clinically and led to a normal infant [22].

The lengthy hybridization time required for classical metaphase CGH meant that biopsied embryos by necessity needed to be frozen and cryostored, with transfer delayed to a subsequent cycle, a process which was considered at the time to be suboptimal for biopsied embryos mainly due to the impact of traditional freezing methods on embryo viability [23]. Furthermore, when comparing CGH to FISH, the test preparation and laboratory personnel skill base needed for testing the multiple embryos available in PGD cycles was more complex, time-consuming, and expensive than the use of FISH which was more readily applied to multiple biopsy specimens simultaneously using suitably trained staff available in most laboratories. CGH for preimplantation embryo karyotyping languished clinically. In 2008, however, Fragouli, Wells, and colleagues [24] (still using classical metaphase CGH techniques) gave the first indications that the potential of total chromosome screening of day 5 blastocysts [25] in combination with vitrification (a refined method of freezing embryos [26]) could realise the improvement sought—but had proven elusive—using FISH [20, 21].

In summary, the key developments that made karyotyping preimplantation human embryos a routine clinical prospect with improved outcomes have comprised (1) the demonstrated safety and reliability of trophectoderm biopsy at the stage of blastocyst [4, 6, 27–29]; (2) the advent and application of an efficient vitrification process for the storage of biopsied embryos [26]; (3) the improved reliability of whole-genome amplification (WGA); and (4) the reduction in cost and improved utility of comprehensive molecular cytogenetic methods that employ array CGH or single nucleotide polymorphisms [23–25, 30–32] and more recently next generation or second generation sequencing [33].

This study was aimed at comparing two methods of molecular karyotyping (microarray analysis vs. partial genome sequencing) in assigning the chromosomal status of embryos that were otherwise defined as clinically useable by traditional embryologic criteria.

Methods

Embryo Culture and Biopsy

All embryo analyses were carried out under a National Health and Medical Research Council (NHMRC) licence for human embryo research (Licence 309702B) and under a protocol approved by Genea's formally constituted and NHMRC-registered Human Research Ethics Committee. Seven couples donated 25 clinically useable

frozen embryos that had become excess to their reproductive needs. Embryos had been stored for up to 9 years in liquid nitrogen.

Patients had been stimulated for multiple egg retrieval using standard protocols [29]. Embryos were cultured to blastocysts in MINC incubators (Cook Australia Pty Ltd) under 89 % nitrogen/5 % oxygen/6 % CO_2; excess blastocysts were cryopreserved using standard slow-freezing protocols. Stage-specific culture medium (Sydney IVF Media Suite version 2, Cook IVF, Eight Mile Plains, Queensland) was used for each step. After thawing, embryos were allowed to re-expand in blastocyst medium. Embryos were removed from the zona pellucida, biopsied according to standard protocols [27] and, where possible, the ICM was identified and kept as a discrete sample for analysis (there was no attempt at removing any attached trophectoderm cells as visually they were considered numerically much less than the ICM cells).

Whole-Genome Amplification

In total, 176 tissue samples were isolated from the 25 embryos, each consisting of about 8–10 cells. All samples were placed into individual PCR tubes and subjected to whole-genome amplification (WGA) using PicoPLEX (Rubicon Genomics, Inc. Ann Arbor, MI). After purification of amplified products (QIAquick PCR purification kit, Qiagen), the WGA product was quantified (NanoDrop 1000, Thermo Scientific); 168 amplifications were considered to have amplified effectively and yielded the manufacturers' suggested final DNA amount (3–6 μg per amplified sample). Two samples from each embryo were selected for both array and NGS analyses.

Array CGH Analysis

Two samples from each embryo were compared by array CGH—ICM (where available) and one trophectoderm product. An aliquot of purified labelled WGA was hybridised to Agilent 8x60k oligonucleotide arrays using standard protocols. The WGA product (600 ng) was labelled using the Agilent ULS labelling system (Genomic DNA ULS Labelling Kit, Agilent Technologies) and 300–400 ng used for each subarray. Control DNA was similarly whole genome amplified, purified, labelled with the alternative ULS fluorophore reagent, combined in equal amount with WGA product, and hybridised for 16–20 h at 65 °C. After washing (Oligo aCGH Wash Buffer 1, Agilent Technologies) at room temperature for 5–10 min and then washed at 37 °C (Oligo aCGH Buffer 2, Agilent Technologies) for 1 min, slides were scanned at 3 μm (Agilent G2565CA Microarray Scanner, Agilent Technologies). The resultant TIFF image was extracted (Feature Extraction 10.7.3.1, Agilent Technologies) and analyses performed using Agilent Genomic Workbench (Version 7.0.4.0, Agilent Technologies) (Moving average: triangular algorithm,

20 Mb window; ADM-2 aberration algorithm; fuzzy zero; Normalisation: GC correction 10 Kb). The plotted microarray outputs for each of the embryo biopsy samples were read visually and independently by at least two trained observers. These reads were used to assign the ploidy status for each piece with the embryo status considered to be the result of the ICM when it was available. aCGH moving average plots for example embryos are presented in Fig. 12.1a–d with individual biopsy pieces from each single embryo overlayed.

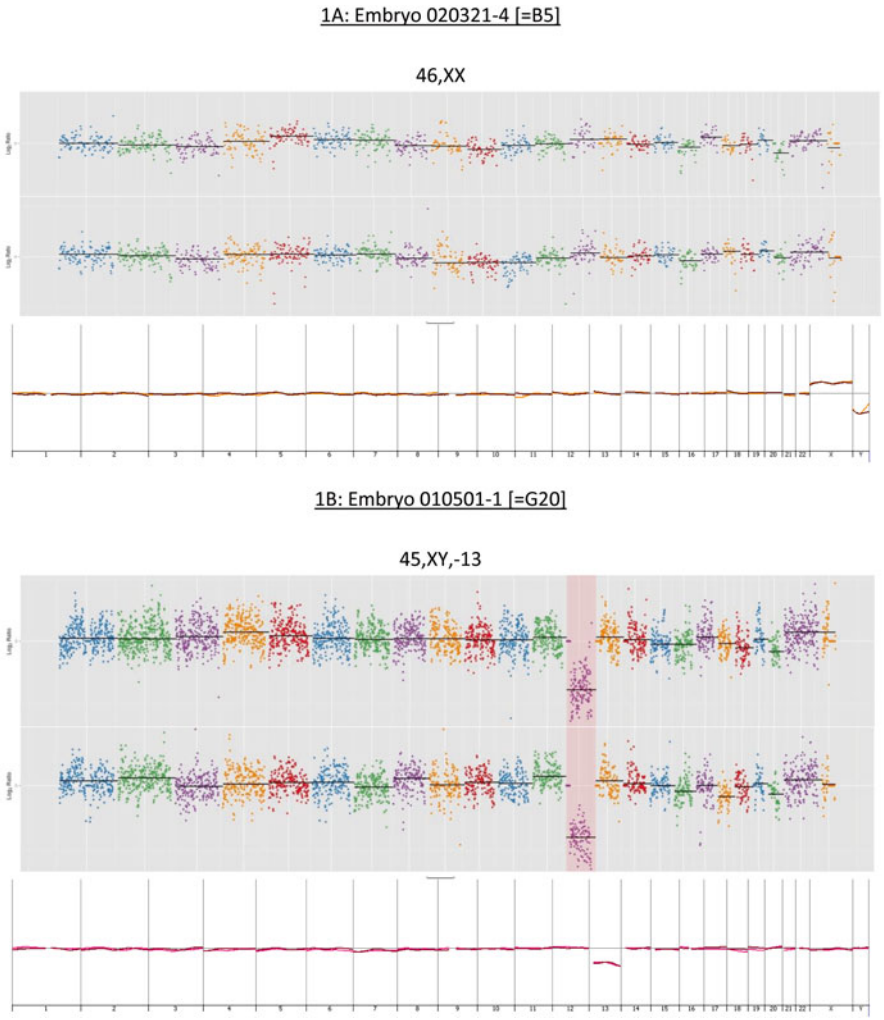


Fig. 12.1 (**a–d**) Upper panel in each section shows chromosome profiles determined by partial genome sequencing. The lower panel in each section shows overlaid array CGH profile

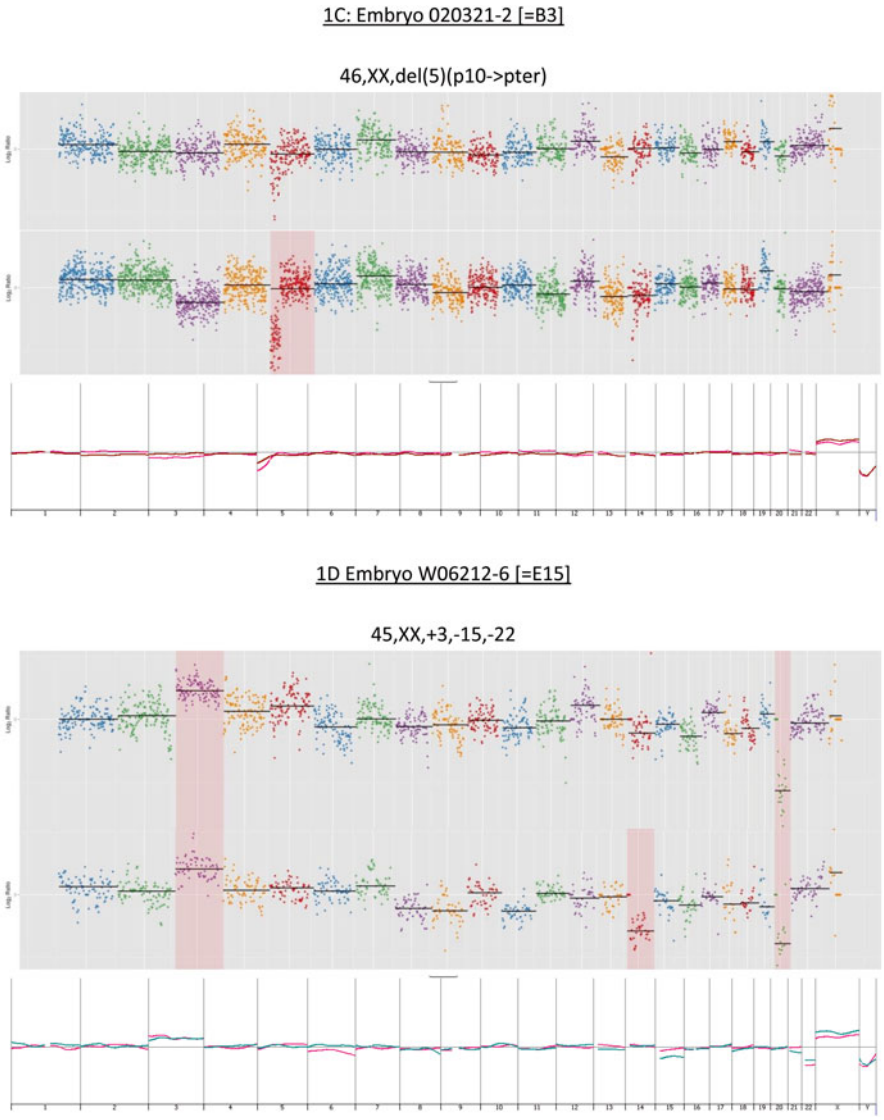


Fig. 12.1 (continued)

Partial Genome Sequencing: Low Depth Sequencing

A second aliquot of WGA product from 50 of the initial samples (two samples from each embryo) was used for sequencing using the Ion Torrent Personal Genome Machine (PGM) system (Life Technologies, Melbourne, Australia). WGA from each biopsy piece produced a range of long amplicon products that were then

fragmented (Ion Xpress Plus Fragment Library Kit, Life Technologies) to yield blunt-ended DNA fragments of c. 250 base pairs. Fragments were then 'library prepared' (Ion Plus Fragment Library Kit, Life Technologies) and indexed using Ion Torrent barcodes (Ion Xpress Barcodes 1-48, Life Technologies). Template preparation was carried out using the OneTouch System (Ion OneTouch 200 Template Kit, Life Technologies) and sequenced using the 200 base read kit (Ion Xpress 200 Sequencing Kit, Life Technologies).

We analysed the initial data using the standard software supplied with the Ion Torrent Suite 3.2 PGM sequencer. The cumulative sequence reads for individual chromosomes were plotted (Fig. 12.2). Each autosome chromosome cumulative score was obtained from the Ion Torrent Suite output and characterised as a simple

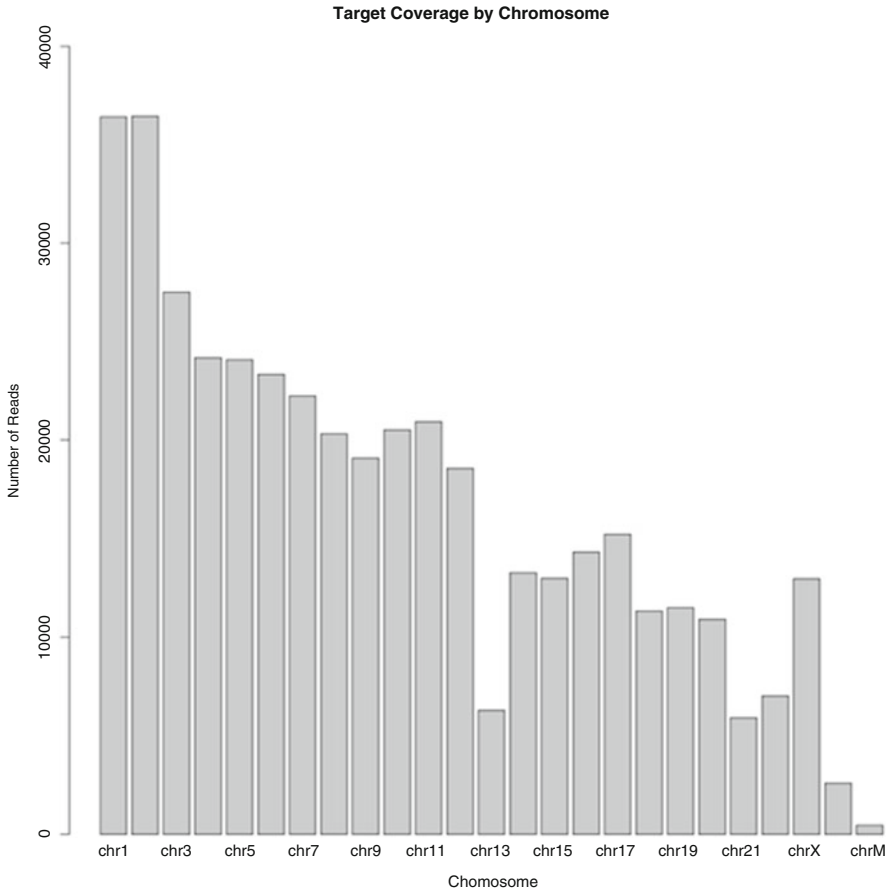
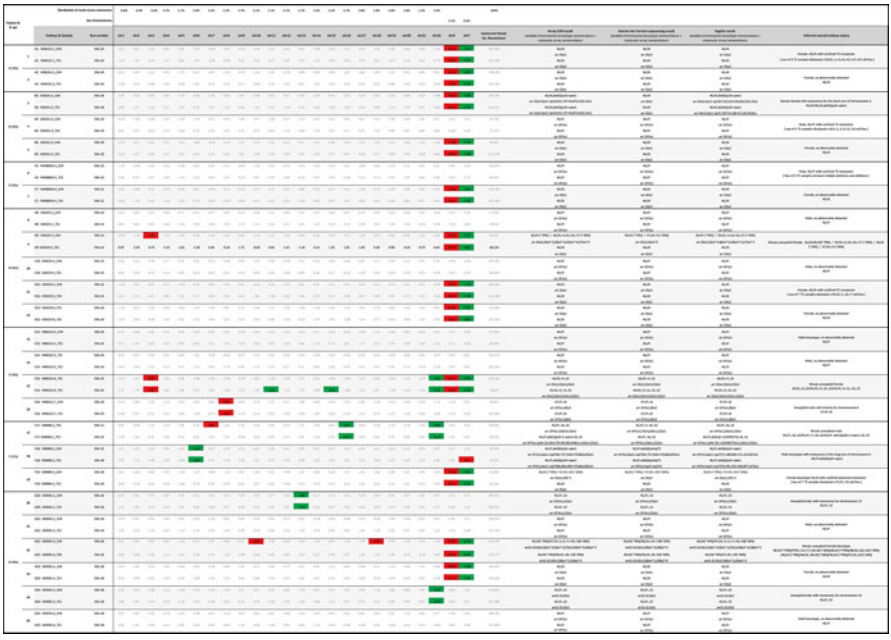


Fig. 12.2 Preliminary chromosome coverage output (Ion Torrent Suite 3.2) for the inner cell mass of embryo G20. Partial sequencing reads are arranged in conventional order of chromosome number (1–22), followed by the sex chromosomes (X and Y) and mitochondrial chromosome (chrM). There is monosomy of chromosome 13

Table 12.1 Partial genome sequencing *Z*-score table. Significant deviations from the population means derived from normal chromosomes shown in *red* (>3 STD above the mean) and *green* (>3 STD above the mean) boxes. Karyotypes and array outputs accord with 2013 International Standing Committee on Human Cytogenetic Nomenclature recommendations [44].



fraction of the total autosome read from that biopsy piece. Mean reads and standard deviations (SDs) for each chromosome from each run were calculated (with the previous array-identified abnormal chromosomes being excluded from individual chromosome normal range calculations) and a *Z*-score table was generated. The Ion Torrent sequencing data and the reads obtained are presented in Table 12.1. These preliminary analyses on their own were found to be adequate for simple chromosome aneuploidy assessment for clinical diagnostic purposes but were insufficient for some segmental losses (and presumably gains). Therefore, we devised and applied further algorithms.

SeqVar Algorithm

Our SeqVar algorithm set was adapted from an open-sourced algorithm [34] that calculates the Poisson probability of difference between two samples of a number of mapped reads in small windows that tile each chromosome. SeqVar thus detects significant over- and under-representation of mapped reads of the sample under test compared to the control sample and adjusts for global variation between the test and control samples across all chromosomes using Poisson distribution. The software

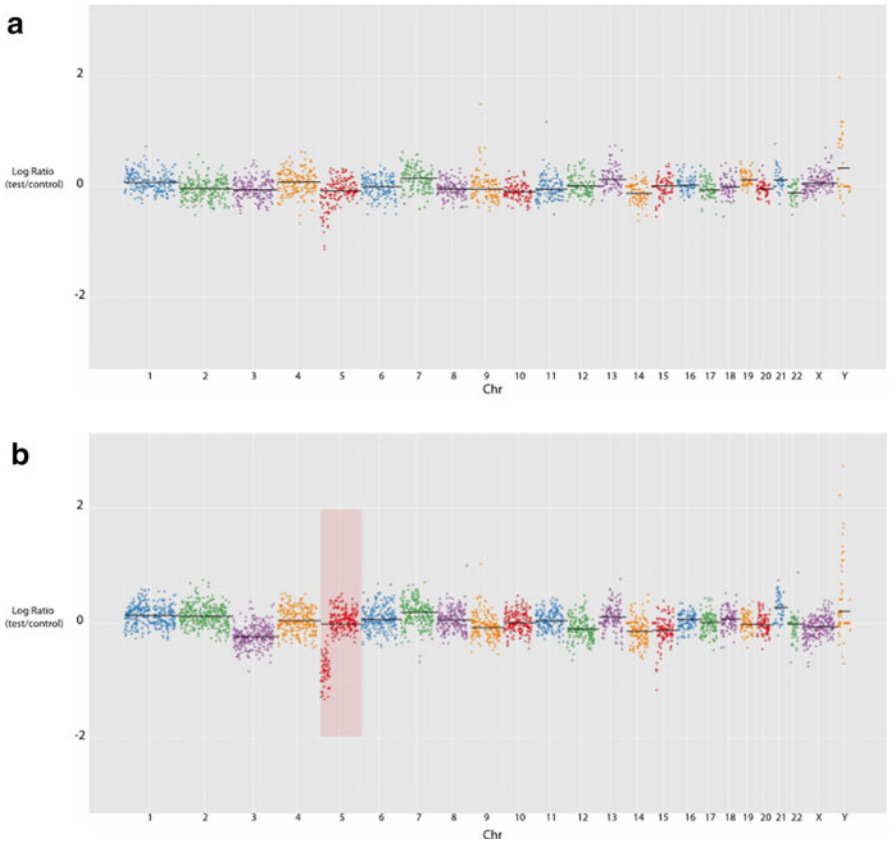


Fig. 12.3 SeqVar output indicating a segmental deletion at *5p* in two samples from embryo B3. (**a**) At a total genome coverage of c. 245,000 reads, (**b**) at c. 400,000 reads. PGS methodology readily permits increasing the resolution in additional examinations of remaining extant sample to clarify areas of ambiguity or concern

output includes visual plots of segmental gains and losses respectively above and below a threshold value on any chromosome and marks the chromosome when the difference is significant (Fig. 12.3).

Detection and Display of Segmental Variations

The procedure for detecting segmental variations involves two steps:

1. *Aligning the reads to the February 2009 human genome reference sequence GRCh37/hg19*. A higher number of reads mapped to the genome increases the statistical power of variation calls and enables the detection of smaller deletions. The 3-stage Ion Torrent Mapping Alignment Program was used to align the

sequence and to filter putative sequencing errors. This was able to align an average of 91–193 % of sequencing reads that passed the Torrent Quality Control step.

2. *Detecting segmental variations from the mapped reads*. Our approach employs a sliding window (of varying size depending on the total number of mapped reads) across the entire genome using the Poisson distribution for subsequent calculation. In each window, the number of reads counted that mapped to the input sample and a normal reference sample were used to calculate the probability that any difference between the input and reference samples is statistically significant. Because the Y-chromosome (chrY) is small, the number of reads that map to it can vary materially between samples. Within each sample, however, the ratio between the number of reads that map to chrY and the total number of mapped reads is reasonably consistent: for *male samples*, median ratio Y/Total = 5.7×10^{-3}, $\pm SD = 4 \times 10^{-4}$; for *female samples*, median = 1.6×10^{-3}, $\pm SD = 3 \times 10^{-4}$. To further reduce false-positive chrY calls, the SeqVar detection algorithm checks this ratio before calling a copy number variation on chromosomes X and Y.

In the absence of an accepted nomenclature for sequence-derived karyotyping, comparable results for aCGH and PGS are given here principally in the familiar banded-chromosome nomenclature of classical cytogenetics. Chromosome-mapping outputs obtained from sampling across the genome are based on relative imbalances in DNA copy number for both CGH and PGS, however, and produce visibly comparable chromosome-based displays. The current international nomenclature for reporting virtual karyotypes from arrays can therefore be provided in addition to the banded-chromosome-based terminology.

Results

Among the 25 available embryos from the seven patients (average age = 34.4, range = 29–40 year), we observed an effective euploidy rate of 15/25 (60 %), a prevalence comparable to that reported for blastocysts from similar age cohorts by other authors [31, 32, 34, 35]. Control normal and abnormal karyotypes obtained by array CGH are illustrated in Fig. 12.1a–d. The median number of embryos per patient was 3 (range = 2–6). Of the 25 embryos, 11 were uniformly normal. Seven embryos were uniformly aneuploid and three embryos that displayed mosaicism across TE and ICM were also considered abnormal.

Four embryos revealed one or more mosaic aneuploidies confined to TE, a phenomenon that (a) generally indicates isolated mitotic aneuploidy generally arising from anaphase lag during rapid cleavage [15, 36]; (b) is usually overlooked clinically when, at the earlier, 8-cell-or-earlier stage, only one cell is sampled for preimplantation testing; or (c) through divisional disadvantage is ordinarily followed by cell-line dilution and extinction [37], or lingering low-level placental mosaicism of uncommon clinical importance [38]. Alternatively, discrete cell analysis of a multicellular TE biopsy (as occurs with FISH), by revealing occasional aneuploid cells, can be over-responded to if it is elected not to transfer the blastocyst on this basis [14].

Initial sequence data were obtained using software supplied with the Ion Torrent Suite 3.2 (Fig. 12.2). Individual partial genome sequence results for each sample were de-convoluted for each chromosome and scored. Total sequence reads per chromosome for each embryo were converted to a fraction of the total sequence reads for the autosomes from that embryo and a *Z*-score table was constructed (Table 12.1). Most analyses were performed with c. 40,000–c. 250,000 such hits per sample. The fractional reads per chromosome were then used to calculate a fraction mean and the standard deviation of the fraction mean. A score greater than 3 standard deviations (SDs) above or below the population mean for the particular chromosome was considered a necessary and sufficient deviation to indicate highly probable aneuploidy (trisomy or monosomy, respectively). In cases of doubt (Fig. 12.3), the already amplified DNA was tested again at resolutions up to c. 800,000 hits. All aneuploidies identified on array CGH were confirmed by NGS, with typical individual array-based aneuploid ascertained chromosome fraction scores appearing 4–8 SDs away from the fraction mean.

The $\log_2$ ratio between the human genome reference sequence and the 'test' sequence in the SeqVar methodology led to identification of every loss and gain detected by aCGH. On aCGH, two blastocysts (8 %) showed an intrachromosomal segmental aneuploidy with a uniform loss of a substantial part of one chromosome: one case of loss of *5p* and one case of loss of *6q14-tel*; a similar segmental aneuploidy prevalence among blastocysts has been reported by others [32]. Mean hit analysis using SeqVar readily detected the significant proportional deviation for the *6q* deletion analysis, but the *5p* loss was equivocal at c. 245,000 reads; testing at increased resolution rendered this segmental aneuploidy obvious. As is the case with aCGH, NGS output plots were visually inspected for anomalies, paying particular attention to focal or segmental within-chromosome losses or gains that reach $\log_2$ ratios outside the range of −1.0 to +0.58 or −1.0. The Agilent CGH array employs a software-based centralization algorithm that balances overall gains and losses and renders the sample's most common ploidy the new zero point—a step acknowledged to lead to erroneous calls for highly aberrant genomes (Agilent Genomic Workbench 7.0 handbook: CGH Interactive Analysis, p. 476). This step is not required with the Ion Torrent/SeqVar direct sequencing strategy, where limits are precisely predefined numerically prior to analysis.

Discussion

From a simple biological perspective, the unsuitability for transfer of any embryo that has significant chromosomal imbalance(s) is unquestioned. What has caught the attention of clinics throughout the world, however, is the relatively high level of aneuploidy amongst otherwise clinically suitable embryos as well the diverse nature of the chromosomes involved. The use of whole chromosome analysis methods is having a significant impact on the ability to identify and transfer genetically suitable embryos with subsequent implantation rates compared to their standard IVF patients nearly doubling in some clinics. The application and benefits of CGH are now

Fig. 12.4 Workflow schedule for partial genome sequencing and for aCGH sequencing and alignment timing is for low hit analysis. As resolution need is increased, then sequencing and alignment times increase to approximately 8 h

receiving worldwide acknowledgement. The use of commercial microarrays has simplified the approach to total ploidy analysis and has permitted many laboratories to offer this service. However, the cost of array CGH can be prohibitive and potentially excludes an even wider uptake, at least in some countries around the world. New technologies such as NGS are now offering a different approach to the same solution of total chromosome analysis. Currently, we show the process timing for arrays and NGS is similar (see Fig. 12.4).

Employing massive parallel sequencing with an average of 8–12 million reads per sample of embryo trophectoderm, Yin and coworkers showed that next generation sequencing technologies can reveal aneuploidies and unbalanced chromosomal rearrangements; their methodology, however, required 10–17 days of lab time [33]—a time frame that while suitable for IVF/cryocycle transfer is not appropriate for fresh transfer. We report the similar use of NGS technology but using a reduced-representation ('partial') approach (see Simpson et al. [39] for a methodological review) to comprehensively study morphologically normal human IVF embryos with a sample of trophoblast and to disclose chromosomal aneuploidies utilising economically low numbers of reads across the genome. The methods employ commercially available NGS chips and equipment available to most IVF laboratories experienced with molecular genetic testing for preimplantation genetic diagnosis. Using different modes of analysis, NGS is also able to identify segmental chromosome losses as well as quantify, in an objective way, the relative abundance of individual chromosomes and so disclose mosaicism to various levels.

We show that complete karyotypes via NGS for biopsied blastocysts can also be available overnight, as is the case with microarrays based on CGH, the present standard [25], while potentially providing some useful advantages.

First, by electively increasing the number of hits per genome or chromosome, we can flexibly increase intrachromosomal resolution. For clinically infertile couples undergoing IVF, as few as 40,000 reads per whole genome enable reliable counting

of whole chromosomes to avoid transferring grossly aneuploid embryos. A clinical need for higher levels of within-chromosomal resolution can become apparent during low-resolution screening sequencing (Fig. 12.3) or can be planned in advance for preimplantation genetic diagnosis in families with a known intrachromosomal segmental CNV or small segment reciprocal translocations. We show that 400,000 or more reads detect relatively small segmental losses within chromosomes and also may enable greater discernment of blastocyst mosaicism.

Second, as equipment manufacturers produce improvements in NGS chip capacity, the number of sequencing tests performed per fixed price lab NGS run is increasing with little change in cost of materials. The Ion 316 chip we used delivered about 2.5 million mappable reads, enabling simple but full karyotypic analysis of up to 50 indexed embryos in one sequencing run. Process improvements in commercially available sequencing kits that decrease the time needed for testing to a single day can be expected in due course, enabling potential for same-day results and the transfer of the embryo or embryos starting with the fresh treatment cycle in which eggs have been retrieved and fertilised. Routine CGH with IVF thus offers the promise of clinical scale karyotyping of all embryos before transfer or cryostorage, at an increasingly economical cost.

Finally, it could be that in some circumstances NGS with the Ion Torrent/SeqVar algorithms is able to resolve genomic complexities beyond the resolution of standard aCGH and reduce the necessity for array customisation in such cases or when there are highly aberrant genomes such as the mosaic states seen in blastocysts [37]. We are presently investigating this possibility further by applying array aCGH and NGS in parallel to a series of aneuploidy-exclusion trophectoderm biopsy cases in our clinical service.

These developments bring blastocyst-based clinical IVF to the point where whole-genome karyotyping can be used to potentially screen all embryos before transfer and thus to substantially decrease chromosomally abnormal conceptions from compromising reproductive objectives. Early experience revealed that whole-genome preimplantation screening for aneuploidy had the capacity to increase pregnancy rates to over 50 % per embryo transferred [23]. By reducing or eliminating chromosomally abnormal embryos [9, 10], the routine use of CGH can be expected also to reduce miscarriage risk by approximately half. These predicted outcomes represent significant improvements over standard IVF practice and even over natural conception [40, 41]. Moreover, by ensuring high rates of implantation, the transfer of chromosomally normal embryos one at a time ought to lead IVF practitioners to limit multiple embryo transfers and thus to reduce IVF-associated perinatal mortality and morbidity from multiple pregnancy [3, 5, 28].

Which approach to take—array or NGS? There are different laboratory technical and equipment requirements for the arrays compared to the sequencing approach, and these differences may be one of the deciding factors on which technology a clinic can or should employ. It is conceivable that array implementation (aCGH) is the best approach for some small to medium clinics with variable loads and insufficient resources to support specialised scientists for NGS, whereas partial genome sequencing (NGS) may be more suited to a bigger clinic or even a service centre

with greater resources in staffing. The final decisions may need to be based on what is most appropriate for the individual clinic. Either array-based or NGS-based embryo molecular karyotyping has the opportunity to improve transfer outcomes for most clinics. With regard to transfer of a tested embryo, which is best—fresh or frozen? Recent reports seem to suggest that a cycle involving embryo storage and subsequent transfer in a non-stimulated situation possibly offers the best outcomes with highest implantation rates and healthiest pregnancies, as the impact of the stimulation protocol on endometrial receptivity may play a larger part on final cycle outcomes than was attributed previously [42, 43]. This would mean that immediate requirements for a speedy analysis protocol may be of lesser importance as would any consideration of protocol changes for biopsy on day 5 compared to day 6. In addition, biopsy followed by vitrification would permit even larger numbers of laboratories to outsource total aneuploid screening through service suppliers and avoid incurring the added burden of expensive capital equipment acquisition and maintenance or supporting further specialised staff.

Note: Life Technologies has now released a software package called 'Ion Reporter' that performs similar functions to the bioinformatics reported herein. This means even more laboratories can now readily access analysis platforms for aneuploidy and segmental chromosome assessment by sequencing.

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Conflict of Interest The authors declare no conflict.

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Chapter 13
Current and Future Preimplantation Genetic Screening (PGS) Technology: From Arrays to Next-Generation Sequencing

Gary L. Harton and Dagan Wells

Introduction

Aneuploidy is a broad term used to describe gross chromosomal imbalance in an organism. For the sake of this chapter, only aneuploidy in an embryo will be considered. Aneuploidy typically presents as either an additional chromosome (e.g., trisomy) or a missing chromosome (e.g., monosomy). Such abnormalities arise during cell division (either meiosis or mitosis) when chromosomes fail to separate equally between the two new daughter cells [1]. Aneuploidy may be present in all cells of the embryo (uniform aneuploidy) or be confined to a subpopulation of the cells (mosaicism). Aneuploidy in embryos has varied effects during reproduction, from early embryonic arrest and lack of implantation, pregnancy loss (spontaneous abortion) with trisomy 16 being the most common chromosome abnormality seen in products of conception (POC), to live born trisomic births with varying phenotypic abnormalities, the best known being Down Syndrome (trisomy 21). Aneuploidy originates during the meiotic divisions (principally in the ovary) and the early cleavage divisions (mitotic) of the preimplantation embryo. Nondisjunction, precocious separation of sister chromatids, and anaphase lag are thought to be the most common causes of aneuploidy during gamete formation and embryogenesis [1]. The impact of aneuploidy in families can be devastating, with patients being faced with the potential of pregnancy losses, stillbirths, and/or a severely affected child. In all cases, aneuploid embryos result in an unfavorable outcome for the family in

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question and are a major contributing factor to the relatively low fecundity of humans when compared with other species.

Preimplantation genetic screening (PGS) is increasingly used during in vitro fertilization (IVF) treatment and involves the cytogenetic analysis of polar bodies biopsied from oocytes, single cells (blastomeres) removed from cleavage-stage embryos, or small numbers of trophectoderm cells derived from embryos at the blastocyst stage. The intention of PGS is to reveal whether an oocyte or embryo is chromosomally normal or aneuploid, ideally allowing a single euploid embryo to be prioritized for transfer to the uterus. In theory, this strategy should lower the risk of some of the problems discussed above and improve the success rates of assisted reproductive treatment (ART). PGS is not a new concept, it was proposed alongside the earliest developments of preimplantation genetic diagnosis [2]. The ability to count chromosomes effectively in small numbers of cells from early embryos has required an evolution of diagnostic technologies, combining speed, accuracy, reproducibility, and reliability. To date, only direct analysis of chromosome copy number through embryo biopsy, and analysis of the complete chromosome complement has shown positive results in terms of improved ART outcomes [3, 4]. Indirect approaches (e.g., metabolomic and proteomic analysis of embryonic products and detailed morphokinetic analysis using time-lapse imaging technology) have yet to be convincingly associated with aneuploidy incidence across multiple laboratories.

Prior technologies aimed at counting chromosomes (e.g., fluorescence in situ hybridization (FISH) applied to polar bodies and embryonic cells) failed to show a clinical benefit in multiple randomized controlled trials (RCTs) (see meta-analysis [5], with only one RCT demonstrating an improvement in the results of ART [6]. The challenges facing FISH-based technology applied to human cleavage-stage embryos are well documented [7] and focus primarily on the safety of embryo biopsy, the importance of low diagnostic error rates, and the need to assess the copy number of all chromosomes, not just the 8–12 possible using FISH. While 24 chromosome FISH is now possible with recently released probes and protocols, technical issues related to signal interpretation and hybridization efficiency, coupled with poor clinical trial data, have signaled the end of FISH testing in eggs and embryos and its replacement with alternative methods. In particular, the advent of robust and relatively inexpensive microarray technologies, allowing rapid evaluation of all 24 chromosomes has led to arrays superseding FISH in most laboratories around the world. Despite the superior technical capabilities of array-based testing methods compared with FISH and several prospective clinical trials showing the benefit of array-based testing, the policy position of both professional and regulatory bodies on PGS has not been revised and continue to take an extremely cautious line, typically referring only to the historic and flawed FISH approach [8, 9].

Current clinical applications for PGS include first polar body, combined first and second polar body, cleavage stage, as well as trophectoderm biopsy [10]. To date, cleavage-stage biopsy has been most widely applied. Biopsy at this stage of development has long been considered to be harmless [11, 12]; however, more recent work has shown that it may reduce implantation potential especially when two cells are biopsied [13]. Concerns about the impact of cleavage-stage biopsy and the

accuracy of genetic diagnosis based upon a single cell have led to an increased clinical utilization of blastocyst analysis. The invasive nature of oocyte and embryo biopsy has led to PGS historically being used to target specific high-risk patient groups (advanced maternal age; repeated implantation failure; recurrent pregnancy loss, and severe male factor infertility). More recently, PGS has been applied to patients considered to have a good ART prognosis in an attempt to improve the success rates of IVF treatment in cycles involving single embryo transfer (SET) (a strategy used to reduce the risks of multiple gestation) [3]. Considering that PGS has a financial cost and that embryo biopsy is invasive and potentially associated with a small risk to the embryo, a robust cost–benefit analysis is essential to confirm whether or not a given patient benefits through the use of PGS [14]. This chapter explores the current methodologies employed for the purpose of PGS using microarray CGH and looks into the future to describe new technologies such as next-generation sequencing (NGS) and how this technology will shape the future of preimplantation testing.

Methods

Biopsy Strategies

A number of different embryo biopsy strategies have been used clinically for PGS. For oocyte testing, first polar body (PB1) biopsy alone and combined PB1 and PB2 strategies have both been used clinically. However, it has become clear that PB1 alone has limited applicability for PGS as up to 30 % of maternal aneuploidy will not be diagnosed if only PB1 is analyzed [15–19]. As precocious separation of sister chromatids appears to be the predominant cause of maternal meiotic aneuploidies, it is critical to biopsy PB2 as well to accurately identify all maternal aneuploidies and ensure that abnormal segregations in PB1 are not corrected in the second meiotic division. The timings of both PB1 and PB2 biopsy are also critical to the efficiency of diagnosis. This was relevant when aneuploidy screening utilizing FISH technology was popular [20] and is equally critical when using array CGH [21, 22] or newer technologies for polar body testing.

Blastocyst stage biopsy may be the optimal stage for aneuploidy screening as it partially negates the problem of mosaicism, allows analysis of meiotic aneuploidy from both the maternal and paternal complement, detects post-zygotic events (mitotic errors), and appears to have minimal impact on the developing embryo. Historically, blastocyst testing necessitated the use of embryo cryopreservation to allow enough time for testing; however, newer methodologies provide results in approximately 12 h from sample receipt permitting fresh transfer. Cryopreservation may have been viewed as a detriment to testing in the past, although it is becoming increasingly apparent that vitrification is a viable strategy to maintain or even potentially increase live birth rates following biopsy [23]. In addition, the routine use of embryo vitrification may allow clinicians and patients to overcome logistic issues

with sample transportation and diagnostic testing. It should be noted that not all embryos created during an IVF cycle successfully develop to the blastocyst stage, so not all patients will produce embryos suitable for biopsy [24]. Therefore, patient education and management of expectations are important components of PGS.

Principles of Array Comparative Genomic Hybridization

Comparative genomic hybridization (CGH) was originally designed for molecular karyotyping of tumor cells [25]. It is a method where the chromosomal count of a cytogenetically uncharacterized DNA sample can be inferred according to its ability to hybridize to target DNA sequences affixed to a solid support, such as a microscope slide, in competition with a reference DNA of known (normal) karyotype. The CGH procedure can be performed using target DNA composed of (1) metaphase chromosomes from a karyotypically normal reference male (metaphase CGH) or (2) a series of specific DNA probes derived from sites along the length of each chromosome spotted onto a glass slide [array CGH (aCGH)]. A schematic representation of the principles of CGH is shown in Fig. 13.1. Metaphase CGH is time consuming, taking 3–4 days to complete one experiment; however, it has been used clinically for PGS [26, 27]. All CGH methods require nanogram to microgram quantities of DNA for optimal performance, whereas a typical single cell contains approximately 6 pg of DNA. Consequently, whole genome amplification is required prior to the CGH procedure itself.

In the case of array CGH, the DNA spotted onto each slide can be from bacterial artificial chromosome (BAC), DNA clones (typically longer sequences) from defined chromosomal regions, or specific oligonucleotides (shorter DNA sequences). This chapter will focus mainly on the BAC clone approach as this system is the most well validated of the methods and has been used for well over 400,000 clinical preimplantation genetic samples to date. The most widely used array, 24Sure™ (Illumina Inc., San Diego, CA) contains nearly 3,000 DNA spots spaced approximately 1 Mb apart. Each clone was chosen based on minimizing copy number polymorphisms, and its location has been confirmed via reverse painting and FISH mapping.

Embryo Biopsy and Sample Handling

Following biopsy of the egg (polar body 1 and/or 2), embryo (cleavage stage), or blastocyst, the sample is washed through a number of droplets, most often phosphate buffered saline (PBS) with an additive such as polyvinyl alcohol (PVA) to reduce cell stickiness. The sample is then picked up in a small volume (<2 μL) and placed into a sterile 0.2 mL Eppendorf tube for transport to the laboratory for testing. Most embryologists perform a quick step to ensure that the cellular material and all of the fluid are collected together at the bottom of the sample tube. This can be accomplished by centrifugation or a quick flick of the tube to collect the sample

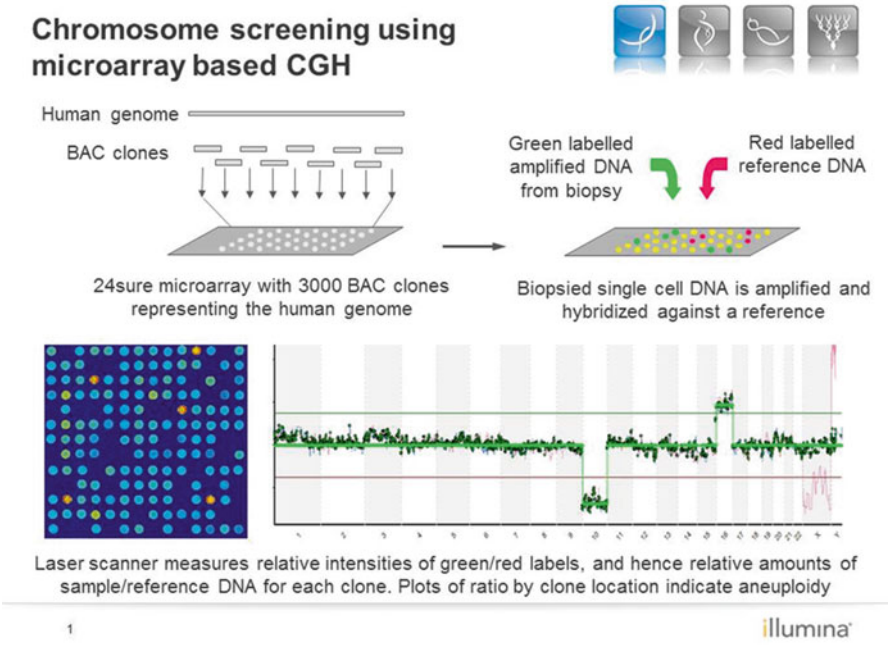

Fig. 13.1 Application of microarray technologies for PGS. Following biopsy, DNA is amplified using WGA, and then each biopsy sample is labeled with *green* fluorescent tags. *Green* biopsy sample is mixed with normal male DNA labeled with *red* fluorescent tags in equal proportions. The mixture is hybridized onto the BAC array and, following stringent washing, the slide is analyzed for the proportion of *red* and *green* fluorescence on each spot. Computer algorithms are used to assess each spot and call any gains or losses of chromosome in the test sample as compared to the normal DNA

in the bottom. Depending on the length of time the sample will have to travel, ambient temperature or wet ice (blue ice packs) can be used for shorter trips, while dry ice may be used for longer distance trips, especially in locations with warm weather at certain times of the year. While mineral oil may be used as an overlay in molecular biology experiments that require polymerase chain reaction (PCR), it should be noted that mineral oil should never be used prior to whole genome amplification (WGA) and aCGH as it inhibits the amplification process and will yield samples with no results. Of course, each laboratory will have its own standard operating procedure for sample handling ahead of transport to the testing laboratory.

Whole Genome Amplification and Labeling

A number of different WGA methods have been used historically for array CGH experiments, with the current, most often utilized method being SurePlex™ (Rubicon Genomics Inc., Ann Arbor, MI, USA and BlueGnome). This WGA kit is fragment amplification based, where self-inert degenerative primers are annealed at multiple

sites along the genome. This system was chosen because it produces optimal fragment sizes, which have been found to be reproducible between samples and are optimized for array CGH. Many of the other WGA techniques have been adapted for use in array CGH but were originally used for other purposes (e.g., single locus PCR and mutation detection). SurePlex™ is suitable because of its simple, short protocol and highly representative amplification.

Following sample receipt and accessioning in the lab, each tube is opened in a dedicated DNA amplification clean room, under a laminar flow or PCR-dedicated hood. Amplification is performed according to the manufacturer's instructions as the SurePlex™ kits have been validated for use in single cells. When using the SurePlex kit, the first step is lysis/extraction step (15 min), followed by pre-amplification steps (90 min), and finally amplification (30 min). To reduce possible contamination issues and eliminate the risk of accidental sample switches, all steps in SurePlex are performed in a single tube. The procedure is performed in a PCR machine as each step is temperature and time dependent.

After SurePlex, agarose gel electrophoresis is performed to confirm successful amplification. As the arrays can be quite expensive, it is best to ensure amplification prior to taking the sample further through the process. A smear of DNA near the top of the gel is indicative of good amplification; low molecular weight DNA or no DNA would be indicative of poor/no amplification. Following agarose gel verification of good amplification, the WGA product is labeled through nick translation with either Cy3 (green) or Cy5 (red) fluorescent tags.

Hybridization

In traditional aCGH, embryo biopsy samples labeled in one fluorescent color (e.g., green) and control reference DNA (typically a karyotypically normal male) labeled in an alternative color (e.g., red) are denatured at 74 °C to make the DNA single stranded. The single-stranded DNAs from the sample and control are then mixed together in equal proportions in hybridization buffer containing formamide and cot-1 human DNA before being adding to each 24Sure™ microarray. Microarrays are hybridized at 47 °C for at least 4 h or overnight in a humidified chamber. The length of hybridization time varies depending on the timing of biopsy, the number of samples in the lab on any given day, staffing levels, and shift patterns. During validation of the array in the lab, hybridization times as short as 3 h and as long as 16 h (overnight) were tested with no differences in diagnostic accuracy noted [28]. On the basis of these results, hybridization for at least 4 h and no longer than 16 h is deemed to be interchangeable. It should be noted, however, that shortening both labeling and hybridization may lead to suboptimal results; therefore, any protocol used clinically should be robustly validated prior to use on actual human samples.

More recent advances have led to so-called single channel aCGH. With this method, control DNA is not hybridized on each array, rather the control DNA is hybridized in each fluorescent color (Cy 3 and Cy 5), for both normal male and normal female, on separate arrays run during each experiment. Therefore, each

experimental array in single channel aCGH contains two embryo biopsy samples, one labeled in Cy3 and another labeled in Cy5. When the analysis is performed, each experimental sample is compared in silico to the male and female reference separately. Single channel aCGH allows for more samples to be run per experiment and reduces the amount of control DNA necessary in each experiment. This has also allowed the price per sample to be lower than in conventional aCGH.

Post-Hybridization Washing

Following hybridization, each microarray is washed as follows: 10 min in 2×SSC/0.05 % Tween 20 at room temperature, 10 min in 1×SSC at room temperature, 5 min in 0.1×SSC at 60 °C, and 2 min in 0.1×SSC at room temperature to remove unbound DNA.

Scanning

Each microarray slide is scanned using a dual channel fluorescent laser scanner in order to create TIFF images (e.g., ClearScan™, Illumina) showing green fluorescence at 632 nm and red fluorescence at 587 nm associated with hybridization of embryo and reference DNA samples, respectively. Raw images are loaded automatically into analytical software such as BlueFuse™ for evaluation of fluorescent signals (ratio analysis).

Scoring

Sample scoring is typically performed by a trained technologist who assesses traces for all 24 chromosomes, noting all gains and losses, as well as determining the sex of each sample. A second technologist then scores the sample blindly, with no knowledge of the initial scoring. The final result for each sample is then assigned by comparing the two scores. If discrepancies are noted between the two assessors, they are typically adjudicated by a third technologist and/or the laboratory supervisor or director. It should be noted that the current version of the BlueFuse™ software allows for automated calling of whole chromosome gains and losses; however, most laboratories do not rely on this for clinical diagnosis.

Reporting

Once results for all samples from each patient are finalized, a diagnostic report is prepared, signed off by an appropriately qualified person (on site or remotely), and shared with the referring laboratory and physician prior to embryo transfer.

Discussion

Validation

In extensive validation using single cells from known cell lines against the gold standard of karyotyping, 24Sure™ demonstrated 98 % accuracy [29]. The use of cell lines does, however, have drawbacks. During this validation, mosaicism was seen in most cell lines meaning that any one cell in the culture may or may not always have the same molecular genotype. Validation for embryo aneuploidy is perhaps even more difficult as truth data (i.e., definitive proof that the sample used as an unknown is actually the genotype that you expect it to be); this is difficult to obtain for human embryos grown in culture (due to mosaicism, for example). Human oocytes offer an interesting method for validation. One can biopsy the first and second polar body and use array CGH to analyze the chromosome complement in each sample individually. This method allows a laboratory to look at trios of data, comparing the first and second polar body to the oocyte. The expectation is to see reciprocal chromosome gains and losses from aneuploid polar body(ies) and oocyte [30].

Limitations of Array CGH

While array CGH has been shown to be highly accurate and reproducible in multiple validation studies and has been used on hundreds of thousands of embryo samples, it still has drawbacks that must be understood prior to clinical use. For example, aCGH cannot discriminate between maternal and paternal errors; it can simply elucidate chromosome gain and loss. It remains to be determined whether knowledge of the parental source of error has clinical value. Array CGH cannot distinguish between meiotic and mitotic errors of chromosome segregation; however, again the data on whether this is an important factor remains unclear. Perhaps the most clinically relevant limitation of aCGH is the fact that it cannot distinguish a euploid embryo from certain forms of triploidy (i.e., 69,XXX chromosomes) or tetraploidy (i.e., 92 chromosomes). Purely triploid and tetraploid embryos often implant, leading to pregnancy loss prior to delivery.

Competing Technologies

While array CGH has become the gold standard and most widely used method for counting chromosomes clinically, there are a number of competing platforms that could challenge this position. As with all competing technologies, there are advantages and disadvantages to each [31, 32]. Comprehensive chromosomal screening

using multiplex quantitative PCR (qPCR) [33] has been proposed as a faster and less expensive means of detecting aneuploidy. However, the qPCR systems optimized for embryo analysis have not been fully commercialized, restricting availability. Furthermore, existing qPCR systems are only applicable to trophectoderm samples and cannot be used for the analysis of polar bodies or single blastomeres. Chromosome counting can also be performed through the use of single nucleotide polymorphism (SNP) arrays. Using a combination of loss of heterozygosity, quantitative SNP calling, and analysis of patterns of SNP inheritance from parents to embryos, it is possible to detect chromosomal gains and losses [34–37]. SNP-based arrays do offer the ability to detect the parent of origin in aneuploidy cases and have been validated to reliably detect inheritance of specific genotypes allowing for nearly universal detection of many single gene defects [34]. However, SNP arrays also have a much longer protocol (24 h+ in most cases), are typically more expensive than alternative methods, and typically require parental DNA ahead of testing adding to the cost and time needed for this type of array.

Noninvasive Indirect Methods of Determining Aneuploidy

It is appealing to consider noninvasive approaches to embryo selection. Weak correlations exist between the presence of embryonic aneuploidy and morphological aspects of embryo development following retrospective analysis [38, 39]. These findings have stimulated the field of morphokinetic analysis during IVF, with an attempt to identify aneuploidy in a real-time clinical setting. Analysis of time-lapse imaging during embryo growth demonstrates that certain morphologic features and/or developmental timings of the embryo may have some relationship to aneuploidy [40, 41]. This data, if confirmed in larger data sets with appropriate subgroup analysis stratified by maternal age and in multiple clinics, may provide some useful information to place embryos in the order of priority for transfer. However, it does not appear that morphokinetics will have the capacity to provide the same level of specificity and accuracy yielded by aneuploidy testing using array CGH. Another promising morphokinetic approach is to assess dynamic fragmentation patterns within early embryos but again, this currently does not identify specific aneuploidies and only provides a relative risk of abnormality for each embryo [42]. Currently, no morphokinetic parameter or set of parameters has been shown to be able to discriminate between euploid and simple aneuploid (e.g., trisomy 21) embryos. In addition to morphokinetics, measurement of specific metabolites or combinations of biologically relevant molecules in culture medium has been suggested as a method to predict the viability of an embryo. However, none of these methods have been proven to be able to differentiate between general chromosomal aneuploidy and specific aneuploidy in prospective controlled studies.

Next-Generation Sequencing for Chromosome Counting

Next-generation sequencing (NGS) may supercede all other methodologies as it promises several advantages over all other techniques [43]. The term *next-generation sequencing* (NGS) describes several distinct methods that share in common an ability to provide huge quantities of DNA sequence data from the samples analyzed, rapidly, and at relatively low cost. There are two ways in which NGS can be employed for the detection of aneuploidy screening. The first involves biopsy of cells from embryos followed by whole genome amplification, after which the DNA is broken into small fragments and then subjected to NGS. The sequence of each fragment is compared to the sequence of the human genome, allowing its chromosome of origin to be determined. The relative proportion of fragments attributable to each chromosome is indicative of its copy number—e.g., an increase in the proportion of DNA fragments derived from an individual chromosome (relative to a chromosomally normal sample) is evidence of a trisomy. The second way that NGS can be used for aneuploidy detection involves the use of multiplex PCR (rather than WGA) to simultaneously amplify multiple specific loci on each chromosome. After amplification, the mixture of DNA fragments is analyzed using NGS, and the number of sequences attributable to each chromosome is calculated. A deviation from the expected number of DNA fragments for a particular chromosome is indicative of aneuploidy.

NGS promises several advantages compared with other technologies for screening aneuploidy in embryos, but perhaps the most important is its potential to reduce costs. Although each NGS experiment remains relatively expensive, costs per sample can be lowered significantly by simultaneously sequencing large numbers of embryos, thus sharing expenses across multiple samples. This strategy also has the effect of reducing the proportion of the genome analyzed from each embryo, which may mitigate ethical concerns related to NGS, as it prevents reliable analysis of individual genes. However, at this time, the cost to analyze a single sample from an embryo by NGS is comparable to the cost of analyzing the same sample by current methods like aCGH. This is likely to change over time as sequencing costs continue to drop as the technology improves.

Following extensive validation, NGS has been used to screen embryos in clinical cycles leading to the birth of healthy children in the United States [30] and China [43]. In addition to the clinical utility, a recent paper details the validation of NGS-based PGS in one laboratory as compared to the current standard of care aCGH (24sure) in one laboratory [44]. In the future, the extraordinary power of NGS may be used to evaluate additional aspects of embryo biology, relevant to viability assessment. Furthermore, as NGS provides DNA sequence information, it also has the potential to be used for the targeted detection of specific mutations responsible for inherited disorders at the same time as screening for aneuploidy.

With innovations such as NGS, we are entering a new and exciting era in preimplantation genetics which is well positioned to enable greater use of the single embryo transfer strategy for IVF patients. The next few years will see less expensive tests and analyses that provide a more detailed insight into embryo viability than

those currently available. Superior embryo viability screening and lower costs, resulting in increased patient access, will likely contribute to a significant improvement in the success rates of IVF. A number of randomized clinical trials are already underway to confirm whether or not this prediction is correct.

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Chapter 14
SNP Array, qPCR, and Next-Generation Sequencing-Based Comprehensive Chromosome Screening

Nathan R. Treff, Eric J. Forman, and Richard T. Scott, Jr.

Introduction

In the last several years, an explosion of new technologies for interrogating DNA has occurred. The field of preimplantation genetic screening (PGS) of aneuploidy is one of many that has benefited from these new technologies. Methods of comprehensive chromosome screening (CCS) have incorporated metaphase comparative genomic hybridization (CGH) [1], single nucleotide polymorphism (SNP) arrays [2], bacterial artificial chromosome (BAC) arrays [3, 4], oligonucleotide arrays [5], quantitative real-time PCR [6], next-generation sequencing (NGS) [7], and an ever-growing public database on the human genome sequence and population level variants [8, 9].

The ability to characterize all 24 chromosomes (CCS) has overcome at least one of the major limitations of historical methods of aneuploidy screening where only a subset of chromosomes could be evaluated. Multiple randomized controlled trials have demonstrated improved success of IVF with the incorporation of CCS, primarily when applied to trophectoderm biopsies from the blastocyst stage embryo [10–12]. The success with blastocyst biopsy alludes to the fact that it may also represent the safest point in preimplantation embryonic development to perform an embryo biopsy [13]. In addition to understanding the safety of embryo biopsy, the clinical predictive value of an aneuploidy diagnosis should also be considered to avoid the disposition of reproductively competent euploid embryos given a false-positive

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diagnosis of aneuploidy [14]. Finally, as scientists explore additional biomarkers of reproductive potential, controlling for aneuploidy through the incorporation of CCS into study designs remains critical to success [15].

This chapter focuses on developing strategies for preclinical and clinical validation, the ongoing limitations associated with existing technologies, and the future possibilities associated with CCS-based selection to achieve success with elective single euploid embryo transfer.

Whole Genome Amplification

One feature which sets the field of PGS apart from other areas of genomic medicine is the paucity of original material available to sample. Since most methods to characterize DNA were developed for large starting amounts of DNA (i.e., DNA isolated from whole blood), this poses a challenge that must be adequately addressed whenever new technologies are applied to the analysis of preimplantation embryos. Multiple displacement, PCR-based, and other methods of whole genome amplification (WGA) have aided in the ability to apply conventional methods to evaluate aneuploidy in the embryo by significantly increasing the quantity of DNA available for analysis.

However, all methods of WGA are prone to introducing bias. This is due to a number of factors including the fact that typical quantities of DNA necessary to perform array-based or NGS-based assessment range from a few nanograms to micrograms of DNA and that a single cell contains only 6 pg of DNA. Therefore, WGA methods need to copy the DNA at least 1,000 times and accomplish this uniformly across more than three billion basepairs in the human genome. The complexity of the human genome sequence includes varying GC content within specific chromosomes that impact polymerase efficiency, repetitive elements that are difficult to amplify, and chromatin structure that may impede enzymatic access to certain portions of the genome and in unique ways depending upon the cell type. These and many other factors prevent reproducible and uniform amplification of the entire genome via WGA [16].

Despite the challenges associated with single cell WGA, there are a number of factors that favor the ability to detect aneuploidy in the preimplantation embryo. One advantage is the fact that there are thousands of basepairs present on each chromosome such that many independent measures of the same chromosome are possible in order to accurately count whole chromosomes. Even when many individual measures are inaccurate when taken separately, by combining thousands per chromosome, it becomes more feasible to obtain an accurate count. This strategy appears to be successful with many combinations of WGA and array-based methodologies [17].

For example, the use of GenomePlex WGA4 (Sigma Aldrich Inc.) in combination with SNP arrays that contain 262,000 SNP probes (Affymetrix Inc.) demonstrated 99 % accuracy in predicting the copy number of chromosomes across the human genome [18]. This method involves Gaussian smoothing across a large

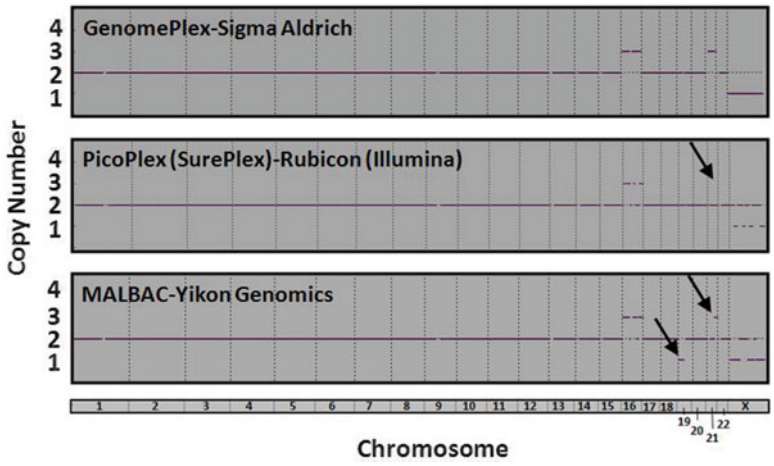

Fig. 14.1 SNP array-based single cell copy number analysis plots using three different WGA methodologies. The Sigma Aldrich GenomePlex WGA4 provided the most accurate copy number assignments when compared to Rubicon Genomics' PicoPlex (also repackaged as SurePlex by Illumina) or Yikon Genomics' MALBAC WGA kit. Both false-negative and false-positive imbalances were detected by the latter two WGA methods

window (five million basepairs or five megabases) to overcome errors in copy number assignments at each individual SNP. That is, the data for five megabases (Mb) surrounding each individual SNP are used to make the final assignment of copy number for that individual SNP. This strategy helps overcome the bias at individual loci introduced by WGA from single cells. Interestingly, when applying a variety of methods of single cell WGA, not all methods perform well (Fig. 14.1), suggesting that unique combinations of WGA and array technology may be more suitable than others.

SNP Array-Based CCS

There are many array-based methods available to interrogate WGA DNA for CCS, including array CGH with BAC arrays or oligonucleotide arrays and various SNP arrays from a number of manufacturers. As array CGH is discussed at length elsewhere in this book, here we will focus on SNP array technologies.

There are two major ways in which SNP arrays have been utilized to interrogate aneuploidy in the human embryo. One involves the use of genotyping data and inheritance patterns to indirectly predict chromosome copy number [19, 20]. Mendelian inheritance rules of bialleleic SNPs and linkage within haplotype blocks are important to the success of these methods. For example, when each parent possesses homozygosity for opposite alleles (i.e., mother is AA and father is BB), all

normal embryos will inherit an AB genotype. When the observed embryonic genotype differs from AB, it is an indicator that aneuploidy exists. For example, if the embryo was found to have a BB genotype, it would indicate that only the paternal chromosome was inherited and that a monosomy of maternal origin was present. Additional criteria such as crossover frequency and position and probabilities of inheriting blocks of SNPs from each parent based upon published frequencies from the HapMap project also impact the predictions of aneuploidy in some instances. At least two groups have developed this type of approach which appears to be better suited for MDA-based WGA instead of PCR based, particularly since MDA provides better genotyping accuracy.

Another way that SNP array data can be used to evaluate aneuploidy is through direct analysis of signal intensities. That is, at a given position (SNP loci), if the signal intensities are higher in an embryo than in a known normal sample it would indicate a trisomy state, or if the signal were lower, a monosomy state. A number of groups have utilized this strategy, most of which involve the use of PCR-based WGA, which gives better copy number accuracy than MDA. Accuracy of evaluating single cell aneuploidy in at least one case was established using cell lines already known to possess specific abnormalities. This study demonstrated a 99 % level of accuracy and represented an important preclinical validation step [18].

One advantage of using SNP arrays (compared to array CGH) is the availability of information from genotypes. For example, the ability to track which embryo implants after multiple-embryo transfer using SNP-based DNA fingerprinting has proven useful in a number of clinical trials, including studies on the clinical impact of embryo biopsy [13] and oocyte vitrification [15]. Other applications include determining the origin of aneuploidy [21], cell division origin of polar bodies [22], the ability to detect uniparental disomy [23], and confirmation of success of somatic cell nuclear transfer in stem cell research [24, 25].

SNP arrays have also been extensively evaluated for clinical utility. For example, outcomes following trophectoderm biopsy and blastocyst vitrification included a live birth rate of 71 % among patients where mean maternal age was 37.8 years [2]. A prospective, blinded, non-selection study demonstrated a 96 % predictive value of an aneuploidy diagnosis for a negative clinical outcome and that trophectoderm-based CCS had significantly better positive predictive value than did blastomere biopsy-based CCS [14]. Finally, an ongoing randomized controlled trial has demonstrated significantly improved success rates with the incorporation of SNP array CCS and blastocyst vitrification compared to fresh transfer of unscreened blastocysts [26].

SNP arrays have also been used to evaluate translocations in embryo from carrier patients [27–31]. This involves interrogating subchromosomal imbalances on each side of the breakpoints from cases with reciprocal translocations. Studies have demonstrated resolution to segments as small as 2.3 Mb. However, the ability to detect de novo segmental imbalances remains a matter of debate as many findings may represent artifacts of the methodology.

qPCR-Based CCS

While the use of SNP arrays was shown to improve outcomes compared to traditional morphology-based embryo selection, the per-embryo cost of WGA and SNP array chips is significant. Due to the processing time required for SNP array, trophectoderm biopsy and fresh embryo transfer within the same cycle would not be feasible within the window of time that the endometrium was receptive. These factors provided the impetus to develop more rapid methods of CCS. qPCR is faster and less expensive than all array-based methods of CCS which represents the driving force behind its development [32–34]. One of the ways in which costs are reduced is through avoiding the need for WGA. Instead, the embryonic DNA is amplified by multiplex PCR of specific targets in the genome, four per chromosome. Each target is then individually quantified in a subsequent qPCR reaction on a 384-well plate. Relative quantities of each target are determined through comparison to a known normal sample. Because the method only requires two PCR steps, it is extremely easy to perform in the laboratory, requires very little specialized equipment, and can be completed within 4 h of embryo biopsy. In addition, because the initial amplification uses conventional PCR, additional primers can be added which work under the same conditions and allow for specific targets such as single gene disorder loci to be interrogated in parallel [35].

Preclinical experiments have demonstrated 99 % accuracy of predicting 24-chromosome aneuploidy using qPCR [34]. In addition to demonstrating equivalence to G-banding in cell lines and SNP array testing in embryos, two randomized controlled trials showed significant improvements in the success of IVF when incorporating qPCR-based CCS [10, 11]. The first of these studies demonstrated a 17.2 % increase in delivery rates with the use of qPCR CCS compared to controls. The second study demonstrated equivalent success rates and no twins when a single qPCR euploid blastocyst was transferred compared to a control group that received two unscreened embryo transfers, having a twin rate of 53 %. Together, these studies appear to establish that elective single qPCR euploid blastocyst transfer is an effective methodology and follow-up studies indicate improved neonatal outcomes [36].

Next-Generation Sequencing-Based CCS

Similar to the driving force behind qPCR CCS development, next-generation sequencing (NGS) provides an even greater opportunity to reduce testing costs. The main premise of NGS-based CCS is the ability to produce massively parallel sequencing, which can then be computationally aligned to the published human genome sequence. The number of sequence reads which align to each specific chromosome can be counted and then normalized to a known normal sample so that each chromosome can be quantified. The ability to add molecular barcodes to each embryo

allows for many embryos to be sequenced in the same reaction. The molecular barcode is essentially a specific unique sequence of bases that can be artificially synthesized and added to the ends of the PCR products of each embryo. The labeled PCR products can then be pooled together for sequencing and then computationally segregated back according to which embryo it came from.

Some groups have begun to develop NGS for CCS using whole genome amplification (WGA) which may allow for whole genome sequencing (WGS) depending on the number of bases sequenced for each sample [37, 38]. However, counting chromosomes requires far less sequence per sample to accomplish than does WGS. Sequencing at low coverage and depth also helps avoid the potential for incidental findings, while maintaining an appropriate cost per sample [39].

Another approach to NGS-based CCS involves targeted multiplex PCR in place of WGA [38]. Similar to qPCR, a subset of the genome with distribution of targets on each chromosome can be amplified by PCR. This further enhances the cost reduction benefit of NGS since PCR is much less expensive than any commercially available WGA methodology. Another advantage is the ability to add primers for specific single gene disorder targets for simultaneous PGD and CCS [35].

Mosaicism

Mosaicism represents an important challenge to the success of CCS. Currently there is no CCS method that provides an opportunity to diagnose mosaicism in a preimplantation embryo. This is due to the unavoidable sampling error associated with CCS, since a representative biopsy is used to make a diagnosis. For example, in the event that a mosaic embryo is biopsied and the biopsied portion of the embryo is aneuploid, that embryo will be diagnosed as aneuploid, when in fact there may be euploid cells in the remaining embryo. In addition, if the biopsied portion is euploid, the embryo will be diagnosed as euploid despite the fact there may be aneuploid cells in the remaining embryo. In both cases, an accurate diagnosis would have been mosaic.

Since mosaicism cannot be accurately diagnosed, it is important to consider what the prevalence might be. However, prior studies have been limited either by a lack of comprehensive analysis of all chromosomes (i.e., FISH), sample selection bias (i.e., abnormal or discarded embryos), or the use of methodologies of CCS having unproven diagnostic consistency. For example, SNP array-based analysis of blastocysts indicated a mosaicism rate of 24 %, but this was derived from the analysis of 50 embryos that had been given an aneuploid diagnosis by cleavage stage FISH [40]. Therefore, these data may not reflect the rate of mosaicism present in the larger population of embryos from patients seeking CCS due to selection bias. Another set of data that might be used to infer the prevalence of mosaicism comes from the evaluation of the origin of aneuploidy. That is, aneuploidy derived from postzygotic mitotic errors would by definition result in a mosaic embryo. For example, data from sequential analysis of polar bodies and the embryo indicated a non-maternal

meiotic origin of aneuploidy rate of 33 % [21]. However, since the contribution of paternal meiotic aneuploidy could not be determined, a more precise estimate of the frequency of mitotic errors was not possible.

Another important consideration when performing CCS at the blastocyst stage is sensitivity to detection of aneuploidy within a mosaic trophectoderm biopsy. Previous data suggest that the sensitivity of array CGH is approximately 50 % [40], while SNP arrays are 40 % [41]. That is, when 40–50 % of the cells within a biopsy have the same aneuploidy, these methods may be able detect the aneuploidy successfully. Some authors have proposed that when an embryo has greater than 50 % euploid cells, then a euploid diagnosis is accurate, and that when an embryo is more than 50 % aneuploid, an aneuploid diagnosis is accurate [42]. However, this declaration on ploidy is based on arbitrary thresholds unsupported by any objective, scientific evidence.

With all of these factors in mind, it becomes important to consider how often mosaicism might lead to misdiagnosis. As discussed previously, one method to evaluate the predictive value of CCS is through a non-selection study where embryos are biopsied, but the CCS data are not used to select the embryos. This study allows one to evaluate whether CCS would have correctly predicted the actual clinical outcome. In the only such study, SNP arrays demonstrated a 96 % negative predictive value, indicating that 4 % of embryos diagnosed as aneuploid still possessed euploid cells sufficient to develop into a chromosomally normal child [14]. In this study, there were no false negatives (i.e., diagnosis of euploidy with subsequent implantation of an aneuploid gestation). However, the sample size to evaluate false negatives may not have been sufficient since most aneuploidies are incompatible with implantation or development into a clinically recognized pregnancy. Instead, a retrospective analysis of a large number of euploid embryo transfers can be performed. Indeed, such a study has indicated a clinical error rate of 0.21 % in 4,794 euploid embryo transfers, with all follow-up analyses of products of conception indicating the presence of mosaicism (unpublished observations).

Discussion

Considerable progress has been made over the last several years to establish evidence that supports the use of CCS to select embryos and improve the success of IVF. Additional factors such as the costs associated with the procedure and the applicable patient populations represent the current focus of ongoing research and development. Solutions to the problems associated with mosaicism may present themselves in the form of new noninvasive methodologies such as time-lapse morphokinetics. In addition, future work to develop additional biomarkers of reproductive competence of the human embryo stands to benefit significantly from the incorporation of aneuploidy screening in study designs. Specifically, it will be important to control for aneuploidy when evaluating the predictive value of new biomarkers since it is clearly one of the most important restraints on reproductive potential.

Finally, as we continue to benefit from the advances made in assessing and understanding the human genome sequence and move towards the era of personalized medicine, future studies to identify the genetics of aneuploidy risk and prevention represent an exciting opportunity to continue to improve the treatment of infertility.

Conflict of Interest The authors declare no conflict.

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Chapter 15
Expanding PGD Applications to Nontraditional Genetic and Non-genetic Conditions

Anver Kuliev, Svetlana Rechitsky, and Oleg Verlinsky

Introduction

Preimplantation genetic diagnosis (PGD) is presently an established clinical option in reproductive medicine [1–4]. Thousands of PGD cases are being performed, allowing at-risk couples not only to avoid producing offspring with genetic disorders but also to have single embryo transfers leading to unaffected healthy babies of their own without facing the risk of pregnancy termination after traditional prenatal diagnosis.

The application of PGD has further expanded to late-onset diseases with genetic predisposition, a novel indication never previously considered for the traditional prenatal diagnosis. For the patients with inherited pathological predisposition, PGD provides a realistic reason for undertaking pregnancy, with a reasonable chance of having an unaffected offspring [5].

Another unique objective of PGD is for HLA typing; this was not originally considered in traditional prenatal diagnosis schemes [6, 7]. In these applications, PGD offers not only preventative technology to avoid affected offspring but also a new method for treating (older) siblings with congenital or acquired bone marrow diseases, for which there is still no available effective therapy.

This chapter presents a different perspective on single embryo transfer. This chapter is devoted to these emerging options, especially when there is no opportunity to diagnose serious disease until it is fully realized, such as in cases of inherited

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cardiac diseases leading to premature or sudden death. The current experience of PGD for HLA is also described, which may in future be applied to any condition that can be treated by embryonic stem cell transplantation.

PGD for Inherited Predisposition to Common Late-Onset Disorders

Indications for PGD were initially parallel with prenatal diagnosis, and the technique was offered to at-risk couples who could not accept pregnancy termination—an outcome expected in 25–50 % of cases following prenatal diagnosis, depending on the mode of inheritance. However, these indications have been extended beyond those for prenatal diagnosis and currently include conditions with low penetrance, late-onset disorders with genetic predisposition, and HLA typing with or without testing for a causative gene. The list of disorders for which PGD has been applied, according to our experience, now comprises nearly 400 disorders, with the most frequent ones still being cystic fibrosis (CFTR), hemoglobin disorders, and some of the conditions caused by dynamic mutations, such as myotonic dystrophy [3, 4]. How patients choose between conventional prenatal diagnosis vs. IVF+PGD is chiefly influenced by the patient's attitude to pregnancy termination. Understandably, this position is strongly affected by social and religious factors, although steadily it is becoming a part of family planning for couples at risk to ensure having only an unaffected pregnancy. This is especially true when there is a risk of having offspring with severe late-onset common disorders with a strong genetic predisposition [5].

The diseases with genetic predisposition have not traditionally been considered an indication for prenatal diagnosis because this could lead to pregnancy termination, which may hardly be justified on the basis of genetic predisposition alone. Conversely, the possibility of choosing one embryo free of genetic predisposition for single embryo transfer (SET) would obviate the need for considering pregnancy termination, as only the potentially normal pregnancy is established. PGD for such conditions appeared to be acceptable on ethical grounds because only a limited number of the embryos available from hyperstimulation are selected for potential transfer or cryopreservation anyway.

Inherited Predisposition to Cancer

Cancers represent the largest single group of conditions with genetic predisposition for which PGD was performed. Our unit currently registers the world's largest series for this technique, involving PGD for 315 PGD cycles performed for 159 couples at risk for producing 18 different inherited cancers including BRCA 1 and 2, Li–Fraumeni disease, familial adenomatous polyposis (FAP), familial colorectal cancer, hereditary nonpolyposis coli (HNPCC) (types 1 and 2), Von Hippel–Lindau

syndrome (VHL), familial posterior fossa brain tumor (hSNF5), retinoblastoma (RB), neurofibromatosis 1 and 2 (NF1 and NF2), nevoid basal cell carcinoma (NBCCS) or Gorlin syndrome, tuberous sclerosis (TSC type 1 and type 2), ataxia teleangiectasia (AT), multiple endocrine neoplasia type 1 and type 2 (MEN1 and MEN2), and Fanconi anemia (FANC) (Table 15.1) [5]. This has resulted in transfer of 448 (1.7 embryos on average) unaffected embryos in 254 transfer cycles, yielding 112 (44 %) unaffected pregnancies and the birth of 128 healthy children free from predisposition to the screened familial malignancies [3, 8]. Of note, these disorders are all relatively rare autosomal-dominant conditions, with prevalence of 1 in 5,000 in the American populations for FAP, 1 in 15,000 for RB, 1 in 36,000 for VHL, and even less common for others.

The first PGD for inherited predisposition to cancer was performed for couples carrying p53 tumor-suppressor gene mutations [9], a cellular marker associated with a strong predisposition to many malignancies. Two couples presented for PGD, one with the maternally and one with the paternally derived p53 tumor-suppressor mutation. The father had a mis-sense mutation due to a G to A transposition in exon 5 of the p53 tumor-suppressor gene; this resulted in a change from Arginine to Histidine at amino acid residue 175 of the protein [10]. When he presented to our center, the patient was aged 38 and had been diagnosed with Li–Fraumeni syndrome (LFS). Moreover, he was diagnosed with rhabdomyosarcoma of the right

Table 15.1 PGD for cancer predisposition (based on [4])

Disease	# Patient	# Cycle	# Transfers	# Embryo transferred	Pregnancy	Birth
AT[a]	1	3	2	3	1	1
BCNS (gorlin)	5	6	5	9	3	3
Brain tumor	1	1	1	1	0	0
BRCA 1	25	40	31	54	18	27
BRCA 2	14	36	23	39	10	13
FANC	20	62	37	60	11	11
FAP	10	23	21	38	5	3
HNPCC 1	1	2	2	4	2	2
HNPCC 2	3	7	7	14	3	4
LFS	4	6	5	9	2	2
MEN1	4	12	11	18	4	4
MEN2	2	3	3	5	2	3
NF1	32	56	52	88	22	24
NF2	4	7	7	15	5	7
RB1	11	19	17	32	8	7
TSC1	13	19	18	37	11	15
TSC2	3	5	5	9	0	0
VHL	6	8	7	13	5	6
Total	159	315 (1.98)	254	448 (1.76)	112 (44 %)	128 (50 %)

[a]See abbreviations in the text

shoulder at the age of two followed by right upper extremity amputation. At age 31, he was also diagnosed with a high-grade leiomyosarcoma of the bladder and underwent a radical cystoprostatectomy. His mother was diagnosed with leiomyosarcoma at the age 37.

In the other couple, the 39-year-old mother with LFS was a carrier of 902insC mutation of the p53 tumor-suppressor gene, representing an insertion of C in exon 8. She was diagnosed with breast cancer at age 30, followed by bilateral mastectomy. She also had thyroid cystic carcinoma, which was also excised. Her mother died from a stomach cancer at age 51. One of her sisters diagnosed with breast cancer at 48 followed by mastectomy also died at age 51. Two of her four brothers were diagnosed with bone or brain tumor in their teens.

PGD for the maternal 902insC mutation was done by DNA analysis of PB1 and PB2, removed sequentially following maturation and fertilization of oocytes. The paternal G524A mutation was tested by DNA analysis of single blastomeres, removed from eight-cell embryos. Based on both mutation and STR analysis, unaffected embryos were preselected for transfer back to the patients. PGD resulted in a singleton pregnancy and birth of a mutation-free child in a couple with the paternally derived G525A mutation, demonstrating a potential for the preselection of the mutation-free embryos and the establishment of an unaffected pregnancy, rather than testing and termination of an ongoing pregnancy in utero. Because many at-risk couples have had such an unfortunate experience of repeated prenatal diagnoses and termination of affected pregnancies, naturally they regard PGD as their only hope for having healthy children of their own, despite having to undergo IVF.

Another cancer for which embryo testing with PGD has been successfully applied for many years is neurofibromatosis (NF). This is a relatively common autosomal-dominant neurological disorder with at least two distinct major forms, including NF type I (NF1), which is more common (1:4,000) and characterized by fibromatous skin tumors with cafe-au-lait spots ("Von Recklinghausen disease"), and NF type II (NF2), which is less common (1:100,000) and characterized by bilateral acoustic neuromas, meningiomas, schwannomas, and neurofibromas [11].

In our experience, of 36 couples who presented for PGD specifically for NF, 32 were at risk for producing a child with NF1 and 4 couples were at risk to have offspring with the NF2 mutation. As a result of IVF and PGD at this center, 31 healthy babies were born demonstrating the acceptable diagnostic accuracy of PGD of NF1 and NF2. So genetic counseling services may consider informing patients at risk of having children with a strong genetic predisposition to NF about the availability of PGD, without which these couples may remain childless because of their hesitancy to avail of standard prenatal diagnosis and possible pregnancy termination.

At present the most common cancer for which PGD has been performed is inherited breast cancer [3, 4, 12, 13]. Almost half of inherited breast cancers are caused by BRCA1 and BRCA2, and these mutations represent the primary indication for 76 PGD cycles here (Table 15.1). A total of 93 embryos free from these mutations were preselected for transfer in 54 cycles, resulting in birth of 40 children without predisposition to breast cancer.

As seen in Table 15.1, the other frequent indication was FAP. Patients with FAP usually present with colorectal cancer in early adult life, secondary to extensive adenomatous polyps of the colon, determined by mutation of adenomatous polyposis coli (APC) gene located on chromosome 5 (5q21-q22). Over 826 germline mutations have been found in families with FAP, causing a premature truncation of the APC protein (through single amino acid substitutions or frameshifts), with most common mutation being a 5-bp deletion resulting in a frameshift mutation at codon 1309. These APC mutations lead to a premalignant disease with one or more polyps progressing through dysplasia to malignancy with a median age at diagnosis of 40 years. Because the mutations in APC gene are almost totally penetrant (although with striking variation in expression), even presymptomatic diagnosis and treatment of carriers cannot exclude the progression of polyps to malignancy, thus making PGD an attractive approach for couples carrying APC mutations.

Eight cycles have been performed for VHL. These treatments resulted in the birth of six babies free of genes predisposing to VHL—a severe cancer syndrome with age-related penetrance characterized by hemangiomablastomas of the brain, spinal cord and retina, bilateral renal cysts, renal carcinoma, pheochromocytoma, and pancreatic cysts. Depending on the combination of these clinical features, four different types of the disease have been described. The gene responsible for VHL syndrome consists of three exons and is located on chromosome 3 (3p26-p25). Specific VHL gene mutations have been correlated to clinical phenotype. Its normal gene product is a tumor-suppressor protein, which is expressed in most cells and has a variety of functions, including transcriptional and posttranscriptional regulation. More than 300 germline mutations have been identified in families with VHL syndrome, consisting of partial or complete gene deletions, and frameshift, nonsense, mis-sense, and splice-site mutations, most commonly affecting codon 167. Mutations in the VHL gene either prevent its expression completely or lead to the expression of an abnormal protein. Because 80 % of VHL cases are familial, PGD is clearly an attractive option for couples carrying these mutations to avoid transmission of these serious tumor-suppressor gene errors to their offspring.

Nineteen cycles have been completed for inherited predisposition to RB, caused by the germline mutations in the RB1 gene located on chromosome 13 (q14.1-q14.2). RB is a malignant tumor of retina, which occurs in cells with cancer-predisposing mutations usually before the age of 5 years. More than half of patients have the unilateral RB, which may be diagnosed at 24 months, while the bilateral RB is recognizable as early as at 15 months, using direct ophthalmoscopy. The majority of cases are due to a point mutation in coding regions of the RB1 gene, while partial deletions of the gene have also been described. Over 200 distinct mutations have been reported, with the majority resulting in premature termination codon, usually through single base substitutions or frameshift or splice mutations, scattered throughout exon 1 to exon 25 of the RB1 gene and its promoter region. Such mutations result in loss of the cell cycle regulation function of the RB1 protein and are nearly completely penetrant in nonsense and frameshift mutations, making PGD an important option for couples at risk of this disorder.

At our institution, IVF with PGD has been undertaken for 11 patients at risk for producing offspring predisposed to RB. Unaffected embryos were preselected and transferred, resulting in clinical pregnancies in seven cases, with birth of seven healthy babies free from the mutant gene predisposing to RB.

A single PGD cycle was performed for a patient carrying the hSNF5 mutation, which predisposes to a very rare type of brain tumor found in sporadic rhabdoid tumors of the central nervous system [8]. Rhabdoid tumors are known to be highly malignant neoplasms usually occurring in children under 2 years of age. Although rhabdoid tumors determined by truncating mutations of the hSNF5 gene are mainly de novo and therefore were not previously thought to be present in parents of affected children, a first familial case of posterior fossa brain tumor has been described in two generations [14]. The proband presented at the age of 18 months with a cerebellar malignant rhabdoid tumor. Although the parents were healthy, the child's maternal uncle died at age 2 from a posterior fossa choroids plexus carcinoma, and her grandfather's sibling also died as an infant from a brain tumor, suggesting the presence of a germline mutation. The couple presented for PGD in order to have a pregnancy free from hSNF5 mutation, also avoiding the birth of a second child with a brain tumor. The mutation was due to G to A substitution in a donor splice site of exon 7, which alters the conserved GT sequence at the beginning of the intron violating the GT rule for splice-site recognition. In this unique case, the mother was unaffected but her daughter who inherited the mutation had a brain tumor. Because the mutation was also detected in DNA from her uncle's tumor, this suggested the risk of transmitting the mutation to the next child. Accordingly, PB1 and PB2 were removed in this case to identify mutation-free oocytes during IVF.

As summarized in Table 15.1, the range of PGD specifically for malignant disease is expanding and most IVF cycles incorporating this technique result in birth of children free of genes which predispose to these hereditary disorders. With current advancements in molecular diagnosis of such cancers (including sequencing of the genes involved in malignancy), it is likely that this application for PGD will become even more prominent. Despite extensive discussions concerning the ethical and legal issues involved in PGD for late-onset disorders with genetic predisposition, an increasing number of patients have come to regard the procedure not just as their best option—but as their *only* option—to experience a pregnancy of their own, without reliance on donor gametes. Thus, IVF with PGD in these cases permits patients to sidestep a potentially difficult decision to terminate a pregnancy at high risk of being affected with a heritable cancer.

Of note, because such diseases present beyond early childhood and even later may not be expressed in 100 % of the cases, the application of PGD for this group of disorders remains highly controversial. However, initial experience with PGD specifically in these settings shows that the availability of this technology can allow couples to undergo single embryo transfer and have a healthy baby. Otherwise, these patients would have never attempted pregnancy without PGD. This may be further demonstrated by PGD performed for genetic predisposition to Alzheimer disease (AD) [15].

Alzheimer Disease

Alzheimer disease (AD) is a rare autosomal-dominant familial predisposition to a presenile form of dementia. Three different genes have been implicated in this form of AD, including amyloid precursor protein (APP) gene, which is well known for its role in the formation of Amyloid deposits found in the characteristic senile plaques seen in AD [16–18]. The early-onset dementias associated with βAPP mutations are nearly completely penetrant and, therefore, are potential candidates not only for predictive testing but also for PGD. Of 10 APP mutations presently described, mutations in the exons 16 and 17 have been reported in familial cases with the earliest onset. One such mutation with onset as early as the mid or late 30s has been reported to be due to a single G to C nucleotide substitution in exon 17, resulting in a valine-to-leucine amino acid change at codon 717 (V717L). This mutation was identified in three of five family members tested (all siblings), one of whom presented for IVF and PGD. This treatment resulted in a pregnancy and delivery of a healthy baby, free from APP mutation [15].

The patient was a 30-year-old woman with no signs of AD but who carried the V717L mutation. This resulted in a G to C substitution in exon 17 of the APP gene. Predictive testing in the patient was sought because of early-onset AD in her sister carrying this mutation; the sister developed symptoms of AD at age 38. Their father had died at the age of 42 and had also a history of psychological difficulties and marked memory problems. The V717L mutation was also detected in a brother of the proband. His mild short-term memory problems began at age 35, and this was accompanied by declines in new learning and sequential tracking in the subsequent 2–3 years. Other family members were asymptomatic. Although predictive testing was done only in sisters, all appeared to be free from mutation in the APP gene.

Two PGD cycles were performed to test for the maternal mutation using DNA analysis of PB1 and PB2. A total of 23 oocytes were available for testing from both IVF cycles, of which 15 were tested by both PB1 and PB2 (13 in one cycle and 2 in the other). The mutation and linked marker analysis revealed six normal and nine affected oocytes (two in one cycle and seven in the other). Following embryo transfer, a singleton clinical pregnancy was followed by birth of an unaffected child.

PGD for early-onset AD may therefore provide a nontraditional option for appropriate patients, especially those who wish to avoid transmission of a mutant gene which predisposes to early AD in offspring. Because this condition may not be expressed in 100 % of cases and never presents at birth (or even early childhood), the application of PGD in this setting is controversial. However, with no current prospect for treatment of AD (a debilitating disorder that can appear despite presymptomatic diagnosis and follow-up), PGD seems to be the only relief for the at-risk couples. Indeed, this approach was used for a total of seven IVF cycles with genetic risks for AD or dementia, and the technique was considered to be an acceptable option by patients.

Inherited Cardiac Diseases

PGD applications have also expanded to cover inherited serious cardiac disease, which may occur despite presymptomatic diagnosis and follow-up. The first case of PGD for inherited cardiac disease was described for a couple at risk for producing offspring with Holt–Oram syndrome (HOS).

This is an autosomal-dominant condition determined by mutation in *TBX5* gene [19]. HOS is characterized by atrial septal defect, cardiac conduction disorders, and upper extremity malformations. These clinical manifestations may be extremely variable and are often not evident at birth (sinus bradycardia may be the only clinical sign which is frequently unnoticed).

Since some inherited cardiac disorders may never manifest clinically even during an entire lifetime, the application of PGD is controversial in these contexts. This likely explains the limited application of PGD for inherited cardiac diseases at present. Yet most inherited cardiac disorders are dominant conditions and unfortunately no cure exists as their first and only clinical presentation may be sudden, premature death. One such condition is hypertrophic cardiomyopathy (HCM), which clinically manifests at different ages. It can be clinically silent for years until provoked by different factors, such as excessive exercise. PGD was applied to different genetic conditions leading to HCM, including HCM4 and HCM7. HCM4 is caused by mutation in the gene *MYBPC3*, encoding the cardiac isoform of myosin-binding protein C, exclusively in heart muscle. HCM7 is caused by a mutation in *TNNI3* gene, leading to asymmetric ventricular hypertrophy and deformed interventricular septum associated with high risk of cardiac failure and sudden death.

Another condition for which PGD with single embryo transfer has been highly useful is dilated cardiomyopathy (CMD). This is an autosomal-dominant disease caused by various mutations in the *LMNA* gene, situated on chromosome 1. This disorder is characterized by ventricular dilation and impaired systolic function, resulting in a heart failure and arrhythmia and ultimately premature (or sudden) death. While the large phenotypic variability of patients may be determined by different mutations in *LMNA* gene, differences from one family to another may be observed within the same mutation, with possible involvement of skeletal muscles associated with generalized muscular weakness.

The relevance of PGD in this context may be demonstrated by the PGD case where it was determined that the male partner carried a *LMNA* mutation predisposing to CMD. He first experienced cardiac symptoms (palpitations) at age 22, with eventual diagnosis of ventricular tachycardia by a 48-h Holter monitoring 4 years later. It was known that this patient's family included numerous relatives with lethal cardiovascular problems; his father had died from heart failure at age 32 (based on these factors, our patient underwent placement of an implantable cardioverter-defibrillator). This patient joined others with similar genetic predisposition to cardiac disease to form a cohort of nine at-risk couples, for whom 18 IVF cycles were completed. PGD enabled preselection and transfer of embryos without the cardiac disease predisposition mutation in 15 of them. Nine pregnancies were established and seven healthy babies (without the relevant mutation) were born [20].

In nine cycles performed for four patients with CMD, 15 mutation-free embryos were preselected for transfer in eight IVF cycles. This led to the birth of three healthy babies (i.e., no mutation predisposing to CMD). In PGD for CMD where a dominant mutation in *LMNA* gene was identified, 10 of 11 embryos were confirmed to have this mutation and four linked polymorphic markers. Two embryos were found to carry a *R335T* mutation in the *LMNA* gene, while the remaining eight embryos had no *R335T* mutation. At the patient's request, two of these unaffected embryos were transferred, resulting in a singleton pregnancy and birth of a healthy child with no *LMNA* mutation.

In another patient group, four IVF cycles were performed for three couples at risk for producing offspring with CMH. Three embryos were preselected for transfer in two cycles, resulting in a singleton pregnancy. Of seven embryos tested, three had a frameshift mutation *D1078*fr in the *MYBPC3* gene, three were unaffected, and one did not amplify (no signal). An unaffected singleton pregnancy was achieved after FET.

In a PGD cycle for a patient at risk for producing offspring with CMH7, 3 of 11 embryos tested for CMH7 were unaffected after testing for the mutation and six polymorphic markers. Because these embryos were also tested for 24-chromosome aneuploidy by array-CGH analysis at the blastocyst stage, the embryos were placed in cryostorage. Subsequently, one was thawed and transferred.

Of three cycles performed for cardioencephalopathy, seven unaffected embryos were found unaffected and transferred, resulting in two unaffected pregnancies and birth of a healthy child free from infantile cardioencephalomyopathy. Of two PGD cycles performed for EMD, five disease-free embryos were preselected for transfer, yielding an unaffected pregnancy in each cycle and birth of two EMD-free children.

The above results show that PGD is an important therapeutic option for couples at risk for producing offspring with serious heritable cardiac disease. Inheritance of such susceptibility factors place the individual at risk of serious cardiac disease which may clinically manifest either as early as the first year of life (i.e., cardioencephalopathy) or later in life, with the only clinical realization of tragic pathology being premature or sudden death (i.e., CMD or CMH).

Which At-Risk Patients Should Consider PGD?

Among conditions in a couple's family history that suggest a possible need for embryo screening with PGD may include myocardial infarction and sudden death at young age. Family members with pacemakers or internal cardiac defibrillators, arrhythmia, and heart surgery, especially if young (age <40 year), may indicate a genetic predisposition to cardiovascular disease which should be evaluated. If present, a mutation would increase the risk that offspring from these patients will develop the same heart disease, although this will vary depending on the particular mutation, mode of inheritance, and penetrance. Of note, penetrance is often difficult to estimate because many inherited cardiac conditions are difficult to diagnose and will develop with age and may be induced by certain medications or activities.

In some cases, more common and apparently milder disease susceptibility genes may contribute to premature death, major disability, or hardship in a family. However, only an individual's own personal experience may influence their decision to undertake PGD. Many couples already going through IVF may have questions about the implications of genetic susceptibility factors for offspring, the option to test embryos, and the appropriateness of using PGD in testing for susceptibility to inherited cardiac disease.

Because symptoms of inherited cardiac disease may be easily overlooked as described in the cases above, the family history may provide the only reason to test for the presence of predisposing gene mutations. Using PGD in such settings has been regarded as a life-saving intervention for individuals at risk. So with further research and identification of additional genes which predispose to inherited cardiac disease, PGD will become an even more useful tool for couples at risk to avoid transmission of these disorders to the next generation.

Preimplantation Embryo Testing for HLA Typing

Preimplantation HLA typing was first proposed as a treatment for couples who desired an unaffected (younger) child free from the same genetic disorder which was present in an older sibling. This has been done in combination with IVF and embryo mutation analysis for Fanconi anemia, resulting in the birth of an unaffected child whose cord blood was then transplanted to the affected older sibling, saving her life [6].

Of note, preimplantation HLA matching has been used for many other congenital and acquired bone marrow diseases to obtain a disease-free offspring who can then become a potential donor for bone marrow transplantation treatment [7, 21–23]. At present, preimplantation HLA genotyping in combination with PGD has found clinical application in more than 1,000 IVF cycles. This has resulted in IVF with preselection and transfer of HLA-matched and unaffected embryos in 17.5 % of embryos tested. While the number of requests to perform PGD in combination with HLA typing has been increasing, a considerable proportion of patients request preimplantation HLA typing alone, without PGD. To date, our experience includes >3,000 PGD cycles performed for almost 400 different conditions, including single-gene defects, dynamic mutations, and some medically relevant genetic variations, of which >12 % of which have been performed for HLA typing (Table 15.2). Overall, 374 cycles for 163 patients have been performed, resulting in transfer of 351 HLA-compatible embryos in 230 cycles. This has yielded 72 clinical pregnancies and birth of 62 healthy children, all of whom were potential donors of HLA-compatible tissue for affected siblings requiring stem cell transplantation.

Another large series of PGD for HLA typing has been reported from Turkey, where 236 PGD cycles were performed resulting in birth of 70 disease-free and HLA-matched children [24–26]. While the majority of the cases in both series were undertaken for preimplantation HLA genotyping in combination with PGD for

Table 15.2 Preimplantation HLA typing with and without PGD [4]

Disease[a]	Gene	# Patient	# Cycle	# Transfers	# Embryo transferred	Pregnancy	Birth
HLA + adrenoleukodystrophy	ABCD1	2	5	1	1	0	0
HLA + Diamond–Blackfan anemia; DBA	RPS19, RPS24, RPL35A	6	10	7	10	3	3
HLA + DMD + Glanzmann's thrombasthenia	ITGA2B, DMD	1	2	2	4	1	0
HLA + dystrophia myotonica 1	DMPK	1	2	1	2	1	1
HLA + ectodermal dysplasia, hypohidrotic, with immune deficiency	IKBKG	2	9	6	8	2	3
HLA + FANCA	FANCA	16	46	28	40	9	6
HLA + FANCC	FANCC	3	6	6	9	2	2
HLA + FANCD2	FANCD2	1	3	2	3	1	1
HLA + FANCF	FANCF	1	3	2	3	0	0
HLA + FANCI	FANCI	1	2	2	3	0	0
HLA + FANCJ	FANCJ	1	3	1	3	0	0
HLA + granulomatous disease, chronic, X-linked; CGD	CYBB	4	10	7	10	3	2
HLA + hemoglobin—beta locus; HBB	HBB	61	137	78	121	20	15
HLA + immunodeficiency with hyper-IgM, type 1; HIGM1	CD40LG	6	11	7	11	4	3
HLA + Krabbe + aneuploidy	GALC	1	1	1	2	1	2
HLA + sickle cell anemia	HBB	6	9	5	9	1	1
HLA + thrombotic thrombocytopenic purpura, congenital; TTP	ADAMTS13	1	2	2	4	1	1
HLA + Wiskott-Aldrich syndrome; WAS	WAS	1	1	0	0	0	0
HLA + PKD1 + aneuploidy	PKD1	1	1	1	2	1	1
HLA genotyping		46	109	70	105	22	18
HLA + piruvate kinase deficiency	PKLR	1	2	1	1	0	0
Total	23	163	374	230 (61.5 %)	351 (1.52 %)	72 (31.3 %)	59

[a]See abbreviations in text

causative genes, including thalassemia, FANC, hyperimmunoglobulin M syndrome (HIGM1), X-linked adrenoleukodystrophy, and WAS, to mention only a few, an increasing number of clinical cycles are being performed for HLA typing without PGD, i.e., the only objective being preselection of HLA-matched progeny for transplantation treatment of siblings with bone marrow disorders [6, 7, 21–26]. The present experience of preimplantation HLA typing as the sole indication has already resulted in the birth of dozens of HLA-matched healthy children to become potential HLA-compatible donors for siblings requiring bone marrow transplantation. The data provide a realistic option for couples desiring to establish a pregnancy with the potential to provide an HLA-matched progeny for the treatment of an affected family member, with the prospect of applying the approach to other inherited or acquired conditions which also depend on HLA-compatible donors for bone marrow transplantation.

Although preimplantation HLA typing is still controversial in some settings, and is not allowed in certain countries, it appears to be so compelling for some couples that they are prepared to travel internationally to achieve their goal. For example, PGD for genetic disease combined with HLA typing has generally been allowed, while HLA typing in the absence of high-risk genetic transmissible disease is prohibited in some jurisdictions [27].

Conflict of Interest The authors declare no conflict.

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Chapter 16
Should Molecular Cytogenetic Techniques Be Applied to Facilitate Single Embryo Transfer in Egg Donation Cases? Assessment of Frequency and Distribution of Embryo Aneuploidy After Anonymous Donor Oocyte IVF

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Introduction

Preimplantation genetic diagnosis (PGD) and preimplantation genetic screening (PGS) are techniques for genetic assessment of embryos prior to transfer into the uterus. These tests offer "at-risk" individuals a greatly improved chance to have an unaffected child. A component of in vitro fertilization (IVF), each is associated with a growing range of uses in clinical fertility practice. Of note, in Europe PGD/PGS is variously prohibited, allowed, or practiced in the absence of legislation, depending on national statues [1]. There are no regulations addressing the provision of PGD or PGS in the United States [2].

In the early 1990s, PGD was first successfully applied to sex determination of embryos to reduce the likelihood of transmitting sex-linked conditions to offspring. In the setting of a family history of any recessive X-linked disease predominantly affecting males (i.e., glucose-6-phosphate dehydrogenase deficiency, Duchenne muscular dystrophy, hemophilia A and B, Wiskott–Aldrich syndrome, etc.), parents

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might elect to undergo embryo screening to identify female vs. male embryos. Then, only an unaffected female embryo would be transferred [3].

From that early success, reproductive medicine has embraced a substantial expansion of applications for preimplantation embryo assessment in IVF. This technology is currently used to identify embryos with hundreds of very serious single-gene disorders like Huntington's disease, as well as to permit embryo sex selection on an elective basis [4]. Moreover, because poor IVF outcomes are often related to embryonic chromosomal abnormalities [5], PGS is increasingly used to screen for aneuploid embryos to optimize pregnancy rates and attenuate the miscarriage rate after in vitro fertilization procedures [6].

Indeed, evidence is accumulating that implantation and pregnancy rates may remain encouraging even for IVF patients using native oocytes up to age 42, with the proviso that only euploid embryos (verified by PGS) are transferred [7]. Such results are consistent with the observation that advancing maternal age is directly correlated with an increasing frequency of chromosomal aberration in embryos [8, 9]. Since up to 60 % of all conceptions (unassisted) result in miscarriage before 12 weeks' gestation irrespective of age [10], it seems likely that ploidy error in human embryos is not a challenge confined only to oocyte sources of advanced age. For example, when selected chromosomes were studied in embryos obtained from donor-egg IVF treatment, the aneuploidy rate in this partial genomic assessment was higher than expected, particularly considering the egg donors themselves had no infertility diagnosis [11].

Building on this earlier pioneering work, our study reviewed use of comprehensive chromosomal screening in the context of anonymous donor-egg IVF. Using increased bandwidth to capture comprehensive screening data on all 23 pairs of chromosomes, this investigation aimed to answer two unresolved questions: (1) What is the true incidence of genetic abnormality in embryos produced from anonymously donated oocytes and (2) what is the gametic source of embryo aneuploidy observed in donor oocyte IVF?

Methods

Study Design

This retrospective investigation reviewed selected data from all in vitro fertilization (IVF) cases from a single institution in California in 2013 to identify the subset of patients where PGS was performed on embryos derived exclusively from anonymous oocyte donors. IRB approval was sought although the proposal was classified as exempt, since the study design reviewed already collected data and no specific patient identifiers were recorded. For our study, 23 cases meeting the eligibility criteria were identified; these patients produced 305 embryos for full molecular karyotyping. This information was collated with parental DNA obtained immediately before IVF (i.e., from the anonymous egg donor and the partner's husband) for chromosome-specific assessments. This approach permitted mitotic and meiotic copy errors to be differentiated for each chromosome among all embryos tested, thus providing information on the specific parental source of embryo aneuploidy.

Oocyte Donor and Patient Selection

Anonymous oocyte donors had completed comprehensive medical and psychological evaluation as described previously [12]. Additionally, donors underwent a genetic evaluation and were required to have a normal result (no mutations) on an expanded carrier test [13] before enrollment. Recipients had their initial reproductive endocrinology consultation and monitoring at our facility, and all baseline laboratory tests were within normal limits. Anonymous oocyte donor counseling was provided by an accredited psychologist before starting gonadotropins. Each recipient selected her anonymous oocyte donor via secure Internet portal with an electronic lockout mechanism to prohibit multiple recipients from accessing the aggregate donor pool at the same time. A dedicated nurse coordinator was available to facilitate oocyte donor selection in all cases. Following registration of each provisional donor–recipient match, the corresponding anonymous oocyte donor entry was deleted from the donor library, thus creating a 1:1 ratio for each recipient and their anonymous oocyte donor (i.e., no two IVF recipients utilized oocytes from the same anonymous donor for this analysis).

The anonymous oocyte donor commenced controlled ovarian hyperstimulation, and transvaginal ultrasound-guided oocyte collection followed 36 h after s.c. hCG administration as previously described [14]. Sperm from the recipient's partner was used to fertilize all freshly retrieved eggs obtained from the anonymous oocyte donor; intracytoplasmic sperm injection (ICSI) was performed in all cases.

For all records reviewed for this study, recipient and partner/husband ages were tabulated, as was age of the anonymous oocyte donor. Husband's sperm concentration and sperm motility were calculated as an average of two semen analyses performed no more than 6 months before treatment. The following laboratory parameters were also evaluated: number of oocytes fertilized (via ICSI), number of 2*pn* zygotes produced, number of embryos biopsied, day of biopsy, and number of euploid embryos. In addition, the number and frequency of error observed in each chromosome was recorded, with reference to the (genetic) parental origin of the abnormality, as described previously [15].

Ovarian Stimulation and Fertilization

Before commencing gonadotropin therapy, oocyte donors underwent transvaginal ultrasound evaluation with remeasurement of serum FSH, LH, and estradiol on day 3 of the index cycle. Pituitary downregulation was achieved with a GnRH agonist administered on day 21 of the cycle immediately preceding treatment, as previously described [14]. Periodic transvaginal ultrasound and serum estradiol measurements were used to track follicular growth and thickness of the endometrial lining. When ≥3 follicles reached the 19 mm mean diameter, periovulatory hCG was administered by subcutaneous injection of recombinant hCG (250 μg Ovidrel®, Merck Serono; Geneva, Switzerland) with oocyte retrieval performed under transvaginal

ultrasound guidance 35–36 h later. Following removal of all cumulus cells, ICSI was performed, and normal fertilization was verified 16–18 h after injection by the presence of two pronuclei and two polar bodies.

Embryology Protocol

Embryo biopsy was performed either on the morning of day 3 or on day 5 (blastocyst stage). Biopsy at day 3 was completed after laser-assisted hatching followed by removal of a single blastomere. Extended embryo culture occurred in Global single-step medium (IVF on Line; Guilford, CT) to blastocyst stage. On day 3 when embryos were at the 6–8 cell stage, a laser (Lycos, Hamilton Thorne; Beverly, MA) was used to create a 6–9 μ circular lacuna in the zona pellucida. This enabled rapid biopsy of trophectoderm (TE) on day 5. Between 3 and 5 herniated TE cells were gently aspirated by a pipette and, when necessary, freed from the blastocyst by application of laser pulses. Harvested TE cells were washed in PBS and placed within a PCR tube with 2.5 μL 1× PBS.

Cell Isolation, DNA Amplification, and Genotyping

Genetic material was obtained from oocyte donors via buccal swabs, from the recipient's husband by peripheral venipuncture, and from the embryos by either single-cell day 3 blastomere biopsy or multicell day 5 trophectoderm biopsy. Single tissue culture (PMNs) and egg donor buccal cells were isolated using a sterile tip attached to a pipette and stereomicroscope (Leica; Wetzlar, Germany). For fresh day 3 embryo biopsy, individual blastomeres were separated via a micromanipulator after laser-facilitated zona hatching as described above; a micromanipulator was also used to isolate individual sperm cells. Except for sperm, single cells for analysis were washed ×4 with buffer (PBS buffer, pH 7.2; Life Technologies, Carlsbad, CA). Multiple displacement amplification (MDA) with proteinase K buffer (PKB) was used for this procedure; cells were placed in 5 μL PKB (Arcturus PicoPure Lysis Buffer, 100 mM DTT, 187.5 mM KCl, 3.75 mM $MgCl_2$, 3.75 mM Tris-HCl) incubated at 56 °C × 1 h, followed by heat inactivation at 95 °C × 10 min, and held at 25 °C × 15 min. MDA reactions were incubated at 30 °C × 2.5 h and then 65 °C × 10 min.

Genomic DNA from buccal tissue was isolated using the QuickExtract DNA Extraction Solution (Epicentre; Madison, WI). Template controls were included for the amplification method. Amplified single cells and bulk parental tissue were genotyped using the Infinium II (Illumina; San Diego, CA) genome-wide single nucleotide polymorphism (SNP) arrays (CytoSNP 12 chip). The standard Infinium II protocol was used for parent samples (bulk tissue), and Genome Studio was used for allele calling. For single cells, genotyping was accomplished using an Infinium II genotyping protocol.

Copy Number and Haplotype Phasing

Because some commercial software packages use heterozygosity to determine copy number and high rates of ADO with preferential amplification in single-cell measurements can cause unpredictable heterozygosity (regardless of chromosome copy number), performance is poor when calling copy number on noisy single-cell data. Accordingly, previous investigators [9] developed a chromosome copy number classification algorithm in MATLAB (MathWorks; Natick, MA), predicated on parental genotypes and the observed distribution of unprocessed single-cell microarray channel intensities collated by parental origin [16, 17].

In brief, this approach is based on prior work [15] whereby the statistical behavior of each parental group differs as a function of the underlying chromosome copy number of the embryo. These changes are predictable and derive from additional allelic content that is contributed by (or missing from) each parent [15]. Moreover, rank statistics are examined for each parental context and compared to the expected orderings under the various chromosome copy number possibilities. Next, the probability is examined for each parental context that could have swapped rank by random chance to establish copy number and calculate confidences [15, 18].

Detection of three unmatched haplotypes adds additional confidence to a trisomy call, as many chromosome copy number errors are meiotic and will be associated with this configuration. Accordingly, this method included parental information with high-confidence disomic single-cell measurements on offspring and recombination probabilities to determine the parental chromosome phase. A maximum likelihood estimator algorithm was used to phase full chromosomes for all parental genotype contexts. Possible haplotypes in single-cell measurements are then evaluated to detect meiotic trisomies.

Segmental copy imbalances were detected by dividing each chromosome into five segments, with the aforementioned algorithm applied to each section independently. If any segments differ in copy number with high confidence, then the corresponding chromosome is flagged. Note that the reported copy number for chromosomes with a segmental imbalance is reflective of the call on the majority of the chromosome, even if part of the chromosome shows gain or loss. Thus, depending on size, segmental copy imbalances may reduce composite confidence of the complete chromosome call. However, confidences on chromosomes with segmental imbalances may still be high if the deletion is relatively small and/or the remainder of the chromosome is called with very high confidence [15].

Individual chromosome means and standard deviations of normalized microarray probe intensities were used to call chromosome copy number. For each single-cell measurement, a training set of single-cell amplification microarray measurements was used to normalize probe intensities across each chromosome. An algorithm was next used to compute the most likely chromosome state for all the single-cell amplification microarray data.

Statistical Analysis

Data were aggregated, analyzed, and visualized with Tableau 8.2 (Tableau Software; Seattle, WA). To estimate a reference population's aneuploidy rate and the donor (maternal genetic) aneuploidy contribution, a binomial proportion confidence interval was used on each proportion estimate using the Wald test. When sample size was small (defined as $\min[np, n(1-p)] < 5$), an adjusted Wald method [19] was used to improve estimate accuracy. For this analysis, the confidence level was set at 95 % by default (90 % for aneuploidy rate comparisons). To compare two sample ratios, the 2-proportion z-test was used for large samples (defined as $\min[np, n(1-p)] \geq 5$); Fisher's exact test was used when sample size was small.

Results

A total of 676 IVF cases proceeded to oocyte retrieval during the 12-month review period ending December 2013. Of these, 50 were anonymous oocyte donors undergoing ovum pickup. The male partners of the intended parents had a mean (±SD) age of 44.3 ± 7.1 (range 25–58 years). Average sperm concentration and motility were 52.8 M/mL (range 2.4–135 M/mL) and 40.8 % (range 2–81 %), respectively.

A total of 428 patients requested PGS during the study interval. Intersecting these two patient subsets identified 24 IVF cases which included both anonymous oocyte donation and PGS (see Fig. 17.1). Analysis of this group revealed that 305 embryos were subjected to biopsy and full molecular karyotyping. The mean (±SD) age of recipient females in this study population was 42.5 ± 4.0 (range 35–52) years. Mean (±SD) age was 24.0 ± 2.7 (range 20–29) years for oocyte donors ($n = 24$).

In this study group, the mean (±SD) number of oocytes which underwent fertilization by ICSI was 17.7 ± 7.8 (range = 6–35), and this yielded an average of 15.1 ± 6.7 2*pn* zygotes per patient (range = 6–32). Most embryos (86 %) were biopsied on day 3, while the remainder (14 %) were biopsied on day 5. Although the number of blastocyst biopsies was relatively small ($n = 44$), it was possible to record embryo ploidy as a function of biopsy timing. Using this approach, we found the incidence of missed calls ("no signal") on chromosomes to be significantly higher among embryos biopsied at day 3, resulting in reduced reporting efficiency for this group compared to the blastocyst biopsy group (92 % vs. 100 %; $p = 0.05$).

Assessment of all embryos produced from oocytes contributed by an anonymous donor identified euploidy in 133 of 284 (46.8 %) embryos with full chromosomal reporting (i.e., zero "no calls"). Complete data on all 23 chromosome pairs was reported for 93.1 % of embryos sampled (284 of 305). Considering all embryo chromosomes, mean error rate was 18 %. A chromosome-specific analysis found error present in all chromosomes; chromosome 22 was most often affected, and chromosome 15 was the least likely to have an abnormality (see Fig. 17.2).

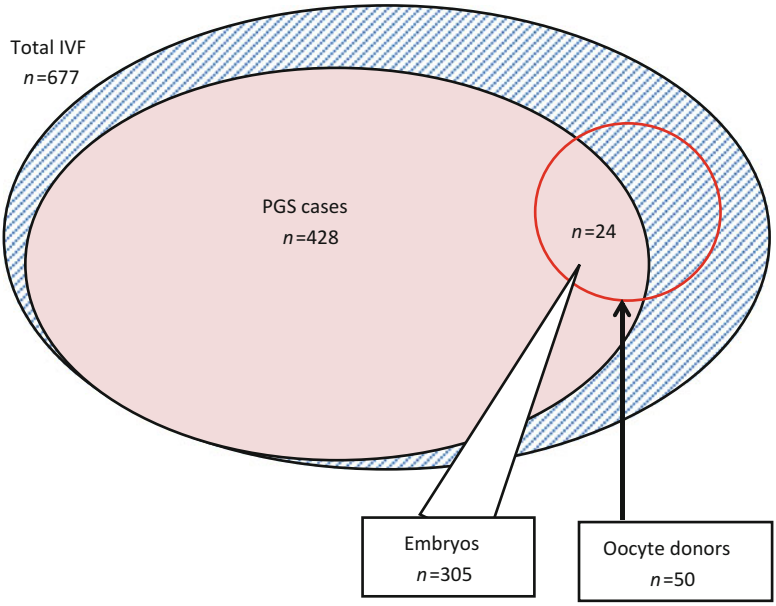

Fig. 17.1 Relational (Venn) diagram showing distribution of study patients and embryos

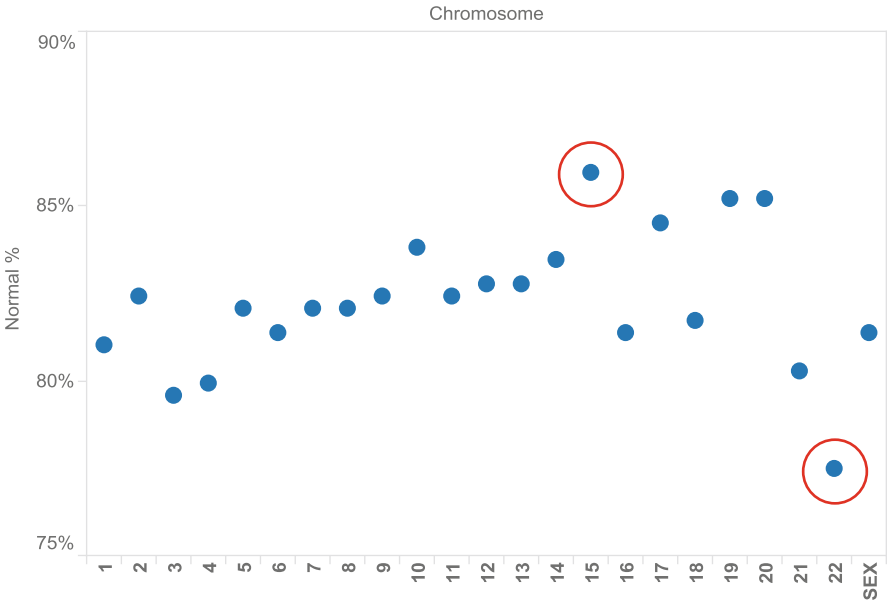

Fig. 17.2 Distribution of aneuploidy as a function of specific chromosomal error measured in embryos ($n = 305$) produced from anonymous donor oocyte IVF cycles

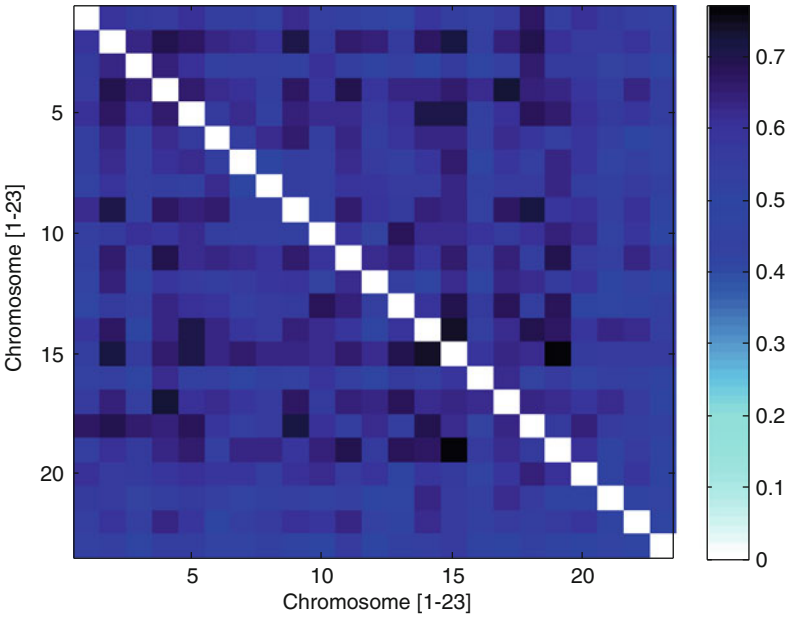

Fig. 17.3 Pairwise correlations of autosomal aneuploidy by mean square contingency (Phi) coefficient, observed in 305 embryos derived from anonymous donor oocyte IVF treatments

The relatively high Phi correlation coefficients (see Fig. 17.3) among embryo chromosome pairs with aneuploidy ($r=0.60$, range 0.42–0.77; $p<0.01$ by chi-square test) indicate that chromosomes tend to have multiple and simultaneous errors (complex aneuploidy).

When analysis was confined only to those embryos with no missed calls for any chromosome, errors attributable to a maternal source (i.e., from the oocyte donor) were noted in 133 of 284 embryos (46.8 %). Conversely, an embryo genetic abnormality of paternal origin was present in 104 of 284 embryos (36.6 %). Among all aneuploid embryos ($n=151$), chromosomal errors from both genetic parents (i.e., oocyte donor and partner's husband) were present in 57.0 % (see Fig. 17.4). While oocyte donor age ranged from 20 to 29 years, some genetically abnormal embryos were produced from donors of each age, and there was no correlation between oocyte donor age and embryo aneuploidy. Likewise, these data did not confirm a correlation between embryo aneuploidy and male partner age or any semen parameter.

Discussion

The role of PGS on the menu of clinical IVF services has evolved substantially in recent years. Although it is tempting to classify PGS applications as simply an accessory to "mainstream" IVF, genetic testing of embryos has been (and will continue to be) a crucial development in the progress of our field. Certainly the

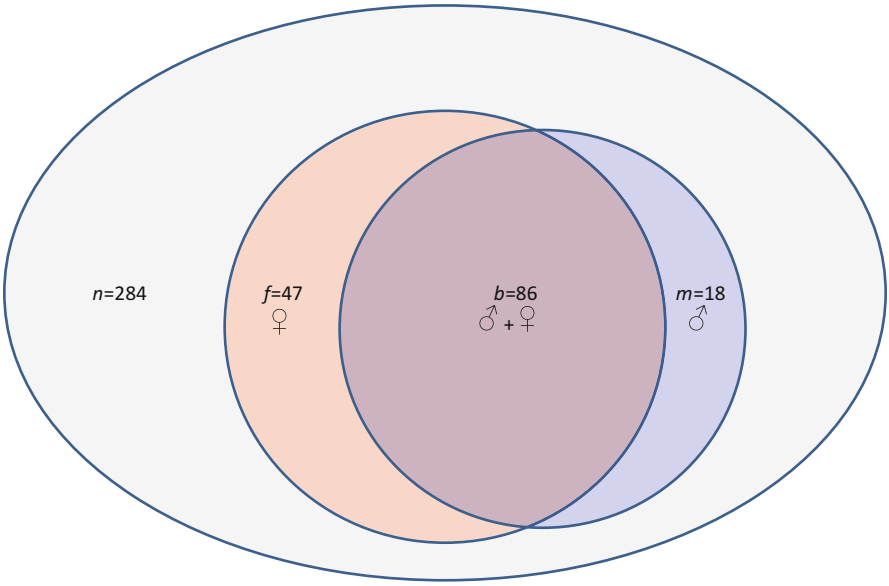

Fig. 17.4 Distribution of aneuploidy origin by gamete source for embryos produced from anonymous donor oocyte IVF

successful passage of the world's first IVF regulatory legislation (Human Fertilisation & Embryology Act, 1990) was strongly influenced and enabled by the arrival of PGD in the United Kingdom [20]; further applications of this technique have continued to push the ethical boundaries for IVF into unfamiliar terrain [21].

In humans, most aneuploidies are triploidies, yet only those involving chromosomes 21, 18, and 13 are compatible with survival to term [22]. Duplication of other autosomes is poorly tolerated and is rarely seen in live births. Viable monosomies are only known to exist for chromosome X, while additional copies of sex chromosomes are developmentally permissive. PGS is a powerful clinical tool to assist in embryo selection to minimize transfer of such embryos, thus improving clinical outcomes.

The arrival of oocyte donation preceded PGS and was originally offered as a treatment for premature ovarian failure or oophorectomy [23]. Egg donation is now commonly in use for many settings besides diminished ovarian reserve, including its use to circumvent transmission of severe genetic disorder(s) in the birth mother to her offspring [24]. While the corrosive effect of age on female infertility can be successfully assuaged for couples using donated oocytes from a younger (presumably more fertile) woman [25], the degree of chromosomal error in embryos derived from such treatment has yielded some unexpected preliminary results [11].

For example, one IVF group recently conducted a 12-year retrospective study on genetic test data collected from anonymous oocyte donor applicants and found that genetic abnormalities caused a significant number of candidates to be excluded from their oocyte donor program [26]. We agree with this approach and, like many institutions, require any potential anonymous oocyte donor to first undergo a careful genetic testing regime before entering the roster of eligible oocyte donors. Indeed,

all of the anonymous donors who supplied oocytes for the current study already had been screened for hundreds of genetic disorders in advance of their accession into our egg donor group. However, despite this reassuring clearance (and in the absence of any obvious reproductive pathology in the oocyte donors) the rate of chromosomal error among embryos produced from their eggs was surprisingly high (e.g., 55 % aneuploidy rate).

Previous research attempted to characterize the role of "defective" gametes resulting in the generation of abnormal embryos using an egg-sharing model (where one IVF patient agrees to share her eggs with another IVF patient) [27]. Unfortunately, this can yield an undesirable outcome for the recipient since what she ultimately gets are simply eggs from another infertile patient. Such a study is unsatisfying experimentally because the variable of oocyte pathology cannot be controlled if all the oocytes for study are generated by other patients with manifold infertility diagnoses.

This problem was also addressed when the aneuploidy rate for eight chromosomes in embryos derived from young (<35 years) oocyte donors using fluorescence in situ hybridization analysis was studied. Using this study approach, all oocytes were provided by healthy women who did not have any infertility diagnosis. The authors reported considerable variation between donor cycles with nearly one-third having <30 % genetically normal embryos [11]. Starting from these data where less than half of the embryo's chromosomes had been evaluated, our work was built on this foundation to screen all 23 pairs of embryo chromosomes in an anonymous donor oocyte IVF setting. Importantly, since the behavior of each parental allelic group is a function of the underlying chromosome copy number of the embryo, and because these modifications may be satisfactorily estimated from additional allelic content contributed by (or omitted from) either the oocyte donor or the recipient's husband (sperm source), we were able to supply additional information on the parental origin of the genetic problems identified in the embryos derived therefrom.

Earlier research has shown a significantly higher observed pregnancy loss rate among IVF patients with age ≥40 compared to women younger than age 40 [28], establishing that the distribution of genetic error in embryos as a function of maternal age is not stationary. This physiologic process of natural ovarian senescence has been sidestepped for many years by using oocytes provided by younger donors [29]. With further refinement of donor oocyte protocols, acceptance of this treatment in routine IVF practice has increased greatly over the last decade, and when donor oocytes are used, the likelihood of an excellent IVF outcome seems independent of recipient age [30]. In the United States, the incidence of twins is markedly higher among anonymous oocyte donor IVF cycles compared to IVF using native (autologous) oocytes (37 % vs. 29 %, respectively), which provides direct evidence that most clinics are not to following a current ASRM recommendation which encourages single embryo transfers when oocyte donor age is young [31]. Indeed, there now appears to be international consensus that elective single embryo transfers are appropriate for oocyte donor–recipient cycles where the donor has good prognosis and when good quality embryos are available [32].

Of note, comprehensive chromosomal screening has not been applied to embryos of donor oocyte origin to quantify the level of genetic abnormality which persists in such embryos until now. If ever the domain of anonymous donor oocyte IVF were regarded as a realm where the role of genetic error in embryos could be dismissed as unimportant, the current study highlights an important supporting role for PGS in this population of IVF patients, too. Moreover, these data provide some fresh observations on human embryo genetics. Here, we focused on the specific topic of parental origin with respect to chromosomal errors which may be harbored by IVF embryos. Our observation that a high rate of embryonic genetic anomaly could be traced back to the oocyte donor was not anticipated. Thus, it appears that the traditional view that most chromosomal errors are of maternal origin caused by malsegregation in the first meiotic division [33] remains valid, even when the age of the oocyte source is very low.

Our report has some limitations which should be acknowledged. Our data come from a retrospective analysis as an initial step to analyze readily accessible existing data. We aimed to produce a hypothesis about aneuploidy rate in embryos derived from anonymous donor oocytes which could then be tested prospectively [34]. Retrospective work has the potential for incomplete documentation, unrecoverable or unrecorded data, and variance in the quality of information recorded. The reliability of data entry is considered as high for this sample, and the proportion of incomplete records was marginal. Also, because our sample was limited and represented the chance event of an IVF patient using anonymous donor oocytes also incorporating preimplantation testing of embryos produced from this treatment, it is uncertain if these findings can generalize to all anonymous donor-egg IVF cases (it should be noted that a secondary chart review for our study population did not reveal any obvious characteristic which may have influenced the patient's decision to include PGS in her IVF treatment). Perhaps the high economic cost of IVF in general (and donor oocyte treatment in particular) introduced some selection bias, since only the most affluent IVF patients could have afforded this treatment [35]. It would be interesting to query the remaining donor oocyte IVF patients in this series who declined PGS ($n=27$), to understand better why they decided not to request genetic testing of their embryos; this represents an area of future research here. Finally, our analysis of male factor data was confined to the age of the recipient's husband and only two semen parameters (sperm concentration and motility). We did not include sperm DNA fragmentation data in this study, although this has not yet been correlated with embryo ploidy [36].

In conclusion, although the problem of embryo aneuploidy does diminish somewhat when anonymous donor oocytes are used for IVF, our results show that it does not disappear entirely even when oocytes from donors as young as 20 years of age are used. Prospective investigations utilizing comprehensive chromosomal screening with larger samples will be welcomed for further study of this phenomenon going forward.

Conflict of Interest The authors declare no conflict.

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Chapter 17
Selecting the Right Embryo in Mitochondrial Disorders

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Authors' Contributions: SCEHS collected and analyzed the data, studied the literature, and developed the chapter; JCFMD provided the data and helped with study design; IFMdC was the lead clinician and referred patients for PND/PGD; CEMdD-S and HJMS supervised the writing and review of the chapter.

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Introduction

Mitochondrial diseases are perhaps the most common of all inborn errors of metabolism [1]. They are highly variable in phenotype, ranging from severe and lethal infant/childhood manifestations to relatively mild symptoms with onset at adult age. Generally, no treatment is available. The (recurrence) risk is dependent on the nature of the underlying primary genetic defect, and so are the available reproductive options. The primary genetic defect can be located either in nuclear or mitochondrial DNA, and this feature will have direct consequences for the recurrence risk. In case of a nuclear gene defect, the disease segregates in autosomal dominant or recessive fashion with recurrence risks of 50 % or 25 %, respectively. De novo nuclear mutations with a lower recurrence risk are rare. For mtDNA defects, the recurrence risk in the family is much more difficult to predict. Age- or drug-induced mtDNA defects (i.e., multiple deletions and mtDNA depletion) occur somatically with no transmission risk at all. However, multiple mtDNA deletions and mtDNA depletion can also be secondary to a primary defect in nuclear genes involved in mtDNA maintenance. In such a case, the recurrence risk is comparable to other nuclear gene mutations. At least 15 % of mitochondrial diseases result from primary mitochondrial DNA (mtDNA) mutations [1, 2] including point mutations and large rearrangements. The recurrence risk of these mtDNA defects can vary between high/unpredictable and very low (in fact even zero for actual de novo mutations) depending on the nature of the underlying defect.

Two characteristics of mtDNA mutations which are key to understanding the complexity of mtDNA disease transmission include mitochondrial heteroplasmy and the genetic bottleneck. The majority of pathogenic mtDNA mutations resulting in severe disease are heteroplasmic, which means a mixture of mutant and wild-type mitochondria within a cell/tissue/individual. Heteroplasmy levels can vary between and within tissues of a carrier. Heteroplasmic mtDNA mutations are characterized by a threshold effect, meaning that there are no symptoms unless the mutant load (proportion of mutant mtDNA) exceeds a certain level. This threshold varies both within tissues and between different mutations and can depend on environmental factors, like the physical condition of the carrier.

Transmission of mtDNA occurs only from females to their offspring and is subject to a so-called genetic bottleneck. During oogenesis, the number of mtDNA molecules to be transmitted is reduced, and the resulting few mtDNAs become the founders for the offspring. In case the transmitting woman carries a heteroplasmic mtDNA mutation, this results in considerable variation in mtDNA mutant load among her individual oocytes [3] and subsequently among offspring. The exact mechanism of the mitochondrial bottleneck is incompletely known, and some controversy exists concerning the content and “size” of the segregational unit [4–9]. This size has been hypothesized to depend on the type of mtDNA mutation [10–13] and to be individual dependent for certain mutations [10], possibly due to individual differences in initial mitochondrial copy number or genetic background. Another

important question is whether the bottleneck really is mutation-specific or that only the degree of skewing is mutation-specific, resulting in apparent differences in bottleneck size. Irrespective of the mechanism, the smaller the lowest amount of the remaining mtDNA (segregational unit), the more rapid a (complete) shift of the mtDNA genotype can occur.

Primary Nuclear Defects

Counseling and Recurrence Risk

About 85 % of mitochondrial diseases is caused by mutations in nuclear genes, which are currently being rapidly resolved by whole-exome sequencing (WES) and which segregate in a Mendelian way with recurrence risks of 25 % or 50 %. In general, they do not affect the mtDNA, although part of the mtDNA defects, like multiple mtDNA deletions or mtDNA depletion, can be due to defects in nuclear genes involved in mtDNA maintenance. Comparable mtDNA defects can also occur somatically due to a nongenetic cause like aging (e.g., multiple mtDNA deletions) or mitotoxic drugs (e.g., nucleoside reverse transcriptase inhibitors can induce mtDNA depletion) with no recurrence risk at all; it is essential to define the cause of these mtDNA defects for proper estimation of the recurrence risk. De novo nuclear mutations with a low recurrence risk are rare.

Reproductive Testing Options

Nuclear gene defects resulting in mitochondrial disease are less complex with regard to reproductive options than primary mtDNA defects. Prenatal diagnosis (PND) and preimplantation genetic diagnosis (PGD) can reliably be offered, provided that the mutation is identified. The latter is the main problem in this category of mitochondrial diseases. Up to now, only in a minority of mitochondrial patients where mtDNA defects have been excluded, the genetic defect has been identified. New sequencing techniques (next-generation sequencing) and unbiased approaches (whole-exome sequencing) are promising in increasing this number, resulting in reproductive options for more at risk couples. In cases where the genetic defect is not known but the mtDNA has been excluded and an enzyme deficiency is detectable in fibroblasts, PND based on biochemical analysis might be an option [14–17]. However, there are some limitations and pitfalls including sensitivity issues, the absence of the enzymatic defect in fibroblasts in 50 % of patients, and limited knowledge on complex assembly and activity during embryonic development [18].

In patient populations where consanguinity is more common, one should be aware that an increased risk of more than one genetic condition may be present. Thus, when offering reproductive options for a mitochondrial defect, there is still a

realistic risk that the resulting child is affected by one or more other genetic abnormalities. Currently, consanguineous couples are empirically counseled regarding genetic risks if no genetic diseases have occurred in their families. In specific ethnic groups, carrier screening is offered for genetic diseases that are frequent in those populations. With the upcoming DNA sequencing techniques, preconception screening will become available on a broader basis.

Familial Primary mtDNA Mutations

Counseling and Recurrence Risk

The most common heteroplasmic mtDNA point mutation is the m.3243A>G mutation in the mitochondrial *MT-TL1* gene, causing MELAS syndrome (mitochondrial encephalopathy, lactic acidosis, and stroke-like episodes), among others. This disorder is characterized by highly variable age at onset, symptom severity, and organ involvement. Correlation between the level of mutant mtDNA in blood and clinical features is poor due to the decrease in mutation load in blood cells with time [19–21]. However, mutation levels in muscle [22] and urine [23–26] seem to be of higher prognostic value. Mutant load in oocytes and embryos of m.3243A>G carriers shows large variation [10, 27] approximating a Gaussian distribution [27], indicating that the level of mutant mtDNA in oocytes and embryos for this mutation is largely determined by random genetic drift [10, 13, 27]. Existing data also point out that although in general a higher mutant load in the mother provides a higher risk of affected offspring, the recurrence risk for an individual m.3243A>G carrier remains very difficult to predict [27, 28].

mtDNA Point Mutations Demonstrating Skewing

Specific mtDNA mutations such as the nt8993 mutations do not show random transmission as with the m.3243A>G mutation, but rather demonstrate skewing. Due to the skewing to the extremes, there is an overrepresentation of oocytes and subsequent embryos with 0 % and 100 % mutation load [12, 27, 29–31]. Accordingly, with these mutations, it is possible for a mother with a high mutant load to have a child with a low mutant load and vice versa [29]. In general, the individual recurrence risk can be better characterized as low (the majority of oocytes not showing the mutation) when the mother's mutant load is low. The proportion of children with a high mutant load increases as the mother's mutant load increases. Other characteristics of the nt8993 mutations are the rather good correlation between mutation load and phenotype [29] and a quite uniform distribution of the mutation in all tissues [32].

Reproductive Testing Options

Prenatal Diagnosis

In general, PND for mtDNA mutations has several limitations. A key problem is the often unreliable correlation between mutation load and disease severity, making it difficult to predict the clinical disease burden for the child and the likelihood of a couple having severely affected offspring [33]. Secondly, mutation load in chorionic villi or amniocytes may not be representative for the mutation load in various fetal tissues. Limited available data suggest that the mutation load of extra-embryonic tissues such as chorionic villi can be considered representative for the mutant load in the fetus [29, 33–35]. However, these data predominantly concern the mutations at nucleotide 8993, which are skewing mutations (as discussed above). Other reports on mtDNA polymorphic variants [36] and on the m.3243A>G mutation [10, 37] indicate that mtDNA mutations may segregate in the placenta, questioning the reliability of (a single) CVS sample analysis for mitochondrial disorders carried out in a PND framework. This is further supported by intra-placental mutation load variations up to 55 % which were reported by Monnot et al. [ESHG2013, Paris]. Finally, the segregation of mtDNA mutations throughout embryofetal development and the distribution of mutation load between different fetal tissues are not fully clarified, although based on existing data from both skewing and non-skewing mutations, these issues do not seem to be a restriction [10, 29–31, 34, 35, 38–47]. Indeed, the data show that the m.3243A>G mutation segregates quite stable throughout the prenatal period, and this is remarkably distinct from postnatal segregation.

Advantages of PND include its relatively low cost and lower physical burden compared to IVF procedures and the fact that no oocyte donor needs to be available. Disadvantages of PND are risk of miscarriage as a result of the invasive nature of the procedure and the decision the couple has to make with regard to terminating the pregnancy if results are unfavorable. The latter is obviously even more difficult when no firm predictions can be offered concerning the clinical outcome of the fetus. It has been reported that for most mtDNA point mutations, a fetus with mutation load below ~30 % or above approximately 90 % could be cautiously predicted to have a low or high probability, respectively, of being (severely) affected [33]. However, such guidelines were not based on a systematic analysis and may not be applicable for all mtDNA mutations. A systematic meta-analysis showed 95 % or higher chance of being unaffected at (muscle) mutation level of $\leq$18 %, irrespective of the mutation [47]. If possible, mutation-specific thresholds should be calculated as has been done with the m.3243A>G mutation (15 %) and the skewing m8993T>G mutations (30 %) (See below). A large range of mutation loads will fall within a "gray zone" with difficult or impossible interpretation, which is also the case for the m.3243A>G mutation. In 13 proven m.3243A>G carriers, a total of 19 (of which 2 occurred in the same twin pregnancy) prenatal diagnoses have been reported [10, 45, 48, 49]. Another four prenatal diagnosis cases were performed in three women without any detectable m.3243A>G mutation in leukocytes in two, and leukocytes

and urine in one of them, but with considerable risk of being carriers [45, 49]. The m.3243A>G mutation was not detected in chorionic villi or amniocytes when the mutation was absent in maternal leukocytes [45, 49]. This was the case in four females (five prenatal diagnoses), one of them having 3 % mutant load in urinary tract cells. PND might indeed be an option for carriers with very low mutation load of the m.3243A>G mutation, although leukocytes seem not to be the best source to determine this—urine or muscle seems more appropriate [19–26]. Moreover, analysis of both chorionic villi and amniocytes in a carrier with 21 % mutant load in leukocytes failed to show the mutation in two fetuses [45]. One of these fetuses was part of a twin pregnancy where mutation loads of 60 % and 63 % were detected in chorionic villi and amniocytes, respectively of the other fetus. The pregnancy was (selectively) reduced for the fetus with the mtDNA mutation. Another example of an m.3243A>G carrier (mutant loads of 1% in blood and 18% in urine) without detectable mutation in chorionic villi was reported by Nesbitt et al. [49]. In two pregnancies of another carrier (with 80 % mutant load in urinary tract cells), mutation loads between 23 % and 35 % were detected; both pregnancies were continued [45]. The PND cases reported by Monnot et al. and Nesbitt et al. included four pregnancy terminations with mutation loads ranging from 59 % to 77 %, whereas, for example, a pregnancy with 79 % mutant load was continued [10, 49]. Chou et al. [48] were confronted with a carrier when she was already 8 weeks pregnant. This case illustrated the limited value of PND for this particular mutation: both of her children harbored similar (high) levels of mutant mtDNA, and the first child was severely affected and died at age 3½, whereas the second child was healthy at age 4.

For the (skewing) nt8993 mutations, PND is more feasible for carriers of a low mutation load, due to the high likelihood of unaffected offspring and a better correlation between mutation load and clinical phenotype. Seventeen cases of PND undertaken in 14 carriers with variable mutation loads of these mutations have been reported [30, 35, 38, 41, 42, 49, 50]. Prenatal diagnosis of another mtDNA mutation, m.9176T>C, in the *ATPase6* gene has been reported in a family after a thorough work-up and counseling [51]. Limited data remain available about this mutation, especially concerning the genotype/phenotype correlation. The fetus appeared to have a mutation load of 87 % (CVS)–88 % (amniocentesis), just below the assumed threshold of expression (90 %). The couple decided to continue the pregnancy. A healthy child was born, not showing any abnormalities at the age of 13. Seven cases of PND in 7 carriers of other mtDNA mutations, namely m.8344A>G, m.13513G>A, m.11777C>A (n=2), m.10191T>C, m.10158T>C and m.3688G>A, respectively, were reported [49]. In four of them the pregnancy was continued (two without mutation in chorionic villi, one with 3% mutant load, one with 54% mutant load), data on pregnancy continuation or termination were not available in the remaining three.

Recently, we performed PND for an unaffected carrier of the m.3303C>T mtDNA mutation (unpublished data). The patient's previous child, who had a nearly homoplasmic mtDNA mutation present in the blood and muscle, died at only age 5 months. Based on limited data from the literature and own experience, the expression threshold for this mutation was considered to be very high (90–95 %), and it was assumed

that fetal mutant load between 0 % and 50 % would likely predict a subsequent child to be unaffected. In amniocytes, a mutant load of ~38 % was detected. The couple decided to continue the pregnancy, which is ongoing and thus far uneventful.

Altogether, PND is not a favorable choice for female carriers of mtDNA mutations with a high or unpredictable recurrence risk and a poor correlation between mutation load and phenotype; this is mainly because of difficulties in predicting the fetal phenotype when a certain mutation load is detected in chorionic villi or amniocytes. Still, when a carrier is already pregnant, PND can be offered with the understanding that a considerable chance exists that no interpretable result can be obtained.

Preimplantation Genetic Diagnosis

Another and fairly new option to prevent transmission of mtDNA mutations is preimplantation genetic diagnosis (PGD) [10, 27, 31, 52–55]. In PGD, embryos obtained after in vitro fertilization (IVF) are analyzed at the blastomere stage (day 3), and only those with amounts of mutant mtDNA below the predicted threshold of (severe) expression are transferred in the uterus. Our threshold for the m.3243A>G mutation (MELAS) is 15 % [22, 56] and 30 % for the skewing mutation m.8993T>G (Leigh) [29]. These guidelines are based on correlations between muscle mutation load and clinical manifestations, assuming that muscle mutation load correlates with the embryonic mutation load. This determination also embraces an arbitrary safety margin to correct for potential errors in determining heteroplasmy levels and for the limited number of data available.

Such thresholds, the preference of individual patients, and input from the clinician all appear to influence the decision on how many embryos to be transferred in the setting of mtDNA disease screening. In the Netherlands, the threshold is determined before a cycle will be started, and the couple agrees that the single best embryo below this threshold will be transferred. In other countries like France, the couple has a more decisive role in choosing the embryo for transfer, even if the embryo manifests a mutation load above the threshold of expression [10, 54, 55]. For most mtDNA mutations, insufficient data are available to establish a mutation-specific threshold level. A systematic meta-analysis showed 95 % or higher chance of being unaffected at (muscle) mutant level of 18 % or less, irrespective of the mutation [56]. This offers a solution for the difficulties in establishing a transfer threshold for mtDNA mutations and implicates that PGD can be offered for any heteroplasmic mutation. Obviously, this meta-analysis is a guideline, and careful counseling is necessary, stressing the limitations of applying these findings on individual cases when data are scarce.

Prerequisites for PGD in mtDNA mutation carriers are the availability of oocytes with mutation load below the threshold for transfer and a comparable mutation load in all blastomeres of an embryo. We performed 14 PGD cycles in six mtDNA mutation carriers so far: four m.3243A>G carriers (a total of 9 cycles), one m.8993T>G carrier (4 IVF cycles), and one m.8344A>G carrier (1 cycle). The m.8993T>G carrier achieved two pregnancies, one resulting in a healthy son and the other preg-

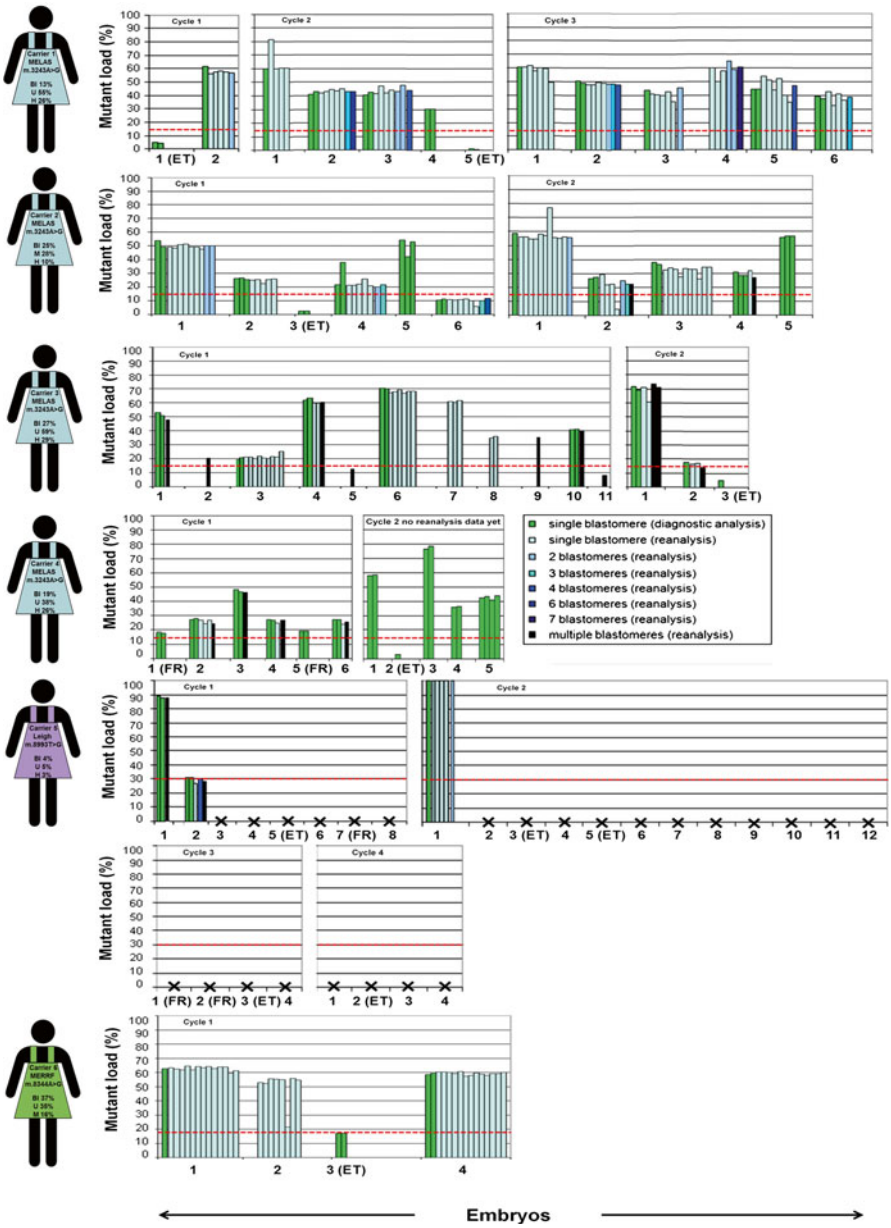

Fig. 17.1 PGD cycles of the respective carrier females, performed in our center. Each cluster of bars represents an embryo with its tested blastomeres. The *red dotted line* represents the threshold level for transfer. For the Leigh carrier, the embryos in which the mutation was not detected are depicted as X. For these embryos, the numbers of analyzed blastomeres are not visible in the figure. *ET* embryo transfer, *FR* frozen, *Bl* blood, *U* urine, *M* muscle, *H* hair

nancy is ongoing [27]. The PGD results from this series are summarized in Fig. 17.1. Here, all carriers did indeed produce oocytes with a mutation load below the threshold, and the blastomere mutation load was generally representative for the whole embryo (although single outliers occasionally occur).

Few additional reports of PGD performed for mtDNA disorders in other centers exist; a total of 12 cycles have been performed in nine mtDNA mutation carriers which resulted in the birth of five children [10, 31, 52, 54, 55]. In general, the mutation loads we observed for m.8993T>G among single blastomeres were concordant with previous reports [31, 52, 57]. Of note, interblastomere differences of 11 % have been noted [57] and fully descriptive data were not provided [52]; in our series, blastomeres/embryos with no mutation were overrepresented (25/28 embryos), making it difficult to draw a general conclusion. Interblastomere variation for the m.3243A>G mutation was generally larger and occurred more often than previously reported for this mutation [10, 54], although Monnot et al. did not perform single blastomere analysis for all embryos [10].

Vanderwoestyne et al. also reported large interblastomere variation of 24 % in an m.3243A>G embryo [53]. As interblastomere variation seems to occur more frequently in certain individuals, this itself might be a phenomenon subject to genetic factors [27] although insufficient data exist for such individual risk stratifications. All data taken together, nicely plotted in a figure by Steffann et al. [55], a generally homogeneous distribution of wild-type and mutant mtDNAs can be seen in individual human blastomeres regardless of the mutation, differing remarkably from data on artificially generated heteroplasmic macaque embryos [55, 58]. Based on human data which shows that single blastomeres can diverge, it is advisable to analyze two blastomeres instead of just one. The adverse risk of removing two cells from the embryo at biopsy is a negative influence on live birth delivery [59], illustrating the difficult balance between a safe and correct diagnosis on the one hand and optimizing the chance of pregnancy on the other.

Trophectoderm biopsy performed at the blastocyst stage provides a larger number of cells for analysis and appears to obviate the negative impact on live birth delivery. This approach would also enable more precise selection of a single embryo based on both mutation load and genetic sex. Male offspring with an mtDNA mutation will not encounter the risk of transmitting the mutation to their offspring. So far, only one blastocyst PGD for an mtDNA mutation (m.3243A>G) has been performed in humans although results were promising with regard to the applicability of blastocyst trophectoderm biopsy and PGD for mtDNA mutation carriers [54] (which had been supported by murine data [60]).

However, recently added follow-up data of the boy born after blastocyst PGD reported clinical symptoms and m.3243A>G mutant loads of 47 % and 46 % in blood and 52 % and 42 % in urine, respectively, at ages 6 weeks and 18 months [61]. The blastocyst mutation load had been only 12 % [54]. This contradicts the original report where no abnormal phenotype was reported and follow-up mutation load was 15 % in buccal mucosa at age 1 month; at ages of 5 and 12 months, the mutation load was measured by a commercial lab and found to be <10 % in blood and undetectable in buccal mucosa and urine [54]. While technical differences do exist between methods used to determine the mutation load, this cannot explain such a

large difference. It is unclear what has happened, and the authors of both papers should work collaboratively to clarify this.

Data on the five children born so far after blastomere PGD at the 8-cell stage are much more reassuring [10, 27, 31, 52, 55]. Besides the balance between safety of embryo biopsy (the number of cells to remove for analysis) and subsequent reproductive outcome, the number of embryos available for analysis also brings some conflicting considerations. From the perspective of a cytogenetics laboratory, the more embryos available for study the better, since a larger sample improves the chances of having at least one embryo suitable for transfer (and thus improves the chances of delivering a healthy baby). However, there is a limit to the hormonal (over)stimulation that can be applied during IVF. and some clinically affected carriers will be found a priori to be poor candidates for PGD/IVF treatment (based on inacceptable health risks). For mtDNA mutation carriers approved to undergo IVF, it is important to realize that PGD for these indications represents a substantial risk reduction but not an absolute risk exclusion. This should be carefully discussed during patient counseling and the informed consent process. A 0 % mutation load only occurs seldomly (except for skewing mtDNA mutations). Furthermore, current data are suggestive, but not definitive, to guarantee that mutation load in the embryo stage will remain constant throughout life without passing the threshold level for symptoms at some later point. Nevertheless, we feel that for heteroplasmic mtDNA mutation carriers who want to have unaffected offspring who are biologically their own (and therefore *not* use donor oocytes), PGD represents the best therapeutic option at present. However, it should be acknowledged that PGD is not permitted in all jurisdictions.

PND Versus PGD: Specific Considerations with Respect to Skewing (8993) mtDNA Point Mutations

Although our considerations might be applicable to skewing mutations in general, only for the 8993 mutations do sufficient data currently exist. The characteristics of the nt8993 mutations make PND a feasible option for female carriers, particularly when mutation load is low. PGD is still an alternative in this group of mutation carriers with medium to high mutant load. The chance of producing embryos without the mutation is generally higher than for non-skewing mutations. In cases of high maternal mutation load, the majority of embryos is expected to have high mutation load although PGD will enable selection of those embryos with no or low mutation load. In contrast, PND would lead to the detection of multiple severely affected fetuses and recurrent pregnancy terminations.

If the maternal mtDNA mutation load is low, the majority of embryos would be expected to be without the mutation [27]. Due to the linear relationship between the mother's and her offspring's mutation load [29], for carriers with intermediate mutation load, the situation will be somewhere in the middle. In the choice between the two reproductive options and pregnancy risks, the burden of PGD treatment will need to be carefully considered.

Oocyte Donation

Perhaps the safest and most reliable method to prevent transmission of mtDNA disease is the use of donor oocytes accompanied by IVF using the partner's sperm. However, the supply of suitable donors may be limited in some locations, and oocyte donation is not lawfully allowed in every country. Maternal relatives such as sisters will generally not be suitable as oocyte donors, as they are at risk of carrying the mutation in their oocytes as well. The latter cannot be excluded based on the absence of the mutation in blood or other tissues. An important personal reason for couples to reject oocyte donation is the fact that the resulting child would not be genetically related to the mother.

Nuclear Transfer

Nuclear transfer (maternal spindle transfer and pronuclear transfer) entails the transfer of the nuclear genome from an oocyte or zygote with mutated mtDNA in the cytoplasm (donor) to an enucleated acceptor oocyte or zygote of a healthy donor (acceptor) with presumably normal, mutation-free mtDNA. This technique is currently under investigation only in a research setting [62–67]. Although promising, the safety and efficacy of nuclear transfer which has been noted in primate models has yet to be shown compatible with humans, so this approach requires further study; important ethical issues also require resolution. Whether this technique will be able to completely exclude the risk of transmitting an mtDNA mutation or attain merely a reduction of this risk to offspring is still unclear, since nuclear transfer cannot avoid the co-transfer of small amounts (<1 % in spindle transfer) of mtDNA from the affected to the donor oocyte/zygote. Nuclear transfer techniques would offer a reproductive option for homoplasmic mtDNA mutation carriers and for heteroplasmic carriers with high mutation load, who might produce no or very few oocytes/embryos with mutation load below the threshold.

De Novo mtDNA Point Mutations

Counseling and Recurrence Risk

Besides being maternally inherited, mtDNA point mutation can also occur de novo in the affected individual, and this distinction makes a big difference for recurrence risk. If a de novo mutation is discovered in a child, this mutation is not expected to be present in his/her siblings. Due to the potential intra- and inter-tissue variability of mtDNA mutations, it can never be completely known for sure that the mother of the affected child does not carry any given mutation (i.e., a mutation load beneath the detection level, or the presence of a mtDNA mutation in any non-tested tissue, particularly the oocytes, would be impossible to exclude). However, proper analysis of multiple

maternal tissues largely diminishes the residual risk of the mother having the mutation. Accordingly, for such de novo mtDNA point mutations, the recurrence risk is low, and the mutation is not expected to appear in a subsequent pregnancy. De novo mtDNA mutations are not rare events [1] Sallevelt et al in preparation, yet many such couples may be counseled incorrectly and given a high recurrence risk (erroneously), based on the high mutation load in the child instead of absence of the mutation in the mother.

Reproductive Testing Options

Given the low recurrence risk of apparently de novo mtDNA mutations, PND is feasible as reassurance Sallevelt et al in preparation. In four apparently de novo mtDNA disease cases based on the absence of the mutation in multiple maternal tissues, we have performed PND in a subsequent pregnancy. The mutation was not detected. In 9 of >100 reported cases describing apparently de novo mtDNA mutations, PND was performed in (a) subsequent pregnanc(y)(ies) with normal findings in the majority [35, 68–71], but recurrence in one family [49]. The latter might be the result of gonadal mosaicism, or of failed detection of very low mutation load in the mother's lymphocytes and/or urinary epithelial cells due to the used sequencing method. This is currently being investigated further. PGD is, considering the burden of the treatment, not a favorable alternative in case of such a low recurrence risk.

mtDNA Rearrangements

Counseling and Recurrence Risk

Large, single mtDNA deletions are generally reported to occur sporadically, therefore having a low recurrence risk [72–74]. Indeed, the available data indicate that a clinically unaffected mother of an affected child has a negligible risk of another affected child [73]. Even for clinically affected mothers with an mtDNA deletion themselves, the risk of having clinically affected offspring is estimated to be low (1:24) [73]. mtDNA duplications are, like mtDNA point mutations, either maternally inherited or de novo and the same counseling aspects apply.

Reproductive Testing Options

PND seems the reproductive testing option of choice for de novo mtDNA rearrangements. Given the low recurrence risk even for women who carry an mtDNA deletion themselves, PND is the most feasible option in these cases, too. mtDNA duplications are, like mtDNA point mutations, either maternally inherited or de novo. For maternally inherited mtDNA duplications, the same considerations regarding reproductive testing options apply as for maternally inherited mtDNA point mutations.

Conclusion

Mitochondrial diseases are common metabolic disorders with potentially high morbidity and mortality. Generally, no treatment is available. Couples with a child affected by a mitochondrial disorder or a positive family history and a high risk of affected offspring may request prevention of transmission to a (future) child. Recurrence risks and the applicable reproductive testing options highly depend on the genetic etiology of the mitochondrial disease. For mitochondrial diseases due to nuclear gene defects, Mendelian segregation results in recurrence risks of 25 % or 50 %. Both PND and PGD are applicable, once the causative mutation has been identified. Recurrence risks particularly for mtDNA mutations should be determined on an individual basis, for example, taking into account the nature of the mutation and the mutation load in the mother. The risk for female carriers of mtDNA point mutations (such as the m.3243A>G mutation) of having affected offspring is often difficult to calculate, but it can be high. In those cases, PND is problematic mainly due to difficulties in predicting the phenotype with a given mutation load. PGD is currently the best reproductive testing option, although it should be regarded as a risk reduction strategy, rather than a method to exclude risk fully. Conversely, PGD is not the reproductive testing option of choice for apparently de novo mtDNA point mutations which have a low recurrence risk, making PND feasible for reassurance. The same is true for (large) mtDNA deletions which occur almost exclusively de novo. PND is also applicable for skewing mtDNA mutations, particularly when the mother has a low mutation load. The development of nuclear transfer technology would complete the portfolio of reproductive choices to prevent the transmission of mtDNA disease.

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Chapter 18
Single Embryo Transfer: Significance of the Embryo Transfer Technique

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Introduction

Elective Single Embryo Transfer

Embryo transfer (ET), an apparently simple technique, constitutes a significant, rate-limiting step that is crucial to the success of any in vitro fertilisation (IVF) cycle. Multiple embryo transfer during IVF increases multiple pregnancy rate, thus also raising maternal and perinatal morbidity [1]. There are several advantages of elective single embryo transfer (eSET); it is the only effective strategy known to minimise the risk of multiple pregnancy that can also be applied to patients aged 36–39 years, thus increasing the safety of ART in this age group [2]. Though a single fresh embryo transfer may be associated with a lower live birth rate than double embryo transfer (DET) [1], no significant differences have been reported in the cumulative pregnancy and delivery rates following eSET compared to DET, accompanied with a significant decrease in the multiple gestation rate with better neonatal and obstetric outcomes [1, 3–5]. Authors have even reported significantly higher cumulative pregnancy rates (54.0 % vs. 35.0 %) and cumulative live birth rates (41.8 % vs. 26.7 %; $p<0.0001$), but lower multiple birth rates (1.7 % vs. 16.6 %; $p<0.0001$) following eSET compared to DET [2]. The comparative efficacy between SET and DET was observed in a natural as well as a hormone-stimulated cycle [1]. In women aged <35 years, a significantly higher rate of 'healthy baby' per transfer cycle has been reported following eSET compared to selective double embryo transfer (sDET), regardless of stage of embryo development [6]. For a woman with a 40 % chance of live birth following a single cycle of DET, the

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chance following repeated SET would be between 30 % and 42 %; for a woman with a 15 % risk of multiple pregnancy following a single cycle of DET, the risk following repeated SET would be between 0 % and 2 % [1].

Factors that Influence the Success of eSET

Failure to achieve a live birth following IVF may be attributed to the embryo transfer stage due to lack of good quality embryo(s), lack of uterine receptivity, or the transfer technique itself [7]. The success of eSET is influenced by the following factors.

Patient Selection

To ensure optimal outcomes with eSET, patient selection plays an important role. Selective application of eSET in a small group of good-prognosis patients may be effective in reducing the overall multiple rate of an entire IVF population without substantially reducing the likelihood of achieving a live birth [8]. Good-prognosis patients may be considered as women aged ≤35 years, in their first or second IVF attempt, and with at least two good quality embryos available for transfer. Women aged 36–37 years may also be considered good-prognosis patients for eSET if good quality embryos, particularly blastocysts, are available for transfer as blastocyst stage embryo transfer generally increases the chance of implantation and live birth compared with cleavage stage embryo transfer. Kresowik et al. [9] reported a live birth rate of 64.6 % and a multiple birth rate of 3.4 % following a mandatory single embryo transfer (mSET) policy for all women aged <38 years, with at least seven zygotes, no prior failed fresh cycle, and at least one good quality blastocyst [9]. In women aged ≥38 years, eSET may result in a significant reduction in live birth rate compared with DET [8].

Embryo Quality

Success with an eSET would be compromised if the embryo quality suffered. Morphological methods used to select the most viable embryos for transfer may be far from predictive of the implantation potential of these embryos. A paradigm shift using morphological factors along with metabolic, protein and genetic markers in culture media aims to enhance embryo selection and IVF success rates [10] and is particularly useful in selecting single embryos for transfer. Several advanced techniques for embryo selection such as rapid polymerase chain reaction (PCR)-based comprehensive chromosome screening (CCS) and trophectoderm biopsy prior to SET have been reported to enhance embryo selection, with a resultant increase in the ongoing pregnancy rate (55.0 % vs. 41.8 %, respectively; $p<0.01$) and a

decreased miscarriage rate compared with traditional blastocyst SET (24.8 % vs. 10.5 %; $p<0.01$). These novel screening techniques may provide a practical way to eliminate multi-zygotic multiple gestation without compromising clinical outcomes per cycle [11]. Image acquisition and time-lapse analysis of the embryos optimise accurate embryo selection of viable embryos by identifying the morphokinetic parameters specific to embryos capable of implanting and thus, make it possible to determine the exact timing of embryo cleavages in a clinical setting [12]. New technology, based on embryo developmental and morphological characteristics, using multilevel images combined with a computer-assisted scoring system (CASS) has the potential to overcome the disadvantages with standard embryo evaluation with a superior ability to predict implantation and live birth [13].

Culture Protocols

Improvements in culture protocols facilitate extended culture to the blastocyst stage and by enabling self-selection of viable embryos and improved uterine and embryonic synchronicity, result in higher implantation rates [14, 15]. Excellent pregnancy rates have been reported with SET blastocyst culture with live birth delivery rates comparable to double cleavage stage transfer (27.2 % vs. 24.8 %) and decreased complications related to multiple births [15]. A significant threefold increase in day 5 single embryo transfers over an 8-year period (4.5 % in 2001 to 14.8 % in 2009; $p<0.0001$) has been associated with a significant decrease in the rate of multiple births from 44.8 % to 41.1 % ($p<0.0001$) [3].

Cryopreservation

Elective SET with cryopreservation has been suggested to be more effective in maximising the cumulative live births and significantly less expensive than DET in good-prognosis patients and therefore, from a cost-effectiveness perspective, should be adopted as a treatment of choice [8, 16]. In order to maintain the reduction in the rate of multiples achieved with fresh eSET, eSET should be performed in subsequent frozen-thawed embryo transfer cycles [8]. Patients should be informed of the reductions in both multiple pregnancy rate and overall live birth rate after a single fresh eSET when compared with DET in good-prognosis patients [8].

Significance of the Embryo Transfer Technique

The significance, growing awareness and positive clinical outcomes obtained following SET mandate the performance of a meticulous, atraumatic ET technique that aims to successfully deliver a single good quality embryo in the uterine cavity

without associated difficulty. The significance of the eSET technique stems from the fact that since the possibility of embryo selection in the uterine cavity is eliminated, efforts entailed in the preceding clinical and laboratory protocols and the embryo selection would be rendered useless and the cycle wasted if the ET technique was suboptimal. This is especially true of a fresh first cycle eSET, which can perhaps be salvaged with additional embryos, if available, but will leave fewer embryos for cryopreservation. However, in the case of the unplanned difficult single embryo transfer, the situation may rarely improve and could also compromise the quality of embryo transferred. Though there is no universally acceptable or standard technique for ET, factors documented to have a positive and negative impact on ET must be strictly respected to achieve the desired outcome.

Factors that impact the clinical outcome following ET include (1) routine evaluation of the uterine cavity to detect abnormalities, (2) mock embryo transfer immediately before the actual ET, (3) evaluation of uterine position and dimensions, (4) ultrasound guidance during ET, (5) depositing embryos in the mid-portion of the endometrial cavity, (6) the use of soft catheters, (7) avoidance of uterine contractions, blood, or mucus on the catheter, (8) ensuring an absolutely atraumatic transfer technique and (9) the experience and skill of the clinician performing ET. Evidence detailing the significance of each of these factors is presented below.

Factors that Play an Important Role Prior to ET

Routine Uterine Cavity Evaluation

A routine uterine cavity evaluation enables a thorough exploration of the uterine cavity to check for abnormalities, such as submucosal leiomyomas, adhesions, polyps and congenital abnormalities, that may interfere with a successful outcome. Endometrial cavity abnormalities have been reported with an incidence of 22.9 % following outpatient hysteroscopy in patients with a previous IVF-ET cycle, the correction of which markedly improves the outcome. Sufficient evidence to support the surgical removal of all abnormalities to improve the IVF-ET outcome and the value of performing this procedure before an initial cycle in patients without previous implantation failure is lacking. However, it would seem logical in an effort to minimise the number of cycles a patient must undergo. Three-dimensional saline sonohysterography may be particularly useful in the evaluation [17].

Evaluation of Uterine Position and Dimensions

Before proceeding to ET, it is essential to have adequate knowledge about the uterine position, anteverted (AV) or retroverted (RV) by ultrasonography (USG). An RV uterus at mock embryo transfer will often change position at real embryo transfer to

become AV [18], changing the course of the ET catheter. Misdirecting the ET catheter can be avoided by accurate knowledge of the uterine position at the time of embryo transfer. Following a comparative evaluation of 996 consecutive mock and real abdominal ultrasound-guided-(USG)-ET embryo transfer cycles, Henne and Milki [18] demonstrated a highly significant ($p < 0.0001$) change in the position of RV uteri at mock transfer (26 % of 55 % ETs) to AV at the actual transfer compared to the conversion of only 2 % of the 74 % of patients with an AV uterus at mock embryo becoming RV at the actual transfer. The change in uterine position was also noted in frozen-thawed embryo transfers (12 % of AV uteri at mock embryo transfer to RV and 33 % RV uteri to AV at real transfer; $p = 0.01$). Accordingly, patients with an RV uterus at mock embryo transfer should still present with a full bladder for embryo transfer, since a significant number will convert to an AV position [18]. Moreover, ultrasound evaluation of the uterocervical angulation and uterine cavity length prior to the actual transfer can optimise the ET technique and may reduce the rate of ectopic pregnancies [19, 20].

Mock/Trial Embryo Transfer

A mock/trial transfer is essential before actual ET as it enables a thorough knowledge of the uterine position (AV/RV), uterocervical length and angulation that may be of value in accurately guiding the course of the catheter during the actual ET. Additionally, it is of value in revealing intracavitary abnormalities that may interfere with pregnancy and in directing possible surgical management prior to ET. While the value of a mock transfer a few days before the actual procedure has been challenged owing to the change in the uterine position [18], a trial catheterization on the day of ET could prevent most of the unanticipated procedural difficulties during the transfer [17]. Moreover, a USG-trial transfer (UTT) in the office, in preparation for an IVF cycle, has shown to be beneficial as significant differences have been noted between patients when comparing difference in length (DL) to previous pregnancy status and the total cavity depth (sounding depth + DL) ($p < 0.05$) [21].

Hysteroscopic Revision of the Cervical Canal

Cervical stenosis may be associated with a technically difficult ET, reducing the chances of pregnancy after assisted reproductive procedures. Hysteroscopic revision of the cervical canal results in easier ET by facilitating the course of the transfer catheter through the cervical canal and thus, improved pregnancy rates in patients with cervical stenosis and histories of difficult ET [22].

Fluid Volume in the Transfer Catheter

The amount of fluid volume for day 3 transfer has been shown to have a significant impact on pregnancy and implantation rates. A high fluid volume (40–50 μL) for loading the transfer catheter resulted in significantly higher pregnancy (40 % vs. 23 %; $p = 0.012$) and implantation rates (24.4 % vs. 14.7 %; $p = 0.011$) compared to a low fluid volume (15–20 μL; $n = 94$) [23].

Removal of Cervical Mucus

Significantly higher clinical pregnancy rates (39.2 % vs. 22.6 %, respectively, $p < 0.001$), implantation (20.5 % vs. 12.2 %, respectively, $p < 0.001$) and live birth rates have been reported following removal of cervical discharge before ET, compared to patients in whom the cervical canal was not cleansed. This suggests that removal of cervical debris prior to ET may have a significant effect on the rate of implantation, pregnancy and live birth [24].

Bacterial Contamination

Microbial examination of samples from the fundus of the vagina, the cervix, the embryo culture medium prior and post-embryo transfer, the tip of the catheter and the external sheet shows that the presence of vaginal-cervical microbial contamination at the time of ET is associated with significantly decreased pregnancy rates (*Enterobacteriaceae*: 22.2 % vs. 51 %; *Staphylococcus* spp.: 17.6 % vs. 44 %; $p < 0.001$) when compared to negative culture groups [25]. While catheter contamination by upper genital tract microbes has been suggested to affect the success of ET and administration of antibiotics like amoxicillin and clavulanic acid before ET can significantly reduce microbial colonisation and catheter contamination rates [26], this intervention did not translate into better clinical pregnancy rates [26, 27]. Hence, the routine use of antibiotics at embryo transfer prior to ET is not recommended [26, 27].

Factors that Play an Important Role During ET

Ultrasound-Guided ET

The use of ultrasound guidance to perform ET has been one of the most significant advances in the ET technique over the traditional ‘blind’ clinical touch method. Despite the lack of a standard evidence-based protocol, there is substantial evidence

that both transabdominal [6, 7, 28, 29] and transvaginal [30] USG-ETs significantly increase clinical pregnancy, embryo implantation, ongoing pregnancy and live birth rates compared to clinical touch alone [6, 7, 28–30]. Occasional studies have demonstrated no benefit with USG-guided ET over the clinical touch method with reference to clinical pregnancy and implantation rates compared to previous ultrasonographic length measurement [31] and in the hands of an experienced operator [32, 33]. However, success in patients with a prior history of difficult uterine sounding or embryo transfer still relied heavily on USG-ET [32]. Of note, the 25 % chance of pregnancy using the clinical touch method alone increased to 32 % (from 28 % to 46 %) when USG-ET was performed instead [34].

Ultrasound-guided ET brings the following advantages to make this an indispensable technique to achieve an optimal outcome:

- It facilitates an accurate evaluation of the uterine position and cavity length before the actual embryo transfer and, hence, the transfer distance from the fundus (TDF).
- It facilitates the correct placement of the catheter in the endometrial cavity.
- It avoids contact of the catheter tip to the fundus.
- It confirms that the catheter is beyond the internal os in cases of an elongated, cervical canal.
- It allows direction of the catheter along the contour of the endometrial cavity, thereby avoiding disruption of the endometrium, plugging of the catheter tip with endometrium and instigation of bleeding.
- The requirement of a full bladder to perform transabdominal USG-ET is itself helpful in straightening the cervical-uterine access and improving pregnancy rates.
- It may facilitate an uncomplicated access through the cervix to access the uterine cavity, thus overcoming cervical stenosis [35].
- It enables visualisation of the catheter tip during ET and the position of embryo deposition [36].
- It significantly increases the frequency of easy transfers [29, 37] and decreases the incidence of difficult transfers and endometrial injury [38] possibly due to a decrease in cervical and uterine trauma [29] compared to the clinical touch method.
- It may be especially beneficial in patients with previously failed IVF cycles or in patients with previous cycles when embryos were transferred by the clinical touch method [30].

Indeed, tactile assessment of ET catheter placement has been considered unreliable as the outer guiding catheter inadvertently abutted the fundal endometrium in 17.4 % of transfers, indented the endometrium in 24.8 % and the transfer catheter embedded in the endometrium in 33.1 % transfers. Unavoidable sub-endometrial transfers occurred in 22.3 % of transfers, while USG-ET avoided accidental tubal transfer in 7.4 % of transfers [39]. Measurement of cavity depth by USG is clinically useful to determine the depth beyond which catheter insertion should not occur. The transfer distance from fundus (TDF = cavity depth minus depth of catheter insertion), measured

by USG, is highly predictive of pregnancy, unlike that measured by mock transfer as cavity depth by US has been reported to differ from cavity depth by mock by at least 10 mm in >30 % of cases [34]. Moulding the embryo transfer catheter according to the uterocervical angle, measured by ultrasound, increases clinical pregnancy and implantation rates and diminishes the incidence of difficult and bloody transfers compared with the 'clinical feel' method. Patients with large angles (>60°) had significantly lower pregnancy rates compared with those with no angle [40]. Significantly higher pregnancy rates per transfer have been reported when ultrasound visualisation was considered to be excellent/good (when the catheter could be followed from the cervix to the fundus by transabdominal ultrasound with the retention of the embryo-containing fluid droplet), compared to fair/poor transfers (where the sequence of events could not be documented). Performance of embryo transfer with a soft catheter under ultrasound guidance with good visualisation resulted in a significant increase in clinical pregnancy rates [36].

Though transvaginal USG-ET may be associated with increased patient comfort due to the absence of bladder distension, the total duration of transfer is statistically significantly higher compared to transabdominal USG-ET [41].

Two-dimensional (2D) USG-ET is the standard for image-guided transfers to monitor catheter passage through the cervix into the endometrial cavity [42], although three-dimensional (3D) USG offers better precision and optimal positioning of uterine catheter tip placement. This is an enhancement in the ET technique and has been shown to improve overall pregnancy rate compared with 2D sonography [42, 43]. Moreover, the disparity of ≥10 mm in transfer distance from the fundus (TDF) between 2D and 3D images may significantly impact the pregnancy outcome [43]. Irrespective of the USG mode used, the important role of USG-ET in optimising pregnancy outcomes warrants perfection in this technical skill.

Catheter Type

The type of catheter used for ET (soft/rigid and echogenic/non-echogenic) may influence the degree of trauma to the endometrial cavity during ET. Significantly higher pregnancy ($p<0.0005$) and implantation rates ($p<0.01$) have been reported with the ultrasoft catheters compared to the more rigid Frydman catheters [35]. A blinded comparison of endocervical and endometrial damage following the use of soft ET catheters [IVF Sydney Set (Cook, Limerick, Ireland), Elliocath (Ellios, Paris, France), Frydman classic 4.5 (CCD, Paris, France)] and rigid ET catheters [Memory Frydman 4.5 (CCD, Paris, France)] demonstrated significantly more frequent endocervical lesions with the soft (63 %) and rigid (85 %) Frydman catheter groups compared to other groups (Elliocath: 29 %, IVF Sydney Set: 26 %; $p<0.0001$). Severe endometrial lesions were significantly less frequently observed when soft catheters were used (85 %, 53 %, 32 % and 11 % for Memory Frydman, Frydman classic, Elliocath and IVF Sydney Set, respectively; $p<0.0001$) [44]. Blood on an ET catheter is a marker for endometrial microtrauma; all ET catheters

can lead to endocervical or endometrial damage, but severe endometrial lesions may less frequently be encountered when soft catheters are used [44]. Though no significant difference in implantation, clinical or ongoing pregnancy rates has been observed following ET with the echogenic catheters (Sure View catheter [44], the echogenic Wallace catheter [45] or the Cook Echo-Tip catheter [46]) and standard catheters without echogenic enhancement, echogenic catheters offer the benefit of superior visualisation due to their ultrasonic contrast properties. This minimises the need for catheter movement to identify the tip [44–46] and significantly shortens the duration of the ET procedure (defined as the interval between when the loaded catheter is handed to the physician and embryo discharge), thus simplifying USG-guided ET [45].

In addition to easy visualisation of the catheter tip, El-Shawarby et al. [47] reported a significantly lower rate of retained embryos in the catheter following ET with the Rocket catheter compared to the Wallace catheter ($p < 0.05$), although there was no difference in clinical pregnancy and implantation rates [47]. The use of a soft pass catheter was the only variable independently and significantly associated with pregnancy success (OR = 2.74) [48].

Depth of Embryo Transfer

Traditionally, ET has been performed blindly with the goal to place the embryos approximately 1 cm inferior to the fundal endometrial surface [49]. The depth of embryo replacement (difference between the cavity depth and depth of catheter insertion) during USG-ET has been shown to have a significant impact on the clinical outcome after controlling for potential confounders [49–53]. Significantly higher ($p < 0.05$) implantation rates (31.3 %, 33.3 % and 20.6 %, respectively) have been reported when embryos were deposited at a distance ≥15 mm (15 ± 1.5 mm or 20 ± 1.5 mm) between the catheter tip and the uterine fundus compared to <15 mm (mean = 10 ± 1.5 mm).

There was no difference between all three transfer groups regarding the main demographic and baseline characteristics of the patients, ovarian response, oocyte retrieval and IVF outcome. Characteristics of embryo transfer and luteal phase support were also similar [49]. While maintaining a uniform method of loading embryos into the embryo transfer catheter and the number and quality of embryos transferred, Pacchiarotti et al. [50] observed significantly higher clinical pregnancy rates (27.7 % vs. 4 %, respectively; $p < 0.05$) when the distance between the tip of the catheter and the uterine fundus at transfer was 10–15 mm compared to ≤10 mm [50]. Tiras et al. [51] buttressed these findings in a large study that included 5,055 USG-ETs in 3,930 infertile couples, observing higher pregnancy and ongoing PRs when the embryos were replaced at a distance >10 mm from the fundal endometrial surface. They suggested that a distance 10–20 mm seems to be ideal for embryo transfer to achieve higher PRs [51]. These findings have been further documented in a very recent study that reported clinical intrauterine pregnancy rates of 65.2 %,

32.2 % and 2.6 % when the distances between the fundal endometrial surface and the tip of inner catheter were <10 mm, 10–20 mm and 20 mm, respectively, suggesting that the optimal distance between the fundal endometrial surface and the tip of inner catheter is 1.5–2 cm [52]. According to Pope et al. [53], for every additional millimetre that embryos are deposited away from the fundus, the odds of clinical pregnancy increased by 11 % [53].

Avoiding Difficult Transfers

It is extremely important to avoid a difficult transfer by preplanning the ET technique, as this may significantly impact the clinical outcome of an eSET. Patients at risk for a difficult ET should be identified so that the ET can be appropriately planned. Embryo transfer is considered difficult if it has been time consuming, the catheter met great resistance, there was a need to change the catheter, sounding or cervical dilatation was needed, there was blood in any part of the catheter [54] or it required at least two attempts [55] and may often be associated with a poor clinical outcome.

In contrast, an 'easy' transfer has been suggested to be an atraumatic insertion of the catheter without touching the uterine fundus. When ET difficulty was evaluated as an independent factor for predicting pregnancy after taking into account the other confounding variables, it was observed that easy or intermediate transfers resulted in a 1.7-fold higher pregnancy rate compared to difficult transfers ($p < 0.0001$; 95 % CI = 1.3–2.2), suggesting that that the degree of difficulty of embryo transfer is an independent factor as regards achieving pregnancy after IVF/intracytoplasmic sperm injection (ICSI) [54]. Hysteroscopic assessment of endocervical and endometrial damage, inflicted by embryo transfer trial, revealed a significant concordance between the perceived difficulty of transfer, presence of blood on the catheter and degree of endometrial damage ($p < 0.05$). There were significantly higher minor and moderate endocervical lesions (35 % and 24 % of cases, respectively) in the difficult transfer group as compared to the easy transfer group (19 % and 3 %, respectively; $p < 0.05$). Within the easy transfer group, 65 % of patients had no endometrial damage, 32 % had minor lesions and 3 % had moderate lesions compared to 42 %, 29 % and 29 % in the difficult transfer group, respectively. Moreover blood on the catheter was noted in 2 %, 56 % and 71 % of the easy, moderate and difficult transfer groups, respectively. The authors concluded that clinical perception of difficulty of transfer and the presence of blood on the catheter are directly associated with endometrial disruption [56].

While the use of external guidance during ET has been shown to significantly reduce live birth delivery rates (LBDR) as compared to an atraumatic ET with a soft catheter (26.0 % vs. 32.5 %, respectively), grasping the portio vaginalis with a tenaculum is reported to result in the lowest clinical pregnancy rates (CPR) and LBDR, compared to ET with a soft catheter, after external guidance or probing of the cervix with a stylet. Though considered to be superior to the use of external guidance in cases of difficult ETs [57], the use of a stylet in the event of a failure of

the soft inner catheter to negotiate the internal os is associated with significantly lower implantation (19.4 % vs. 13.8 %), clinical pregnancy (41.9 % vs. 31.1 %) and live birth rates (37.3 % vs. 27.4 %), compared to ETs without the use of a stylet [58].

Physical contact (such as touching the uterine fundus with the tip of the ET catheter during transfer) results in mechanical stimulation activity of the uterus or junctional zone contractions (JZCs) that may relocate intrauterine embryos. Hence, all efforts should be made to avoid triggering JZCs as this has been implicated in cases of IVF-ET failure or ectopic pregnancy [59]. Embryo transfers that provoke bleeding and those that result in retention of embryos in the cervix and embryo expulsion have all been linked to JZCs [19, 52, 60]. Physicians should use a stepwise approach in difficult embryo transfers [52].

Injection Speed

There appears to be an inverse relationship between ejection speed (i.e., the velocity of discharge of embryo/s plus media from the catheter) and the subsequent development rate of the transferred embryo/s. Thus, reducing the ejection speed of the transferred load may help avoid developmental delay and decreases the associated embryo(s) injury. Specifically, the embryo development rate has been found to be the slowest in embryos exposed to a fast ET with a higher mean apoptotic index of embryos compared to the group exposed to a slow ET (17.6 % vs. 5.6 %, respectively) and the control group (2.58 %). Hence, embryos should be transferred with the lowest possible ejection speed [61].

Experience of the Practitioner

Apart from the numerous factors that should be considered while performing an ET, the most influential factor in the outcome is the operator's experience in the use of each system, and not the system itself [62]. The physician factor is an important variable in the overall ET technique and can result in significant differences in clinical pregnancy rates ($p \leq 0.01$), as shown by comparisons between different providers using the same method of loading embryos into the embryo transfer catheter and the same number of embryos transferred [63]. Desparoir et al. [64] demonstrated pregnancy rates of 29.9 % for attending physicians (>20 years of experience), 28.2 % for assistant physicians (2–5 years of experience) and 19.1 % for resident physicians (<6 months of experience) ($p < 0.05$). Resident physicians used tight difficult transfer (TDT) catheter more often than attending physicians: 42 % vs. 21.3 %, respectively ($p < 0.05$), suggesting that resident physicians require monitoring to avoid lower pregnancy rates [64]. Authors have even suggested that in the hands of experienced, skilled operators, neither the choice of transfer catheter and difficulty of transfer nor observations of blood on the transfer catheter will make any significant impact on pregnancy outcomes [65].

Embryo After-Loading

Despite significantly more transfer catheters with mucus contamination compared to direct transfers (25.58 % vs. 5.95 %), there was a trend towards an increase in clinical pregnancy rate following the embryo after-loading technique compared to the direct technique (52.4 % vs. 34.9 %) [21]. However, more evidence is required to substantiate these results.

Blood on the Catheter

The presence of blood on the transfer catheter may be an indication of a difficult transfer or infection. While some studies have demonstrated a significant decrease in the pregnancy and implantation rates in the presence of blood on the catheter [66, 67] or inside the catheter [68] after ET, others have failed to support the association between the presence of any type of contamination, whether macroscopic or microscopic, presence of blood or mucus and pregnancy outcome [69].

Retained Embryos

Immediate retransfer of embryos retained in the catheter following an initial transfer attempt in the absence of blood and mucus in the transfer catheter and other signs of a difficult transfer does not adversely influence the pregnancy outcome in terms of pregnancy, implantation, and delivery rates per embryo transfer [70, 71].

Recent Advances

Despite attempts to standardise the protocol of manually performed conventional embryo transfers, a comparative study that evaluated the injection speeds of simulated conventional embryo transfers by seven laboratory technicians and a pump-regulated embryo transfer (PRET) device demonstrated a large variation in injection speed in manually performed transfers, even after standardisation of the protocol. The recently developed automated PRET device generates a reliable and reproducible injection speed and therefore, brings new possibilities for further standardisation of the embryo transfer procedure. However, additional studies are needed to confirm if the observation mimics real clinical circumstances and if a standardised injection speed results in more exact positioning of the transferred embryos and therefore, higher pregnancy rates [72].

Conclusion

To maximise pregnancy outcomes with a single euploid embryo, we believe it is mandatory to ensure the atraumatic ultrasound-guided delivery of the embryo with a soft echogenic catheter, at a precise position in the endometrial cavity with a receptive endometrium, in a timely manner. The ET technique deserves dedicated attention owing to the number of parameters involved in ensuring a smooth and successful ET as discussed here. Should these factors be neglected, the reproductive outcome may be compromised. Hence, the ET technique must be preplanned to anticipate and avoid difficult transfers and those associated with a negative outcome. The clinician's knowledge of these factors and skill in performing ET is of paramount importance.

Conflict of Interest The authors declare no conflicts of interest.

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Chapter 19
The Vitrification Component: An Integral Part of a Successful Single-Embryo Transfer Program

Juergen Liebermann

Introduction

In 1996, the Centers for Disease Control and Prevention (CDC) initiated data collection on assisted reproductive technology (ART). In 2011, the CDC reported a national multiple pregnancy rate with ART or in vitro fertilization (IVF) of 30 %, with 27.5 % rate for twin pregnancies and 2.5 % for triplet or higher-order multiples [1]. The original goal of IVF treatment was to maximize the chance of achieving a pregnancy by transferring several embryos, regardless of any known complications created by multiple-order pregnancies [1, 2]. It has since been shown that the risk of multiple-order births increases with the number of embryos being transferred, thus adversely modifying the risk for pregnancy complications [1, 3].

The increased risk of multiple pregnancies is therefore associated with increases in maternal and perinatal mortality and morbidity and increased costs for all parties involved [4–6]. Over the past decade, ART has made progress worldwide in terms of greater infertility treatment success [1, 3]. This can be attributed to the availability of complex culture media, a better understanding of in vitro culture conditions for human embryos, which allows culture to be maintained until the blastocyst stage, and improved cryopreservation techniques for surplus embryos not chosen for transfer [3]. But clinical experience shows that many patients are confronted with dilemmas when deciding whether to choose one or two embryos for embryo transfer. The nature of some of these dilemmas may lie in:

- The emotional stress that a patient may be undergoing (urgency to get pregnant)
- The financial aspects of the treatment (cost to the infertile couple, which increases with no IVF insurance coverage)

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- Educational issues (lack of information about the risks of multiple gestations)
- Statistical concerns (being aware of the low ongoing pregnancy rate per treatment cycle from national data).

The European Society of Human Reproduction and Embryology Consensus Conference [7] raised awareness of the problem of infertility therapy-associated multiple pregnancies, suggesting that the essential aim of IVF "is the birth of a single healthy child, with twin pregnancy regarded as a complication" [7]. Accordingly, in an effort to reduce high-order multiple pregnancies, a growing body of evidence supports reducing the number of embryos transferred and moving toward elective single-embryo transfer (eSET) as a viable alternative to multiple-embryo transfers [8–15]. This may be thought of as moving away from simply "maximizing" an IVF cycle to "optimizing" an IVF cycle by maintaining a balance between the end result and the efforts, costs, and complications of the treatment. Besides carefully selecting the right patient, identifying the features that characterize a top-quality embryo is also crucial for achieving success with eSET [16, 17]. Today, the current established method for embryo selection in clinical application based on static morphologic and physical characteristics identified by light microscopy gets support by a variety of minimally invasive approaches such as time-lapse photography to assess "true" embryonic developmental potential. The application of time-lapse embryo monitoring under clinical application avoids the need to remove embryos from incubation to assess the embryo development on a daily basis. Moreover, by collecting time-lapse images and rewinding them in order to observe morphokinetic details in embryo development, an additional powerful tool for embryo selection exists.

Methods

Decision Making

What patient population would be suitable to offer eSET? The facts show that women with the best chance of getting pregnant after infertility treatment are also those at highest risk to conceive multiple gestations (usually patients who are age <35 years). The CDC revealed in their 2011 report that if patients younger than age 35 undergo a two-embryo transfer, the incidence of twin pregnancies was about 45 % with a occurrence of 1.3 % triplets or more [18]. At our institution, we recommended using eSET for good prognosis patients. The criteria for this recommendation include:

- Age <37 years
- Having their first IVF cycle or having conceived in a previous IVF cycle
- Availability of one or more high-quality blastocysts.

Furthermore, convincing patients to reduce the number of embryos transferred from two to one is effective only when a patient is convinced of the success of eSET. The acceptance of eSET can be supported by a successful cryopreservation program which can achieve outcomes similar to that of fresh transfers.

The Vitrification Procedure

The impact of cryopreservation on the growth and improved efficiency of assisted reproduction in humans has become increasingly appreciated. With approximately one-quarter of a million babies born following cryopreservation, cryopreservation has been shown to increase pregnancy rates while allowing for further selection of embryos. Therefore, it is possible to achieve implantation and pregnancy rates with frozen–thawed embryos as high as those achieved with fresh embryos. Lower numbers of embryos are being transferred, resulting in fewer higher-order multiple gestations and improved implantation rates. Moreover, cryopreservation of embryos is a powerful tool in the prevention of twins. In addition, the true augmentation potential of cryopreserving embryos on the total reproductive potential of a single oocyte harvest can be evaluated.

Today, cryopreservation is one of the keystones of clinical infertility treatment. In particular, an ultrafast cooling technique known as vitrification has become a well-established and widely used procedure that allows important expansion of therapeutic strategies during IVF. Most important, vitrification of human blastocysts allows the potential for conception to be maximized from any one in vitro fertilization cycle and prevents wastage of embryos. The ability to vitrify blastocysts either on day 5 or day 6 opens the opportunity to offer to selected patients the transfer of one elective single blastocyst instead of two, with no decrease in pregnancy rate while also greatly reducing the likelihood of multiple gestation. Making the patient aware, and the key here lies in the importance of patient education in regard to multiple pregnancies, of the nonexistence of different outcome using either fresh or vitrified blastocyst will increase their confidence in the procedure and in their choice to go with one embryo at a time.

Next, the application of vitrification technology for cryopreserving human blastocysts is described in a step-by-step sequence.

Stepwise Blastocyst Vitrification Procedure

Vitrification of blastocysts should be undertaken utilizing a “closed system” (HSV: High Security Vitrification Kit; CryoBio System, L’Aigle, France; FDA 510(k) clearance for cleavage stage embryos in blastocysts) after a two-step loading with cryoprotectant agents at 24 °C. If assisted collapsing is done before vitrification, then the blastocyst should be placed on an inverted microscope equipped with a

laser system (ZILOS-tk, Hamilton Thorne). The junction of two trophectoderm cells in each blastocyst needs to be located and one pulse (100 % power, 500 µs duration) applied. Then the blastocysts are returned to the incubator for 5–10 min. Briefly, blastocysts should be placed in equilibration solution, which is base medium (M199 with 20 % Serum Supplement Substitution, SSS) containing 7.5 % (v/v) ethylene glycol (EG) and 7.5 % (v/v) dimethyl sulfoxide (DMSO). After 5–7 min, the blastocysts need to be washed quickly in vitrification solution, which is the base medium containing 15 % (v/v) DMSO, 15 % (v/v) EG, and 0.5 M sucrose, for 45–60 s and transferred onto the HSV using a micropipette. Immediately after the loading of not more than two blastocysts in less than 1 µl drop on the HSV, the straws can be heat sealed, then plunged in LN_2, and secondarily stored inside 5 ml liquid nitrogen prefilled canes (Visotube Rond, IMV; France). Each component is described in detail below.

1. Aseptic techniques are required at all stages. For equilibration and vitrification procedures ensure the benchwarmer is at room temperature (~25 °C).
2. Take reagents from the refrigerator and allow them to warm to room temperature.
3. Separate the blastocysts to freeze into a separate well. Bring this dish to the inverted microscope and with the embryo positioned with the laser objective use a single pulse to hit the blastocysts between two trophectoderm cells to collapse the embryo. Place the dish back into the incubator for 5–10 min.
4. Label a petri dish with the patient's name under the lid as follows: HTF-HEPES, ES, and VS. Prepare 2 × 50 µl of HTF-HEPES, 2 × 50 µl of ES, and 4 × 50 µl of VS.
5. The vial label should include the patients' first and last name, accession number, MPI#, date plus number and type of embryos.
6. Before vitrification, use a Stripper tip with 200 µm end hole for loading the blastocysts on the top.
7. Fill Styrofoam container with LN_2.
8. Each sample that is vitrified will be done in a separate hood and verified by a second embryologist before proceeding. Vitrify good expanded/hatching blastocysts on day 5/6/7.
9. Remove embryos from culture dishes using a stripper tip into the HTF-HEPES (drop 1), gently aspirating to remove any traces of culture media.
10. Pipette from mHTF (drop 1) to the other drop of mHTF (drop 2) and immediately merge it with the first drop of ES (drop 3). Set timer for 5 min.
11. When the time is up, transfer embryos to the remaining drop of ES (drop 4). Set the timer for 3 min. Place embryos on the top of the drop and let them settle to the bottom.
12. Next, load blastocysts in a VS back-loaded stripper tip and rinse through the four droplets of VS (drops 5–8), between each droplet clean tip.
13. Note that placement into the VS and loading of the cryotop should take <1 min, so that the total incubation time in 15 % VS is 30 s. After 30 s, gently transfer them to the tip of the HSV by using a stripper tip to load the blastocysts in as small volume as possible (i.e., <0.5 µl) onto the edge of the stick.

14. Visually confirm placement.
15. Before loading, apply label to the open end of the empty straw. Load the HSV stick into the empty straw, the side with the embryos first. Use the blue handle to make sure the stick has been fully advanced. Then, using the heat sealer, seal the open end of the stick and plunge the whole straw into the LN_2. Place the straw in a precooled aluminum cane for further storage.
16. Store cane in nitrogen tank.
17. Record cane location on the freezing worksheet and cryo inventory log.
18. Complete all paperwork and recheck that all vial locations are logged into the Embryo Inventory.

Stepwise Blastocyst Warming Procedure

Regardless of the day of cryopreservation of the embryo (whether day 5, 6, or 7), at thawing, blastocysts should be treated as if they had been frozen on the fifth day of development. To remove the cryoprotectants, blastocysts need to be warmed and diluted in a three-step process. With the HSV submerged in LN_2, the inner straw should be removed. The carrier with the blastocysts can then be removed from the LN_2 and placed directly into a pre-warmed (37 °C) organ culture dish containing 1 ml of 1.0 M sucrose. Blastocysts can be picked up directly from the HSV and placed in a fresh drop of 1.0 M sucrose at 24 °C and immediately connected with a drop of 0.5 M sucrose. After 5 min, blastocysts can be transferred to 0.5 M sucrose solution and connected with drops of base medium for additional 5 min. Even when switching the cells between different concentrations of warming solutions, fill up the pipette with the next lower concentration of warming solution before picking up the cells for moving in the following concentration. Then the blastocysts can be washed in the base medium for 3 min and returned to the culture medium (SAGE Blastocyst Medium, Trumbull, CT, USA) until transfer. Each single step is described in detail below.

1. Take reagents from the refrigerator and allow them to warm to room temperature. All cryoprotectants are removed at 25 °C.
2. Place a 200 µl drop of TS on a petri dish and place on a warming plate.
3. Label a petri dish (Nunc) with the patient's name under the lid as follows: TS, DS, and WS. Prepare 1×50 µl of TS, 4×50 µl of DS, and 6×50 µl of WS.
4. Before warming, use a Stripper tip with 200 µm end hole for removing the blastocysts from the top.
5. Fill Styrofoam container with LN_2.
6. Confirm location and identification with a second embryologist before warming any HSV kit. Warm one kit at a time.
7. Each sample that is warmed will be done in a separate hood and verified by a second embryologist before proceeding.
8. With the HSV kit under LN_2, open the kit by cutting the outer straw. Use the blue handle to remove the inner stick.

9. Submerge HSV kit directly in the pre-warmed drop containing TS, which should be as close as possible to the LN_2 styrofoam container. As soon as the HSV kit contents liquefy (within 1 min), try to locate the blastocysts before removing them with a stripper tip. After locating all the blastocysts, remove them from the tip and place them in the droplet of TS (drop A) and connect immediately with the first droplet of DS (drop B). Wait for shrinkage and re-expansion.
10. When they start to wrinkle, connect with the second droplet (drop C) and finally with third droplet of DS (drop D).
11. When they stop reacting and start to reshrink, transfer blastocysts to 0.5 M sucrose (drop E) by placing at the top of this drop so they float to the bottom. When the reaction is complete, connect with first of WS (drop F; wait for about 90 % re-expansion).
12. After 100 % expansion, connect with droplet #2 (drop G) and then with droplet #3 (drop H) of WS. Turn on benchwarmer and finally dilute through a series of three wash drops of HS (I to K).
13. Place the blastocysts into a culture dish and put it in the incubator for subsequent culture.
14. Record the survival and appearance of all blastocysts. Update log with warm data, and notify the physician of result.

Results

Successful Application of eSET

Since 2007, Fertility Centers of Illinois (FCI) offered eSET using morphologic criteria for the selection of good-quality embryos, combined with careful selection of patients. The following report summarizes the results of our study with eSET at this institution.

Between 2007 and April of 2014, we performed 8,192 autologous cycles with embryo transfer of which 3,453 (42 %) embryo transfers were performed on day 3 without eSET, and 4,739 (58 %) embryo transfers were done on day 5. Records of a total of 1,037 autologous eSET on day 5 (~22 % of all blastocyst transfers—1,037:4,739) were reviewed. The CDC reported a national average of 12.2 % for eSET cycles in patients age <35 years in 2011 [19].

The mean (±SD) age of our patient population was 31.8 ± 3.3 years. On average, 18 oocytes per patient were retrieved. Of a total of 18,173 oocytes retrieved, 80.1 % were injected, and 77.0 % fertilized normally (11,207/14,551). The majority (98.0 %) of the fertilized oocytes cleaved on day 2. Of normally fertilized oocytes, 74.0 % progressed to blastocyst stage (Table 19.1). In 1,037 eSET cases, 717 positive pregnancies (69.1 %) were achieved with 642 clinical pregnancies (62.0 %). The implantation rate was 63.5 % (659/1,037), with 579 ongoing pregnancies, yielding a 55.8 % ongoing pregnancy rate. We have now confirmed live births from 883 eSETs done between 2007 and July of 2013. The ongoing pregnancy rate was 53.7 % (474/883), followed by a live birth rate of 51.8 % (457/883).

Table 19.1 Retrospective outcome data from 1,037 autologous elective single-embryo transfers on day 5

Patients, *N*	1,037
Patients' age, years	31.8 ± 3.3[a]
Oocytes retrieved, *N*	18,173
Oocytes injected, *N*	14,551
Oocytes fertilized, *N* (%)	11,207 (77.7 ± 14.6[a], 76.8–78.6[c])
Embryos cleaved on day 2, *N* (%)	10,967 (98.1 ± 5.9[a], 97.7–98.5[c])
Embryos with ≥6 blastomeres on day 3, *N* (%)[b]	9,714 (87.2 ± 15.4[a], 86.3–88.1[c])
Compacting embryos on day 4, *N* (%)[b]	8,219 (74.0 ± 22.0[a], 72.7–75.3[c])
Blastocysts on day 5, *N* (%)[b]	8,270 (74.3 ± 18.3[a], 73.2–75.4[c])
Patients who underwent eSET, *N*	1,037
Implantations, *N* (%)	659 (63.5)
Positive pregnancies/eSET, *N* (%)	717 (69.1)
Clinical pregnancies/eSET, *N* (%)	642 (62.0)
Ongoing pregnancies/eSET, *N* (%)	579 (55.8)
Multiple pregnancy rate, *N* (%)	17 (2.6)
Confirmed live births from 883 eSET between 2007 and July 2013	
Ongoing pregnancies/883 eSET, *N* (%)	474 (53.7)
Live birth rate/883 eSET, *N* (%)	457 (51.7)
Live births, *N*	469 (211 boys and 258 girls)

eSET elective single-embryo transfer, *SEM* standard error of mean
[a]Means ± SEM; [b]%/2 pns; [c]95 % confidence interval

In all of the 1,037 eSET, blastocysts were available for transfer on day 5. Of note, 963 patients had cryopreservation (93 %) whereas only 74 patients (7 %) ended up with no cryopreservation at all. It should be mentioned that 30 % of the "no cryopreservation" group had no cryopreservation because they declined to sign the relevant consent. A total of 4,961 blastocysts were vitrified, yielding an average of five blastocysts per patient.

Applying eSET to a large proportion of patients, more embryos would be available for vitrification which in turn would result in more successful vitrified–warmed cycles. The majority of blastocysts were vitrified on day 5 (67.5 %), whereas only 32.5 % were vitrified on day 6 (see Table 19.2). As shown in Table 19.2, patients with no embryos suitable for cryopreservation had the same chance to get pregnant compared with the group of patients having surplus embryos for cryopreservation.

Results on Blastocyst Vitrification After Failed Fresh eSET

To calculate a patient-specific augmented pregnancy rate, it is essential to include as augmentation only those pregnancies from vitrified blastocysts among patients who did not have a pregnancy after fresh eSET. This represents true augmentation of the patient-specific expectation of pregnancy from the same oocyte harvest. After 1,037 eSET, a total of 464 patients experienced a negative outcome. 320 patients

Table 19.2 Retrospective outcome data from 1,037 autologous elective single-embryo transfers on day 5 with or without having embryos suitable for cryopreservation

	1,037	
Total number of eSETs	With cryopreservation	Without cryopreservation
Patients, *N*	963	74
Patients' age, years	31.7 ± 3.2[a]	32.0 ± 4.3[a]
Blastocysts vitrified, *N*	4,961	–
Average number of blastocyst per patient vitrified	5	–
Day 5 blastocysts vitrified, *N* (%)	3,347 (67.5)	–
Day 6 blastocysts vitrified, *N* (%)	1,614 (32.5)	–
Implantations, *N* (%)	612 (63.6)*	47 (63.5)
Positive pregnancies/eSET, *N* (%)	667 (69.3)*	50 (67.6)
Clinical pregnancies/eSET, *N* (%)	597 (62.0)*	45 (60.8)
Ongoing pregnancies/eSET, *N* (%)	535 (55.6)*	38 (51.4)

*$p > 0.05$
[a]Means ± SEM

Table 19.3 Retrospective outcome data from 333 autologous vitrified–warmed embryo transfers after failed fresh elective single-embryo transfers compared with 1,037 fresh day 5 eSETs

	FET after failed fresh eSET	Fresh eSET
Patients, *N*	333	1,037
Patients' age, years	32.1 ± 3.1[a]	31.8 ± 3.3[a]
Blastocysts warmed, *N*	594	–
Patients taking ONE embryo, *N* (%)	76 (23)	–
Patients taking TWO embryos, *N* (%)	253 (76)	–
Patients taking THREE embryos, *N* (%)	4 (1)	–
Implantations, *N* (%)	278 (46.8)	
Patients with single implantation, *N* (%)	134 (65.0)	
Patients with twin implantation, *N* (%)	73 (35)**	17 (2.6)
Positive pregnancies/eSET, *N* (%)	234 (70.3)*	717 (69.1)
Clinical pregnancies/eSET, *N* (%)	207 (62.2)*	642 (62.0)
Ongoing pregnancies/eSET, *N* (%)	192 (57.7)*	579 (55.3)
Cumulative ongoing pregnancy rate/oocyte retrieval, *N* (%)	192 + 579 = 771/1,037 = 74.3 %	
Added value of cryopreserving embryos (%)	74.3 − 55.8 = 18.5	

FET frozen embryo transfer
*$p > 0.05$; **$p < 0.001$
[a]Means ± SEM

(30.1 %) faced a negative pregnancy test, 75 patients achieved a biochemical or ectopic pregnancy only, and additional 69 patients lost their ongoing pregnancy beyond 7 weeks. To reflect the true augmenting effect of vitrification, only the first transfer of vitrified embryos occurring after an unsuccessful fresh eSET was analyzed. 333 patients returned for a frozen embryo transfer; 70.3 % achieved a positive pregnancy, with a clinical and ongoing pregnancy rate of 62.2 % and 57.7 %, respectively (see Table 19.3).

Because more than 70 % of the 333 patients subsequently elected to undergo a two-embryo transfer, the occurrence of twins increased to 35 %. However, combining the ongoing pregnancy rate from the fresh eSET (579) and the first frozen transfers (192) provides the cumulative expectation of a pregnancy with embryos from the same oocyte retrieval of 74.3 % (771:1,037). As shown in Table 19.3, the added value of cryopreservation is 18.5 % (74.3–55.8). At FCI, extended culture generates high pregnancy and implantation rates, even when we are transferring just one embryo instead of two. After 1,037 eSETs at our center, we did not observe a decrease in the overall ongoing pregnancy rate in our program, although a dramatic reduction of twins was observed (from 48 % in 980 fresh transfers in patients age <35 years with two blastocysts versus to <3 % with fresh eSET) and a complete disappearance of any high-order multiple pregnancies (see Tables 19.1 and 19.3).

Discussion

These data show that successful implementation of eSETs for clinical application can be achieved. Patient education concerning the risk of multiple gestations is important, as is the acceptance of eSET among physicians and embryologists [19]. In many ways, the success of an IVF unit's eSET program is contingent on having suitable cryopreservation skills available in the laboratory. Taken together, attitude, acceptance, and equipment are therefore essential ingredients to implement eSET successfully and to effectively reduce the rate of multiple gestations associated with IVF. Implementation of ESET at our institution has been shown to be a valuable tool, not merely to maximize but rather to *optimize* pregnancy rates. Initiating a patient education program is a top priority to establish a successful eSET program.

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Conflict of Interest The author discloses no conflict of interest.

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Chapter 20
A Review of Luteal Support Protocols for Single Embryo Transfers: Fresh and Frozen

Conor Harrity, Denis A. Vaughan, and David J. Walsh

Introduction

The Normal Luteal Phase

The luteal phase is the period between ovulation and the onset of menstruation or establishment of a pregnancy [1]. During this part of a normal menstrual cycle, there is episodic production and secretion of estradiol and progesterone from the corpus luteum, dependent on continued tonic secretion of luteinizing hormone (LH) from the anterior pituitary [2]. Progesterone concentration typically rises sharply post-ovulation, reaching a peak at around 6–8 days following the mid-cycle LH surge and then falling several days before menstruation [2, 3]. Pulsatile secretion of estrogen and progesterone occurs every 1–4 h, with maximal progesterone secretion being much higher than estradiol, at around 25 mg/day compared to 0.6 mg [2, 3]. The role of estradiol is well known in the follicular phase of the cycle, but controversy persists regarding its function in the luteal phase. Estrogens play a vital role in uterine preparation for embryo implantation by stimulating endometrial proliferation and improving both uterine and endometrial perfusion [4–8]. Progesterone induces the endometrium to undergo secretory change, improving endometrial receptivity.

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The development of a pregnancy leads to human chorionic gonadotrophin production by the syncytiotrophoblast cells and continued stimulation of the corpus luteum, which maintains ovarian progesterone secretion. Then a change from ovarian to placental steroid placental production takes place over a period of several weeks [3]. Placental progesterone production has been detected as early as 36 days following embryo transfer, and the luteoplacental shift occurs around the seventh gestational week [9].

The Luteal Phase in Assisted Reproduction

ART cycles are associated with a luteal-phase defect [10]. The low luteal-phase LH levels during ART cycles may not be sufficient to induce the endometrial maturation required for implantation and support of pregnancy [11]. The use of oral contraceptive pills can prevent corpus luteal function and may adversely affect ovarian responsiveness [12, 13]. Women with diminished ovarian reserve appear to be particularly susceptible to the suppressive effects of these agents, potentially leading to a poor stimulation response and lower oocyte yield [14]. During stimulated IVF cycles the process of controlled ovarian hyperstimulation combined with GnRH agonist or antagonist therapy leads to several changes in the luteal phase. GnRH agonist therapy has been shown to suppress endogenous LH production for up to 10 days following discontinuation of the GnRH analogue [11]. In contrast, release of gonadotrophins from the pituitary recovers rapidly following discontinuation of the GnRH antagonists [15]. It was initially thought that antagonist protocol cycles would lead to less disruption of the luteal phase, but early studies demonstrated a significant reduction in pregnancy rates in IVF cycles without LPS [16, 17]. It has also been suggested that vigorous or repeated follicular aspiration during oocyte retrieval could lead to disruption of granulosa cells [18]. Exogenous gonadotropin therapy leads to multiple follicular development and formation of additional corpora lutea. The supraphysiological steroid concentrations found during the early luteal phase of ART cycles resulting from extra corpora lutea directly inhibit pituitary LH release by negative feedback, rather than a central hypothalamic–pituitary cause or an intrinsic steroid production problem in the corpus luteum [19]. Premature luteolysis due to LH deficiency secondary to high steroid levels is currently thought to be the main cause of luteal-phase defects associated with ART [20]. Estrogen and progesterone levels have also been shown to decline more rapidly following ART than in natural cycles, which is due to a shorter duration of ovarian steroid production following gonadotropin stimulation [3].

In patients at high risk of ovarian hyperstimulation syndrome (OHSS) there has been a trend to reduce the hCG trigger dose from 10,000 iu to 5,000–6,500 iu, which could exacerbate the mid-luteal phase deficiency of LH/hCG [21]. A shortage of mid-luteal LH/hCG changes the progesterone profile, with the highest concentration being found early in the luteal phase, rather than the typical mid-luteal peak found during a normal menstrual cycle [21]. A review by Tsoumpou et al. [22]

identified that most studies reported similar outcomes in women receiving either 10,000 iu or 5,000 iu of hCG for follicular maturation trigger. It has been suggested that a minimum mid-luteal progesterone threshold of around 80–100 nmol/l (25.2–31.4 ng/ml) should be reached, and levels above this range lead to a reduction in miscarriage and increase in live birth rate [21]. Brady et al. [23] looked at serum progesterone levels on the day of transfer in cycles using day 3 embryos derived from donor oocytes and identified that patients with progesterone levels <20 ng/ml (<63.6 nmol/l) had lower clinical pregnancy rates than those above this level (RR 0.75, 0.60–0.98). The authors reported that increasing the progesterone dose following transfer in patients with lower serum levels did not lead to an increase in pregnancy rates and that obese patients could need a higher starting progesterone dose for luteal support [23]. An early rise in progesterone levels, prior to administration of hCG trigger, has been linked to premature luteinization and also reduced pregnancy rates. There is marked variation in reported cutoff levels, ranging from 0.8 to 2 ng/ml (2.5–6.4 nmol/l) [24]. This theory has been controversial, and an early meta-analysis by Venetis et al. [25] did not detect a significant association between the probability of pregnancy and elevated serum progesterone levels. A follow-up analysis by Venetis et al. [26] identified 63 studies eligible for inclusion and concluded that the probability of pregnancy was reduced when progesterone on the day of trigger exceeded a threshold of 0.8 ng/ml (2.54 nmol/l) in fresh cycles, but that it did not appear to have any effect on frozen transfer or donor recipient cycles [26]. The potential to consider elective cryopreservation of all embryos and delayed transfer in a frozen–thawed cycle has been suggested as a possible treatment option when there is early progesterone elevation in stimulated cycles [24].

Luteal Support Regimes

Background

There is no consensus regarding the optimal LPS strategy in ART cycles. The optimal route of administration and total duration of use have been subject to extensive research. Progesterone-based protocols are the most frequently adopted, while alternative regimens including human chorionic gonadotropin (hCG) and GnRH agonists (GnRH-a) remain controversial [1]. At our institution, vaginal progesterone is generally considered the first-line therapy for LPS. The starting time and duration of luteal-phase supplementation after the onset of pregnancy are still debated [1]. A global Internet-based survey by Vaisbuch et al. [27] assessed current trends for luteal support prescriptions in ART cycles by obtaining data from 408 fertility centers in 82 countries, accounting for 284,600 annual IVF cycles. They found that LPS luteal are commenced on day of oocyte retrieval in 80 % of cycles and that vaginal progesterone is used in 94 % of cases (as a solitary progestogen in 77 %, and combined with intramuscular in 17 %) [27]. These data contrasted sharply with a previous Internet survey by the same author nearly 3 years earlier, which

assessed 84 centers in 35 countries [28]. The older survey identified that vaginal progesterone was used in 80 % of cases, as a single agent in 64 %, combined with injectable in 15 %, and along with oral in 1 % [28]. In the first survey, hCG alone was used for LPS in 5 % of cycles, but there were no reported cases of single agent hCG for LPS in the more recent publication.

Duration of Luteal Support

The optimal duration of luteal support following a stimulated ART cycle is controversial. Protocols can vary from 2 to 12 weeks of progesterone use. Once pregnancy-associated hCG production leads to a rise in endogenous estradiol and progesterone, it has been suggested that there is no need to continue pharmacologic supplementation beyond this point [29]. Schmidt et al. [30] reported no difference in ongoing pregnancy rates between patients receiving vaginal progesterone just until a positive hCG result was detected, or those receiving an additional 3 weeks of therapy; however, this early analysis lacked randomization. A more recent meta-analysis by Liu et al. [31] identified six RCTs with 1,201 patients and found no difference in miscarriage, ongoing pregnancy, or live birth rates between early cessation or prolonged duration of progesterone for LPS. The authors suggested that further large RCTs are needed to validate this conclusion. In order to provide contemporary evidence to answer this question, a UK multicenter prospective RCT has been proposed to compare 2 weeks of progesterone after embryo transfer, versus a further 8 weeks of vaginal progesterone treatment [32]. Despite the lack of convincing evidence of benefit, there is still a global trend for prolonged progesterone LPS. Vaisbuch et al. [27] reported that progesterone LPS was continued until a positive fetal heart beat was seen on ultrasound in 22 % of fresh cycles, until 10–12 weeks gestation in a further 67 % of cases, and that it was only discontinued when a hCG test was positive in 12 % of fresh cycles.

Progesterone Therapy in the Luteal Phase

Types of Progesterone

Exogenous progesterone therapy is the main ingredient used for LPS in ART cycles, and it is incorporated into the vast majority of protocols either alone or in combination with other agents. Progesterone leads to a secretory transformation in the endometrium, increases stromal density, promotes local vasodilatation, and prepares the endometrium for implantation [33, 34]. These changes improve endometrial receptivity if administered after estrogen priming [35]. Available progesterone

preparations include vaginal, intramuscular, rectal, and oral options, with parenteral treatments bypassing first-pass hepatic metabolism. Natural progesterone has been traditionally been preferred to synthetic derivatives as it does not decrease high-density lipoprotein levels and lacks potential androgenic or teratogenic side effects [15, 36].

Vaginal Progesterone

Vaginal progesterone administration permits high progesterone concentrations within the endometrial compartment along with low peripheral serum levels due to the uterine first-pass effect, avoiding initial liver metabolism [37, 38]. The vaginal route has certain benefits for patients, including ease of administration, high acceptability, and a low incidence of allergic reactions [3]. Various vaginal preparations have been used, including micronized tablets, bioadhesive gel, gelatin capsules, pessaries, and suppositories.

Crinone® 8 % (Merk Serono) is a bioadhesive vaginal gel which contains 90 mg of micronized progesterone in an oil and water emulsion containing a polycarbophil inert base which adheres to the vaginal mucosa [39]. The advantages of this preparation are a controlled and sustained delivery, low variability in absorption, and a longer half-life than other vaginal therapies. A potential disadvantage of Crinone is that the polycarbophil base is not absorbed and can accumulate causing an unpleasant discharge in some patients [3]. Endometrin® (Ferring), or Lutinus® in Europe, is a micronized natural progesterone effervescing tablet. This preparation adsorbs vaginal secretions and disintegrates into an adhesive powder, facilitating absorption via the vaginal epithelium [40]. Lewin et al. [41] reported sufficient endometrial development with a dose of 100 mg twice daily. A 2012 prospective multicenter RCT involving 2,057 patients compared vaginal gel with vaginal micronized tablets and found no substantial difference in pregnancy rates or live birth rates between the groups [42]. The gel preparation was found to be significantly better in terms of convenience and ease of use [42].

Progesterone suppositories, such as Cyclogest® (LD Collins), contain semi-synthetic glycerides which are made from the interesterification of hydrogenated vegetable oil [15]. A prospective RCT comparing vaginal and rectal administration of progesterone pessaries (Cyclogest 400 mg BD for up to 8 weeks) in fresh antagonist protocol cycles found no substantial difference in serum progesterone concentrations or clinical pregnancy rates between the groups [43]. In terms of side effects associated with pessary use, the vaginal route was associated with more perineal irritation (21.3 % vs. 2.2 %), and rectal administration led to a greater incidence of tenesmus (35.1 % vs. 21.1 %) and rectal itching (26.7 % vs. 2.8 %) [43]. Yu et al. [44] reported no difference in perineal irritation between vaginal micronized tablets or suppositories; however, it was found that tablets were more difficult to administer.

Intramuscular Progesterone

Intramuscular micronized natural progesterone is formulated with ethyl oleate or sesame oil (50 mg/ml) and benzyl alcohol (10 %) to act as a preservative [15]. Preparations of progesterone in oil lead to higher peak plasma concentrations, of longer duration, than aqueous solutions [15]. Potential disadvantages of the intramuscular route include pain at the injection site, difficulty in self-administering i.m. injections, the long half-life of the oil in muscle, and the need for daily administration due to rapid metabolism [3, 15]. Numerous studies have compared vaginal and injectable progesterone to assess their efficacy with no definitive overall conclusion being made. The initial Cochrane review on luteal support in ART [45] suggested a benefit from intramuscular progesterone compared to vaginal administration in terms of ongoing pregnancy and live birth rates. However, this opinion was modified in the updated review [20] which identified four RCTs comparing i.m. progesterone with vaginal or rectal administration, and reported no difference between the groups [20]. Zarutskie et al. [46] also revisited a previous review which had suggested benefit associated with intramuscular administration. Nine RCTs from 1992 to 2008 were suitable for inclusion in a meta-analysis to compare i.m. and vaginal progesterone, with no difference in clinical pregnancy rate or ongoing pregnancy rate between the groups [46]. Three of the trials included in the Cochrane analysis were also in the study by Zarutskie et al. A 2012 RCT by Silverberg et al. [47] reported significantly higher pregnancy rates in patients aged <35 years using vaginal progesterone gel than intramuscular administration, but equal efficacy in older patients. A more recent prospective RCT by Miller et al. [48] also reported no difference in pregnancy rates between vaginal or i.m. progesterone use.

There are fewer publications comparing these preparations in frozen embryo transfer (FET) cycles than fresh stimulated transfers. The absence of corpora lutea and associated endogenous steroid production in medicated frozen cycles means that it may not be appropriate to extrapolate results from stimulated treatments to FET cases [49]. One retrospective study [50] looking at FET cycles of day 3 cleavage stage embryos suggested lower clinical pregnancy rates with vaginal than i.m. progesterone (36.9 % vs. 51.1 %; $p < 0.001$). The study authors hypothesized that in hormonally medicated FET cycles, with minimal endogenous progestrone production compared to stimulated cycles, the pharmacokinetics and pharmacodynamics of intramuscular administration may be beneficial [50]. There is evidence that higher maximum serum progesterone concentrations are found in patients receiving i.m. progesterone, but higher endometrial tissue levels are obtained with vaginal administration [49, 51]. Higher endometrial progesterone levels may lead to early luteinization and altered glandular development; however, studies comparing mid-luteal endometrial histology between i.m. and vaginal use have shown contrasting results [34, 50, 52]. The longer half-life associated with intramuscular administration results in a continuous progesterone release compared to the intermittent peaks associated with vaginal use [49]. Feinberg et al. [53] performed a retrospective analysis comparing vaginal tablet monotherapy with combined vaginal tablet and

i.m. progesterone. There was no difference in pregnancy or live birth rates during fresh cycles, but a significant improvement in FET cycle outcomes after the addition of injectable progesterone.

A recent large retrospective analysis has suggested no difference in outcome in frozen cycles. Shapiro et al. [54] compared i.m. progesterone and vaginal gel in FET cycles leading to blastocyst transfer and reported no difference in implantation, clinical pregnancy, or live birth rates. Further prospective RCTs to assess the role of intramuscular progesterone for luteal support in FET cycles would be beneficial in order to provide additional information to clinicians.

An alternative to injectable micronized natural progesterone is 17-α-hydroxyprogesterone caproate (17-HPC), an ester derivative of 17-α-hydroxyprogesterone produced from caproic acid. This preparation has the advantage of twice weekly administration rather than daily use [55]. A 2001 RCT found no difference in pregnancy rates between injectable 17-HPC and progesterone in oil [55]. Satir et al. [56] compared intramuscular 17-HPC with vaginal progesterone and found a tendency to higher clinical pregnancy rates with vaginal administration (OR 1.85, 1.28–3.03), but no significant difference in ongoing pregnancy rates ($p=0.14$). Contrasting results were reported by Unfer et al. [57] who found better outcomes with 17-HPC than vaginal gel [57]. As most studies on i.m. administration have used natural progesterone, more data is needed to provide evidence on 17-HPC, which has been more widely studied as a preventive measure for preterm labor.

Oral Progesterone

A major disadvantage of oral progesterone therapy is the breakdown into 5α and 5β metabolites by the first-pass hepatic metabolism [58]. This leads to reduced serum concentrations and low bioavailability [40]. Only around 10 % of the administered oral dose circulates as active progesterone [59]. Maximum serum progesterone concentrations are lower following 100 mg of oral micronized progesterone than 90 mg of vaginal gel [60]. Oral dosing regimes therefore need to be modified to compensate for this and may be associated with sedative side effects due to inhibition of the $GABA_A$ receptor complex [15, 61]. Studies have suggested lower pregnancy and implantation rates with oral progesterone compared to vaginal or intramuscular preparations, so the oral route is not typically employed as a first-line therapy. The 2011 Cochrane review did not identify any difference in live birth rates associated with oral progesterone for LPS, but the meta-analysis was limited by a small number of included studies, with only one RCT comparing oral and intramuscular administration, and two for oral versus vaginal [20].

Oral dydrogesterone has been proposed as a synthetic alternative to micronized natural progesterone; it is has good oral bioavailability and few side effects [62]. Compared to other progestogens, dydrogesterone has no androgenic, estrogenic, or glucocorticoid activity and is 10–20 times more potent than natural progesterone [63]. An additional benefit is the lack of androgenic effects on the fetus [63].

An RCT by Chakravarty et al. [64] reported no difference in pregnancy rates between 10 mg b.i.d. oral dydrogesterone and 200 mg t.i.d. of vaginal progesterone, but significantly higher patient satisfaction and tolerability with dydrogesterone. A 2011 RCT involving 1,373 cycles identified similar pregnancy and miscarriage rates with oral dydrogesterone and vaginal micronized progesterone gel and tablets [65]. Similarly a 2013 prospective RCT identified no significant difference in clinical pregnancy rates or miscarriage rates between oral dydrogesterone and vaginal progesterone [66].

Luteal Estrogen Therapy in Stimulated Cycles

While progesterone has been widely accepted as the preferred method of LPS in ART, the incorporation of E_2 into a luteal-phase support protocols during stimulated IVF/ICSI cycles has been the subject of much research and debate. Controlled ovarian hyperstimulation results in supraphysiological estradiol levels, and the consequences of this are controversial. It has been hypothesized that lower serum E_2 levels represent reduced production of E_2 by the growing follicles, which then luteinize poorly after exposure to hCG. Oocytes derived from such follicles have, in turn, a poor fertilization rate [67]. Both low and extremely high levels of estradiol have been suggested to be negative predictors of clinical pregnancy rates in IVF [67–71]. Simon et al. [71] suggested that high E_2 levels are responsible for impaired endometrial receptivity without affecting embryo quality in oocyte donation cycles. Mitwally et al. [72] identified a positive correlation between E_2 and pregnancy rates up to a certain point, after which a negative correlation was found. This suggested adequate estrogenization of the uterus is necessary in preparation for embryo implantation, but a detrimental effect may occur once a threshold is exceeded. A 2013 prospective RCT evaluated the role of estrogen supplementation to LPS using vaginal progesterone and found no difference in clinical pregnancy rate between the groups, but a reduced incidence of luteal vaginal bleeding ($p=0.01$) if estrogen and progesterone were combined [73]. Kutlusoy et al. [74] performed a prospectively randomized study comparing the addition of either 2 mg or 6 mg estradiol hemihydrate to vaginal progesterone LPS regime. They found a significantly higher CPR with a combination of 2 mg estradiol and progesterone than compared to progesterone alone, and no difference between the 2 mg or 6 mg dose of estradiol. The study was limited by small numbers, with 27–35 patients per arm. An earlier prospective case–control study reported no difference in pregnancy rates between vaginal progesterone LPS and a combined protocol using progesterone with 2 mg oral estradiol [75]. A larger 2013 prospective RCT involving 402 patients found no significant difference in CPR if 6 mg estradiol was added to i.m. progestrone for LPS [76]. A Cochrane review compared live birth rate, clinical pregnancy rate, ongoing pregnancy rate, miscarriage rate, and risk of OHSS in studies using either progesterone alone or progesterone and estrogen for LPS. One study [77] compared live birth rates and did not find

a significant difference between groups. Seven studies compared clinical pregnancy rates, and no significant difference was found [20]. Five studies assessed ongoing pregnancy rates, and a meta-analysis showed no significant difference in outcome [20]. Similarly, with regard to both miscarriage rates and incidence of OHSS, there was no difference based on the addition of estrogen [20].

Luteal-Phase hCG

Human chorionic gonadotropin (hCG) has been used as a surrogate for the mid-cycle LH surge to induce final follicular and oocyte maturation during assisted reproduction therapy cycles for many years [21, 78]. The hCG trigger has a dual role, combining both ovulation induction and also early luteal-phase stimulation of the corpora lutea [21]. hCG has an indirect role in luteal support, increasing estradiol and progesterone levels, and leads to rescue of the failing corpora lutea [10]. hCG administration has also been shown to increase the concentrations of relaxin and integrin αν [79]. hCG was commonly used for luteal support in the early days of ART [46], but one concern with use of hCG for LPS was the possibility of increasing the incidence of OHSS [10]. Contrasting information exists on the benefits of luteal-phase hCG use. A 2002 meta-analysis by Pritts et al. [80] identified no difference in clinical pregnancy rates between hCG and progesterone. A further meta-analysis from 2005 by Nosarka et al. [81] found HCG to be more effective than progesterone regarding clinical pregnancy rate (OR 1.71, 1.06–2.76). A prospective RCT by Ludwig et al. [82] identified no difference in clinical ongoing pregnancy rate between luteal support using three doses of hCG, single dose of hCG on the day of ET combined with vaginal progesterone, or vaginal progesterone alone. Mochtar et al. [83] found that the combination of vaginal progesterone and hCG as opposed to a progesterone-only LPS resulted in significantly higher estradiol and progesterone levels and that higher estradiol concentrations appeared to have a detrimental effect on implantation, leading to lower pregnancy rates. The Cochrane review concluded that hCG did not have any effect on clinical pregnancy rate, or live birth rate compared to placebo, but there was a significant improvement in the ongoing pregnancy rate compared to placebo or no treatment [20]. Moreover, when comparing hCG with progesterone for LPS, the Cochrane group concluded that there was no difference in clinical pregnancy rate, ongoing pregnancy, or live birth rate, but a significant increase in OHSS with the use of hCG [20]. A subgroup analysis was performed comparing hCG alone vs. combined hCG and Progesterone for LPS, which identified four suitable RCTs, and no difference in clinical pregnancy rate was noted [20]. There are limited data to assess the effect of supplemental hCG for LPS in FET cycles. Lee et al. [84] performed a retrospective analysis to evaluate any effect of hCG in this setting. The group identified that the addition of two doses of 1,500 iu hCG, the first on the day of transfer and a second 6 days later, had no effect on clinical pregnancy rate or miscarriage rate [84].

GnRH Agonists and the Luteal Phase

GnRH Agonist Triggering

The use of a gonadotropin-releasing hormone agonist (GnRH-a) for final follicular maturation instead of hCG separates the triggering ovulatory signal from the initiation of early luteal-phase support [21]. The GnRH-a flare-up effect produces a short-lasting endogenous LH surge which induces oocyte maturation, but the limited duration has a luteolytic effect, allowing any corpora lutea to regress. This strategy prevents secretion of vasoactive substances such as vascular endothelial growth factor (VEGF), which has been implicated as a major mediator of OHSS [78, 85–87]. Although this approach is effective in reducing early-onset OHSS, initial studies indicated that patients who continued to fresh embryo transfer had inferior clinical pregnancy rates and live birth rates when compared to transfers following the standard hCG trigger [88, 89]. Further research demonstrated that any difference in pregnancy rates disappeared following cryopreservation of embryos and delaying transfer until the following cycle [88, 90, 91]. The unacceptably poor pregnancy rates following transfer after agonist trigger were thought to be the result of a defective luteal phase having a detrimental effect on the endometrium and implantation, which could not be overcome by standard progesterone-based LPS protocols [88, 92]. A segmentation strategy was proposed to avoid the risk of developing OHSS after stimulation, where an antagonist protocol combined with a GnRH agonist maturation trigger is followed by elective cryopreservation and delayed FET [93]. This strategy aims to eliminate OHSS by avoiding either endogenous or exogenous hCG during the initial treatment [93]. There is limited evidence that severe OHSS can still follow this segmented approach using an elective freezing of all embryos without hCG administration, thus demonstrating that although this technique leads to a massive reduction in the incidence of OHSS, it does not completely eliminate the complication [78].

LPS Following Agonist Trigger

Although initial studies demonstrated very poor pregnancy rates following GnRH-a trigger, more recent publications have shown that intensive luteal support protocols can compensate for this. The addition of hCG to the LPS regime following oocyte retrieval in agonist-triggered cycles has the potential to maintain the function of the corpus luteum and enable a fresh embryo transfer [94]. Fatemi et al. [95] demonstrated that early luteal-phase steroid levels were similar following a 10,000 iu hCG trigger with routine LPS and an agonist trigger (triptorelin 0.25 mg) followed by delayed administration of 1,500 iu hCG 35 h later and standard LPS. Many studies have been performed to assess how modified LPS treatment following GnRH-a trigger affects pregnancy rates. Radesic et al. [85] performed a retrospective analysis of patients at high risk of OHSS where oocyte maturation was triggered using an

agonist, and a 1,500 iu dose of HCG was given on the day of oocyte retrieval to support the corpora lutea and enable a fresh transfer. An ongoing clinical pregnancy rate of 51 % per embryo transfer was identified, along with a 1.4 % incidence of severe OHSS. A similar 2013 multicenter retrospective study including 275 women at high OHSS risk also assessed outcomes following fresh transfer after agonist trigger and intensive LPS involving a single administration of 1,500 iu hCG on the day of oocyte retrieval, vaginal progesterone, and oral estradiol. The study group reported a clinical pregnancy rate of 41.8 % per cycle started using this technique and a low incidence of severe OHSS at 0.72 % [94]. Haas et al. [96] assessed the effects of delaying a 1,500 iu hCG bolus until 3 days after oocyte retrieval. This proof-of-concept study was limited by very small numbers, and the control group had intensive luteal support without any hCG administration, but the authors found that the patients receiving hCG day 3 post-OR had higher mid-luteal progesterone levels (127 nmol/l vs. 42.1 nmol/l) and a higher pregnancy rate (40 % vs. 16 %); however, the study did not have enough power to determine if this difference was statistically significant.

Iliodromiti et al. [97] performed a retrospective cohort study to assess if there was a difference in live birth rate between agonist trigger and intensive LPS compared to standard triggering with hCG and conventional LPS in patients considered at high risk for OHSS. There was no difference in live birth rate between the groups (29.8 % vs. 29.2 %; $p=0.69$); however, there were fewer cases of OHSS following GnRH-a trigger (0.3 %) compared to hCG (7.0 %). Although this trial was not randomized, the low incidence of OHSS following delayed hCG administration in patients considered high risk is very reassuring. Studies have been performed to assess the benefits of repeated hCG injections. Kol et al. [98] used a two-dose hCG regime, 1,500 iu on the day of OR and 1,500 iu 4 days later, with no estrogen or progesterone for LPS after GnRH-a trigger in normal responder patients with at least one prior failed cycle using a hCG trigger. Patient numbers in the study were low, but the authors reported 11 positive pregnancy tests following 15 embryo transfers and 7 ongoing pregnancies (46.7 %) [98].

A French study compared intensive luteal support following antagonist trigger using two doses of hCG (1,500 iu on day of OR and 1,500 iu 5 days later) combined with 400 mg progesterone vaginally, versus a single dose of hCG (1,500 iu on day of OR) with 600 mg vaginal progesterone and 4 mg oral estradiol [99]. Ongoing pregnancy rates were the same in both groups; however, 7.7 % patients given two hCG doses developed late OHSS and only 1.5 % receiving a single dose developed early OHSS (and no late OHSS) [99]. This study suggests that a second hCG dose could be harmful, as it increases OHSS without improving pregnancy rates, but further data are needed to confirm this.

In order to proceed directly with a fresh transfer rather than pursue a segmented approach, particularly for SET clear evidence is needed that OHSS risk remains low and pregnancy rates are not compromised. A 2012 observational cohort study compared fresh and segmented transfers in patients considered to be high risk for OHSS, triggered by GnRH-a during antagonist protocol cycles [100]. Live birth rates were 27.1 % after fresh transfer and 20.0 % with FET. The difference was not significant ($p=0.4$). Although this data is reassuring, further randomized trials are needed to provide stronger support for this strategy.

GnRH Agonists as an Adjunct to the Luteal Phase

GnRH agonists have also been proposed as a beneficial LPS adjunct, but the presumed mechanism of action is unclear [101]. Potential effects could include stimulation of pituitary LH secretion supporting the corpus luteum, action on local GnRH receptors in the endometrium, or direct action on the embryo [102]. Luteal-phase administration of a GnRH agonist (0.1 mg triptorelin) 6 days after oocyte retrieval has been shown to increase luteal-phase estradiol, hCG, and progesterone concentrations in both long agonist and antagonist stimulation protocols [103]. Kyrou et al. [104] performed a meta-analysis of six RCTs comparing patients where a GnRH-a was added to the LPS regime to those without, and significantly higher live birth rates were reported in patients using GnRH-a. A 2012 prospective RCT involved 426 patients undergoing a long GnRH agonist protocol, with cases randomized to either progesterone only LPS or progesterone combined with three daily 1 mg doses of luprolide 6 days after OR [101]. In this study, no differences in implantation, clinical pregnancy, or ongoing pregnancy rates were detected [101]. The 2011 Cochrane review identified significantly higher clinical pregnancy and live birth rates when progesterone and GnRH agonists were used together, compared to progesterone alone [20]. More studies are therefore needed to assess the effects of this adjunct before general recommendations can be made.

Considerations for Frozen Embryo Transfer

FET Cycle Options

In contrast to the complex stimulation protocols employed to stimulate follicular growth for IVF and fresh transfer, FET protocols are much simpler and have the primary aim to adequately prepare the endometrium to receive the thawed embryo. Natural cycle FET (NC-FET) is the simplest form, in which the endocrine preparation of the endometrium is achieved by endogenous sex steroid production from a developing follicle. The timing of transfer is determined by detection of the spontaneous LH surge. An alternative approach is to administer hCG to initiate luteinization. Pregnancy rates in NC-FET are heavily dependent on the timing of ovulation and the subsequent period of endometrial receptivity [105, 106]. Thawing and transfer should occur during this period. LH monitoring can be problematic in NC-FET cycles, and it is recommended that levels are checked once to twice daily, typically using urine LH kits. There is significant variation between urinary test kits with high false-negative rates reported, as well as complaints from patients that they can be difficult to interpret [107]. The detection of the LH surge in urine tends to lag behind the serum LH surge by about 21 h, reducing the optimal timing for embryo thaw and transfer [108]. Because of these difficulties, hCG triggering of ovulation can be performed once a dominant follicle of sufficient size has been identified on ultrasound.

In artificial cycle FET (AC-FET), estrogen and progesterone are administered to mimic the endocrine exposure of the endometrium in a normal menstrual cycle. Estradiol is initially administered to cause endometrial proliferation, but this also suppresses development of the dominant follicle. Endometrial proliferation is monitored by ultrasound, and once the Endometrial thickness is 7–9 mm, then progesterone is added to initiate secretory change [109]. This regime replicates the physiological mid-cycle shift from estrogen to progesterone [110]. The timing of initiating progesterone supplementation defines when embryo thawing and transfer occurs. Since estrogen alone does not guarantee complete downregulation in hormonal artificial FET cycles, it has been suggested that luteinization may occur in around 5 % of cases [111]. To counter this, some authors have suggested addition of a GnRH-a to further suppress development of the dominant follicle.

Artificial vs. Natural FET Cycles

There is a shortage of evidence comparing natural and medicated FET treatments. An early study by Tanos et al. [112] showed no significant difference in clinical pregnancy or live birth rate depending on the method of endometrial preparation. A Cochrane review in 2008 [113] reported no difference in clinical pregnancy rate between natural and artificial FET cycles, but only one RCT was included for analysis. A 2013 retrospective cohort study including 2,216 NC-FET and 2,018 AC-FET cycles [114] reported higher implantation and pregnancy rates in the AC group (29.3 % vs. 21.5 %, and 48.7 % vs. 42.7 %, respectively; $p=0.01$ for both). Groenewoud et al. [115] combined eight retrospective studies with the Cattoli RCT to perform a meta-analysis including 8,152 FET cycles. The results demonstrated no difference in clinical pregnancy rates between natural or artificial cycles. ANTARTICA, a randomized multicenter non-inferiority trial, has been design to compare NC and AC FET with regard to live birth and clinical pregnancy rates, but it will also assess cycle cancelation rates and cost-efficiency [116]. Based on the current published literature, it is not possible to recommend one endometrial preparation method in FET cycles over another.

Modified Natural FET Cycles

Some protocols have suggested modifying natural FET cycles with the addition of progesterone for luteal support as a way to improve outcomes. Bjuresten et al. [117] prospectively evaluated live birth rates in NC-FET compared to modified natural cycles using additional luteal vaginal progesterone (400 mcg twice daily) from the day of transfer. The live birth rate was higher among those who received vaginal progesterone (0.3 vs. 0.2, $p=0.0272$). In contrast, a retrospective analysis by Kyrou et al. [118] reported no benefit from progesterone LPS in patients undergoing

modified NC-FET. A prospective RCT was recently completed to assess the role of supplemental i.m. luteal-phase progesterone in NC-FET [119] and found no difference in clinical pregnancy rate between natural and modified natural cycles ($p=0.66$). The meta-analysis by Groenewoud et al. [115] identified two RCTS and three retrospective studies comparing NC-FET suitable for inclusion, accounting for 1,965 frozen cycles. The pooled results identified no significant difference in pregnancy rates between natural and modified natural treatments with luteal-phase progesterone [115]. Certainly for single embryo transfer, there is limited available evidence on the subject of adding supplementary progesterone for luteal-phase support in NC-FET, and this should be a subject of further research before recommendations can be made to support this strategy.

Conclusion

Luteal-phase support is an essential component of assisted reproductive therapy, and a thorough understanding of previous relevant research can help optimize reproductive outcomes with SET. With the exception of natural cycle FET protocols, the addition of a progesterone LPS regime results in significantly better outcomes in terms of pregnancy and live birth rates. Current evidence suggests similar efficacy between vaginal and intramuscular progesterone therapy in stimulated cycles, so the choice of regime should be based on clinician and patient preference. Although hCG alone for LPS has been shown to be better than a placebo, available data do not suggest any additional benefit is gained when it is combined with progesterone therapy. The significant increase in incidence of OHSS associated with luteal-phase administration of hCG limits the potential use of this treatment. More studies are needed to assess adjuncts such as oral estradiol or GnRH agonists to LPS regimes before they can be routinely recommended, but promising results have been reported from initial studies using GnRH agonists. Although some studies have reported improved outcomes in FET cycles, more work needs to be done to evaluate this.

Conflict of Interest The authors declare no conflict of interest.

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Chapter 21
Cost-Effectiveness of Single Embryo Transfers Relative to Higher Embryo Transfer Policies in Clinical Practice: A Population-Based Analysis

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Introduction

An in vitro fertilisation (IVF) treatment cycle can lead to a single live birth, multiple births, or, in most instances, no birth at all. Considerable debate surrounds the issue of whether, after how many treatment cycles, and for whom, certain embryo transfer (ET) policies are cost-effective. Although a mandatory single ET (SET) policy may be inappropriate for all patients, an excessive ET policy will lead to a higher

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proportion of multiple births that are born prematurely and carry significant perinatal and neurological risks. This chapter presents a population-based retrospective analysis using nationwide IVF data from across the United Kingdom (UK) from 1 July 1991 to 31 December 1998. This work aimed to test the hypothesis that the cost-effectiveness of any IVF policy depends not only upon maternal age and number of transferred embryos [1, 2], but also upon the number of IVF treatment attempts.

Persuasive movement towards a SET approach in clinical IVF practice is best facilitated by a correct reckoning of the full economic costs associated with the current clinical practice entailing transfer of multiple embryos per cycle. Accordingly, this chapter estimates the cost-effectiveness of each ET policy for clinically relevant subgroups of women undergoing treatment cycles of IVF and captures data during a very specific phase in the life cycle of IVF patients. For this study, our inclusive time horizon begins with the attempts to achieve pregnancy with IVF and concludes at the end of the fifth year of life for the children ultimately delivered following ET. The analysis embraces all hospital costs for the mother during IVF and delivery and for the child to the end of the fifth year of life. Not included are specific costs related to disability should a child suffer from a condition requiring services that are not provided in a hospital setting.

As the current National Institute for Health and Care Excellence (NICE) guidelines suggest a maximum of two embryos transferred per cycle, it is important to know for each subgroup of women (i.e. older vs. younger women; first-time users vs. repeat users) whether an alternative policy such as SET after molecular screening may be more cost-effective. Central to this debate are the questions: (a) what is the cost-effectiveness of each ET policy, and (b) what would be the cost to achieve an additional live birth event for each group of women, if an additional embryo were offered on a given cycle? Hence a traditional incremental cost-effectiveness analysis was constructed to determine whether improved live birth rates and avoided multiples following SET or alternative ET policies, for that matter, justify their additional costs.

Cost-effectiveness planes were constructed from the incremental cost and marginal effect data presented and combined in Figs. 21.1 and 21.2 of this chapter. These graphical representations show the within-cycle incremental cost-effectiveness of moving between three changes to the number of transferred embryos on the final treatment cycle: from SET to 2ET, from SET to 3ET, and from 2ET to 3ET.

Methods

Data on IVF clinics in the UK were provided by the Human Fertilisation & Embryology Authority (HFEA) to calculate the cost-effectiveness for each ET policy (1 embryo, 2 embryos, and 3 embryos) and treatment history category (1 cycle, 2 cycles, ≥3 cycles). Within-cycle cost-effectiveness was calculated for two age groups (<38 vs. ≥38 years) and compared between three embryo transfer shifts: from SET to 2ET, from 2ET to 3ET, and from SET to 3ET. The incremental cost-effectiveness between cycle groups was not compared in the same way, because patients in different cycle categories are assumed to have different levels of baseline fertility.

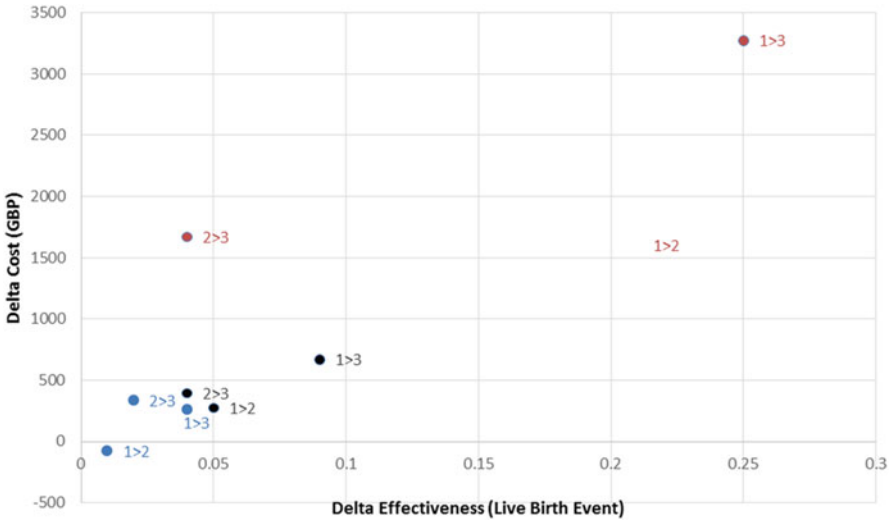

Fig. 21.1 Incremental cost-effectiveness ratios of embryo transfer policies for women <38. *Notes*: Whereas adjustments for inflation would move the ICER ratios upward on the y-axis, the relative difference in the ICERs between alternative embryo transfer policies would likely remain the same. As such, a standard healthcare inflation adjustment (based on published annual healthcare inflation for the UK) can be applied to the final ICER estimates, as well as the "willingness-to-pay' thresholds

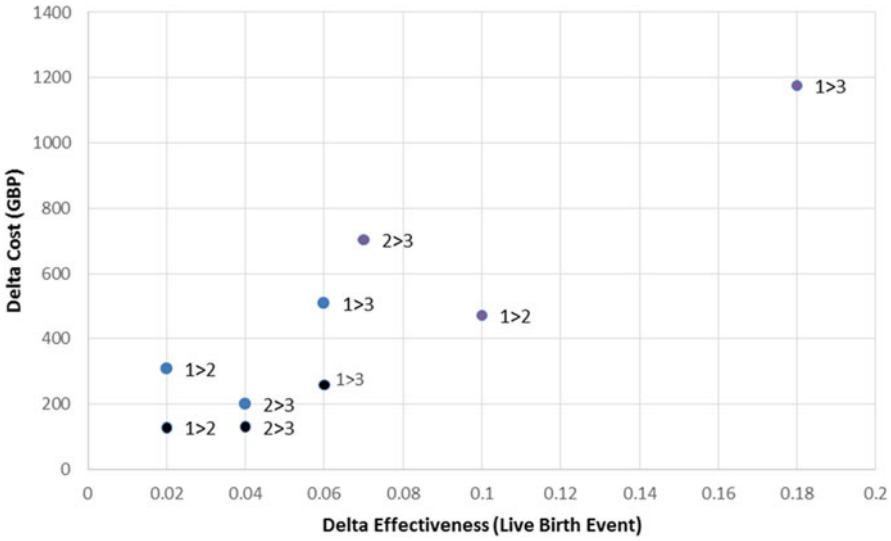

Fig. 21.2 Incremental cost-effectiveness ratios of embryo transfer policies for women 38 years and older. *Notes*: Whereas adjustments for inflation would move the ICER ratios upward on the y-axis, the relative difference in the ICERs between alternative embryo transfer policies would likely remain the same. As such, a standard healthcare inflation adjustment (based on published annual healthcare inflation for the UK) can be applied to the final ICER estimates, as well as the 'willingness-to-pay' thresholds

Study Population

A total of 68 clinics contributed comprehensive data to the HFEA under what can be described as a legislative mandate. The study population comprised all women undergoing at least one IVF treatment cycle with ET in the UK between 1 July 1991 and 31 December 1998 ($n = 174,418$). All IVF treatment cycles (with and without ICSI; fresh and frozen sperm/eggs/embryos; donor and partner's gametes) and outcomes registered during this time were retrospectively reviewed in a non-identifiable, anonymous manner. Cases excluded from this research were all women who received a fertility therapy other than IVF with ET, all women older than 44 years of age at cycle start, and quadruplet deliveries ($n = 4$ sets). We based our calculations on IVF treatments involving only SET, 2ET, or 3ET (higher-order ETs were not tabulated). Accordingly, a total of 74,755 women undergoing 137,307 cycles (79 % of registered cases) met our inclusion criteria. Patients were next stratified by age, number of transferred embryos on their final cycle, and number of treatment cycles.

A health economic evaluation was performed in the form of an incremental cost-effectiveness analysis. Costs included all treatment costs, antenatal costs, and pregnancy and birth costs from parturition to the first 5 years of childhood life (inclusive). Costs were reported according to the period over which the treatments occurred, with subsequent adjustment to 2012–2013 levels using standard healthcare inflationary corrections.

Definitions: Live Birth Rate and Multiple Birth Rate

Our investigation used the standard HFEA definition of a live birth event: a maternity in which the child(ren) survive(s) 27 completed days post-delivery. Because the HFEA dataset does not distinguish between stillbirth and neonatal death, infants who died in utero or who did not survive through 27 competed days post-delivery per pregnancy were not included for analysis.

The live birth rate (LBR) as used in this study includes most cases familiar to clinicians, but the 'average live birth rate per IVF patient' is used here to normalise the live birth rates between increasing cycle categories. This form of LBR is often referred to as the 'take-home' baby rate.

Two forms of measuring the multiple birth rate (MBR) were used: the first is calculated as the MBR divided by the total number of IVF cycles, and the second method is the MBR divided by the total number of live birth events. The former statistic represents the per cycle incidence of multiple births. The latter estimation is more useful, since it represents the proportional incidence of multiple births as a function of all live births.

Sensitivity Analysis

There was uncertainty regarding values of several estimated parameters in our analysis. Sensitivity analysis allows assessment of robustness of conclusions to changes in key parameters by assigning varying ranges to uncertain parameters over realistic ranges and re-evaluating the conclusions for different combinations. This can be accomplished by using a one-way sensitivity analysis, where only one variable is changed at a time. In probabilistic sensitivity analysis, parameter ranges are used to estimate likelihood of cost-effectiveness. In multi-way sensitivity analysis, several variables are changed concurrently. Finally, in the extreme scenario analysis a nominal estimate of cost-effectiveness is determined, and uncertain parameters are varied using their extreme 'maximum' and 'minimum' values. This latter approach was utilised for the present analyses (i.e. low cost/low resource use vs. high cost/high resource use) to estimate the extent to which the conclusions in this chapter may change with maximum and minimum variations in the cost and resource assumptions.

Data organisation and Presentation of Statistical Significance

Our initial analysis was confined to records of IVF patients who completed no more than three treatment cycles because >90 % of the national study population underwent only one, two, or three treatment cycles. All results are reported as exact (or mean) values. Differences in live birth rates and multiple birth rates between subgroups of women were compared by Student's *t*-test, with differences considered significant if two-tailed *p*-values were ≤ 0.05.

Sources of Cost Estimations

For purposes of this analysis, the estimated average cost per IVF cycle is £2,876.26 (±681.63) excluding medications. This estimate was derived from the author's (CAJ) 2003 telephone survey of charges in the 70 UK clinics that provided IVF services [3]. The number of treatment cycles (and thus cycle costs) will change for different patient populations (1 cycle, 2 cycles, and ≥3 cycles).

Average antenatal bed days were estimated by Henderson et al. [4] at 1.09 days (SE = 0.01) for women expecting a singleton child, 8.35 days (SE = 0.51) for those expecting twins, and 32 days (SE = 11.22) for those expecting triplets. A cost per bed day of £277.40 (±41.53) was based on figures provided by the Oxford Radcliffe Hospitals NHS Trust (www.orh.nhs.uk) multiplied by the average number of bed days reported by Henderson et al. [4]. This cost per bed day was compared to a national estimate of £318.93 in the sensitivity analysis. Resulting antenatal costs were calculated at £302.37 (SE = 2.77) per singleton delivery, £2,316.28 (SE = 141.47) per twin delivery, and £8,876.77 (SE = 3,112.41) per triplet delivery.

'Cost of the first 5 years of child life' was adapted from a report on long-term health service costs for hospital stays associated with singleton, twin, and triplet births up to 5 years of age [4] . That computation was derived from the Oxford Record Linkage Study (ORLS), which recorded health data on all women and infants who lived and delivered in Oxfordshire or West Berkshire between January 1, 1970, and December 31, 1993. Their study included all delivery costs for the mother, as well as hospital service utilisation costs from birth through baby's first 5 years of life. These costs were adjusted for inflation estimated at £2,345.69 (±12.50) per singleton delivery, £11,715.88 (±80.46) per twin delivery, and £37,462.66 (±467.13) per triplet delivery.

For each IVF case, the hospital costs described above were added to IVF treatment costs to generate a total cost. To bring these historical economic figures in line with current levels, all costs were inflated by £2,004 using NHS Hospital and Community Health Services pay and price deflators provided by the UK Department of Health (www.statistics.gov.uk/statbase). This total cost included the cost of IVF treatment (without gonadotropins and other medications), hospital visits during the antenatal period, intrapartum care, and any paediatric hospitalisation from birth through the first 5 years of life. Mode of delivery was included neither in the HFEA dataset nor in the report by Henderson et al. [4]. Accordingly, delivery costs were excluded from our analysis.

Measuring Incremental Cost-Effectiveness

Here, effectiveness is defined as the average number of live birth events per woman in each category as classified by age, cycle, and number of transferred embryos. The incremental cost-effectiveness ratio (ICER) is calculated for each group of women as the cost of achieving an additional live birth event in a higher embryo category. ICERs are expressed in terms of (a) maternal age (<38 vs. ≥38 years), (b) number of treatment cycles (1, 2, or ≥3 cycles), and (c) number of transferred embryos (1, 2, or 3 embryos) on the final treatment cycle. The average live birth rate per woman was chosen for effectiveness to normalise the data with respect to differences in the number of women in each age, ET, and cycle populations.

In summary, the variables included in the analysis comprise (a) the respective number of singleton, twin, and triplet live birth events; (b) antenatal cost of each plurality; (c) cost from delivery to the first 5 years of life; (d) total cost for each plurality; (e) total cost of each ET policy; (f) total effectiveness of each ET policy; (g) incremental cost of achieving an additional live birth event in a higher ET policy; (h) incremental effectiveness at achieving an additional live birth event with a higher ET policy; and (i) incremental cost-effectiveness expressed as the incremental cost to achieve an additional live birth event in comparative ET policies. Effectiveness ratios were expressed as the number of live birth events per woman. ICERs were subsequently mapped onto cost-effectiveness planes to graphically illustrate the within-cycle cost-effectiveness of each intervention.

Results

A total of 174,418 IVF treatment cycles occurred in the UK between 1 July 1991 and 31 December 1998. After application of exclusion criteria, 74,755 women undergoing 137,307 cycles (79 % of those registered) were analysed. These 74,755 fertility patients underwent between 1 and 23 IVF cycles where ET occurred. A total of 41,033 women underwent one cycle only, 18,275 two cycles only, and 15,447 three or more cycles. Of these 61,284 were less than 38 years of age and 13,471 were greater than 38 years of age.

SET Versus 2ET Policy

One Prior IVF Cycle

Among 3,089 women aged <38 years who underwent SET after one prior IVF cycle, there were 463 live birth events comprising 450 singletons, 11 sets of twins and 2 sets of triplets. A policy of 2ET was noted to increase the live birth rate by a factor of 2.4 (0.15 vs. 0.36 births/woman; $p<0.05$), although this came at the expense of tenfold rise in multiple births (2.81 % vs. 27.32 %; $p<0.05$). Correspondingly, the incremental cost per additional live birth associated with a 2ET policy was £7,728 in the nominal scenario. This value ranged from £7,450 to £8,023 in minimum and maximum scenarios, respectively.

In 1,270 women aged ≥38 years having SET after one prior IVF cycle, there were 71 live births comprising 69 singletons, 1 set of twins, and 1 set of triplets. In this group, moving from SET to 2ET increased the live birth rate by a factor of 2.7 (0.06 vs. 0.16; $p<0.05$) at the expense of a fivefold increase in the incidence of multiple births (2.82 % vs. 13.89 %). The incremental cost per additional live birth in moving to a 2ET policy in the one prior cycle population was £4,663 in the nominal scenario. This value ranged from £4,537 to £4,794 in minimum and maximum scenarios, respectively.

Two Prior IVF Cycles

In 1,451 women aged <38 years with SET and two prior IVF cycles, there were 64 live births comprising 63 singletons and 1 twin delivery. A policy of 2ET increased the live birth rate by more than twofold (0.04 vs. 0.09; $p<0.05$), accompanied by a ninefold increase in the risk of multiple births (1.56 vs. 14.23; $p<0.05$). Whilst the proportion of multiple births to total births was slightly lower than the younger, one-cycle patients, the live birth rate was almost four times lower for two-cycle compared to one-cycle patients (0.04 vs. 0.15; $p<0.05$). The incremental cost per additional live birth event in moving to a 2ET policy in this population was £5,662 in the nominal scenario, ranging from £5,464 to £5,874 in minimum and maximum scenarios, respectively.

There were 460 women aged ≥38 years who underwent SET after two prior IVF cycles, from which 10 live birth events resulted (all singleton deliveries). A policy of 2ET doubled the live birth rate (0.02 vs. 0.04; $p<0.05$) at the expense of an increase in the incidence of multiple births (0 % vs. 11.76 %) including three sets of twins and one set of triplets. There were no multiple gestations in the SET group with two prior IVF cycles. The live birth rate was three times lower for women undergoing two cycles as compared to women undergoing only one cycle (0.02 vs. 0.06; $p<0.05$). The incremental cost per additional live birth event in moving to a 2ET policy in this population was £8,001 in the nominal scenario. This value ranged from £7,538 to £8,535 in minimum and maximum scenarios, respectively.

Three or More Prior IVF Cycles

There were 49 live births comprising 48 singletons and 1 set of twins in 1,274 women aged <38 years with SET after ≥3 prior IVF cycles. A policy of 2ET accomplished a moderate increase in the live birth rate (0.04 vs. 0.05; $p>0.05$) at the expense of a fourfold increased risk of multiple births (2.04 vs. 7.29; $p<0.05$). Whilst the proportion of multiple births to total births was 30 % higher in comparison to women undergoing only two cycle attempts, the live birth rate was identical. There is an incremental cost savings (indicated by a minus sign) of (–)£6,340 in the nominal scenario per additional live birth event in moving to a 2ET policy in this population. This value ranged from (–)£3,751 to (–)£8,920 in minimum and maximum savings scenarios, respectively.

In 288 women aged ≥38 years with SET and ≥3 prior IVF cycles, there were two live births, both singletons. In this subgroup, a policy of 2ET doubled the very low live birth rate from 0.01 to 0.02. This came at the expense of an increase in the proportion of multiple births with the extra embryo, although it is important to note that there were no multiple births observed among women undergoing SET in this category. The live birth rate was six times lower for women undergoing three or more cycles compared to women undergoing only one cycle attempt (0.01 vs. 0.06; $p<0.05$). The live birth rate was half the rate for women undergoing two IVF cycles compared to those undergoing three or more IVF cycles (0.01 vs. 0.02; $p<0.05$). The incremental cost per additional live birth event in moving to a 2ET policy in this population was £20,906 in the nominal scenario. This value ranged from £16,980 to £24,840 in minimum and maximum scenarios, respectively.

Two Versus Three ET Policy

One Prior IVF Cycle

Four thousand seven hundred ninety-five live births occurred among 13,260 women aged <38 years with 2ET and one prior IVF cycle, comprising of 3,485 singletons, 1,302 sets of twins, and 8 sets of triplets. A policy of 3ET increased the live birth

rate by 10 % (0.36 vs. 0.40; $p<0.05$) at the expense of a 50 % increase in the proportion of multiple births (27.32 vs. 40.97 %; $p<0.05$). Correspondingly, the incremental cost per additional live birth event in moving to a 3ET policy in this population was £45,964 in the nominal scenario. This value ranged from £42,218 to £50,430 in minimum and maximum scenarios, respectively.

Among 2,258 women aged ≥38 years with 2ET and one prior IVF cycle, there were 360 live births comprising 310 singletons and 50 twin sets. A policy of 3ET increased the live birth rate by a factor of 1.4 (0.16 vs. 0.23; $p<0.05$) at the expense of a 1.7-fold increase in the proportion of multiple births (13.89 % vs. 23.87 %; $p<0.05$). The incremental cost per additional live birth event in moving to a 3ET policy in this population was £10,045 in the nominal scenario. This value ranged from £9,501 to £10,668 in minimum and maximum scenarios, respectively.

Two Prior IVF Cycles

Amidst 5,925 women aged <38 years with 2ET and two prior IVF cycles, there were 555 live births consisting of 476 singletons, 78 sets of twins, and 1 set of triplets. In this group, a policy of 3ET increased the live birth rate by approximately 50 % (0.09 vs. 0.14; $p<0.05$) at the expense of 40 % increased proportion of multiple births (14.23 % vs. 19.91 %; $p<0.05$). Whilst the proportion of multiple births to total live birth events was approximately half the value for <38 years/1 cycle patients, the live birth rate was four times lower for two- compared to one-cycle patients (0.09 vs. 0.36; $p<0.05$). The incremental cost per additional live birth event in moving to a 3ET policy in this population was £8,943 in the nominal scenario. This value ranged respectively from £8,406 to £9,570 in minimum and maximum scenarios.

In 895 women aged ≥38 years with 2ET and two prior IVF cycles, there were 34 live birth events comprising 30 singletons, 3 sets of twins, and 1 set of triplets. A policy of 3ET doubled the live birth rate (0.04 vs. 0.08; $p<0.05$) with a slight decrease in the proportion of multiple births in the higher embryo category (11.76 % vs. 10.07 %). The proportion of multiple births to total births was slightly less than the value for one-cycle patients, but the twin delivery rate was higher in women receiving 3ET compared to those receiving 2ET. The live birth rate was four times lower for women with only two cycles compared with those having only one cycle (0.04 vs. 0.16; $p<0.05$). The incremental cost per additional live birth event in moving to a 3ET policy in this population was £3,173 in the nominal scenario. This value ranged from £3,214 to £3,125 in the minimum and maximum scenarios, respectively.

Three or More Prior IVF Cycles

IVF for 4,853 women aged <38 with 2ET and ≥3 prior cycles resulted in 247 live births comprising 229 singletons and 18 sets of twins. A policy of 3ET increased the live birth rate by 60 % (0.05 vs. 0.08; $p<0.05$) with surprisingly fewer multiple births in the higher embryo category (7.29 % vs. 6.44 %, respectively). The proportion of multiple births to total births was approximately 25 % of the value for women who

received three or more cycles compared to those who received only one cycle. The proportion of multiple births was approximately half the value for women who received three or more cycles compared to those who received only two cycles. The live birth rate was approximately seven times lower for women who received three or more cycles compared to women who received only one cycle (0.05 vs. 0.36; $p<0.05$), and approximately half the live birth rate of women who received only two cycles (0.05 vs. 0.09; $p<0.05$). The incremental cost per additional live birth event in moving to a 3ET policy in this population was £14,016 in the nominal scenario, and ranged from £11,431 to £16,619 in minimum and maximum scenarios, respectively.

In 636 women aged ≥38 with 2ET and ≥3 prior cycles, there were 14 live birth events comprising 12 singletons and 2 twin sets. A policy of 3ET trebled the live birth rate (0.02 vs. 0.06; $p<0.05$) with four times fewer multiple births in the higher embryo category (14.29 % vs. 3.41 %, respectively; $p<0.05$). The proportion of multiple births to total births was slightly higher for women who received three or more cycles compared to those who underwent only one or two cycles ($p>0.05$). The live birth rate was eight times lower for women who received three or more cycles compared to those who received only one cycle (0.02 vs. 0.16 births per woman; $p<0.05$), and half that of women who received only two cycles (0.02 vs. 0.04 births per woman; $p<0.05$). The incremental cost per additional live birth event in moving to a 3ET policy in this population was £4,969 in the nominal scenario. This value ranged from £4,304 to £5,632 in minimum and maximum scenarios, respectively.

One Versus Three ET Policy

One Prior Cycle

Sixteen thousand seven hundred fifty women aged <38 underwent IVF with 3ET after one prior cycle. There were 6,680 live births from this group comprising of 3,943 singletons, 2,273 sets of twins, and 464 sets of triplets. A policy of 3ET increased the live birth rate by a factor of 2.7 (0.15 vs. 0.40; $p<0.05$), although this was accompanied by a 15-fold increase in the proportion of multiple births in the 3ET category (2.81 % vs. 40.97 %; $p<0.05$). The incremental cost per additional live birth event in moving from SET to 3ET in this population was £13,440 in the nominal scenario. This value ranged from £12,645 to £14,359 in minimum and maximum scenarios, respectively.

Among 4,406 women aged ≥38 who completed IVF with 3ET after only one prior IVF cycle, there were 1,018 live birth events comprising 775 singletons, 222 sets of twins, and 21 sets of triplets. For this subgroup, a 3ET policy increased the live birth rate by a factor of 3.8 (0.06 vs. 0.23; $p<0.05$) with an eightfold increase in the proportion of multiple births after 3ET (2.82 % vs. 23.87 %; $p<0.05$). The incremental cost per additional live birth event in moving from SET to 3ET for this subgroup of IVF patients was £6,864 in the nominal scenario. This value ranged from £6,566 to £7,196 in minimum and maximum scenarios, respectively.

Two Prior IVF Cycles

Among 7,682 women aged <38 who underwent IVF and 3ET after two prior IVF cycles, there were 1,065 live births comprising 853 singletons, 182 sets of twins, and 30 sets of triplets. Here, a policy of 3ET increased the live birth rate by a factor of 3.5 (0.04 vs. 0.14; $p<0.05$), accompanied by 13 times more multiple births in the higher embryo category (1.56 % multiples in the 2ET group vs. 19.91 % in the 3ET group). The proportion of multiple births to total births was approximately half the value for women who underwent only two cycles compared to those who underwent only one cycle. The live birth rate was approximately three times lower for populations undergoing two cycles compared to populations undergoing only one cycle (0.14 vs. 0.40; $p<0.05$). The incremental cost per additional live birth in moving from SET to 3ET in this population was £7,223 in the nominal scenario and ranged from £6,864 to £7,632 in minimum and maximum scenarios, respectively.

In 1,862 women aged ≥38 years who received 3ET after two prior IVF cycles, there were 149 live births comprising 134 singletons, 14 sets of twins, and 1 set of triplets. A policy of 3ET increased the live birth rate by a factor of 4 (0.02 vs. 0.08; $p<0.05$) at the expense of a tenfold increase in the proportion of multiple births in the higher embryo category (0.0 % vs. 10.07 %; $p<0.05$). The proportion of multiple births to total births was approximately half the value for women with two prior IVF cycles compared to those with only one prior cycle. The live birth rate was approximately three times lower for women with two previous IVF cycles compared to those who had only one prior IVF cycle (0.08 vs. 0.23; $p<0.05$). The incremental cost per additional live birth event in moving from SET to 3ET in this population was £4,519 in the nominal scenario and ranged from £4,356 to £4,697 in minimum and maximum scenarios, respectively.

Three or More Prior IVF Cycles

Among 7,000 women aged <38 who underwent IVF and 3ET after three or more previous IVF cycles, there were 528 live birth events comprising 494 singletons, 31 sets of twins, and 3 sets of triplets. Here, a 3ET policy doubled the live birth rate (0.04 vs. 0.08; $p<0.05$) at the expense of a threefold increase in multiple births in the higher embryo category (2.04 % vs. 6.44 %; $p<0.05$). The proportion of multiple births to total live births for women who had completed three or more IVF cycles was approximately one-sixth that of women who had completed only one prior IVF cycle (6.44 % vs. 40.97 %; $p<0.05$). The proportion of multiple births to live births for patients with a history of three or more IVF cycles was approximately one-third that of the proportion for women who received two cycles (6.44 % vs. 40.97 %; $p<0.05$). The live birth rate was five times lower for women with three or more prior IVF cycles compared to women with only one prior cycle (0.08 vs. 0.40; $p<0.05$), and 43 % lower than the live birth rate of women with only two prior IVF cycles (0.08 vs. 0.14; $p<0.05$). Again, this indicates that patients entering treatment with a history of three or more prior IVF cycles are significantly less likely to

conceive than patients with only one or two prior IVF cycle attempts. The incremental cost per additional live birth event in moving from SET to 3ET in this population was £7,169 in the nominal scenario, ranging from £6,324 to £8,028 in minimum and maximum scenarios, respectively.

There were 1,396 women aged ≥38 years who completed IVF with 3ET after three or more prior IVF cycles. Within this group, 88 live births were recorded, comprising 85 singletons and 3 sets of twins. A policy of 3ET increased the live birth rate by a factor of 6 (0.01 vs. 0.06; $p<0.05$) with the result of three sets of twins. The live birth rate for patients with three or more IVF cycles was approximately one-fourth that of women who had completed only one prior IVF cycle (0.06 vs. 0.23; $p<0.05$), and approximately 33 % of the live birth rate of women who received two cycles (0.06 vs. 0.08; $p>0.05$). The incremental cost per additional live birth event in moving from SET to 3ET in this population was £9,249 in the nominal scenario, ranging from £7,710 to £10,793 in minimum and maximum scenarios, respectively (Table 21.1).

Comparisons of Incremental Cost-Effectiveness Ratio as a Function of Patient Age

In evaluating a policy move from SET to 2ET for IVF patients <38 years of age, the ICER for women who had completed two prior IVF cycles was 27 % less than the ICER for those with only one prior IVF cycle. For women who received one or two cycles where SET was performed, the cost of achieving an additional live birth by moving to a policy of 2ET was less than £8,160. However, the ICER comparing SET to 2ET was negative for women with three or more prior IVF cycles, suggesting that it is actually cost saving to offer this particular population of IVF patients an extra embryo for transfer. The total cost per patient with a history of three or more prior IVF cycles was £6,078 less with 2ET compared to SET. This unexpected finding is the topic of further investigation.

In the case of moving from SET to 2ET for IVF patients 38 years and older, the ICER increased precipitously with increasing IVF cycle attempts. This suggests that for IVF patients aged ≥38, it may be more cost-effective to offer 2ET to those with shorter and less complex treatment histories. Offering 2ET to women who were in the one prior IVF cycle category with SET yielded an ICER of £4,663 per additional live birth event. This ICER doubled (to £8,001) in the case of two prior cycles/SET women and quadrupled (to £20,906) in the case of women who underwent SET with three or more IVF cycle attempts.

2ET to 3ET ICER Comparison

For patients age <38 years who had completed two prior IVF cycles, the ICER was approximately one-fifth the value estimated for those who underwent only one prior IVF cycle and 67 % of that estimated for those with three or more previous IVF cycles.

Table 21.1 Fiscal impact of advancing to a higher embryo transfer policy (i.e. moving from SET to 2ET, and 2ET to 3ET)

	<38 years of age				≥38 years of age			
	ΔLBR	ΔMBR	LBR comparisons	ICER	ΔLBR	ΔMBR	LBR comparison	ICER in nominal scenario
1ET + 1Cycle	2.4× increase (0.15 vs. 0.36, $p<0.05$)	10× increase (2.81 % vs. 27.32 %, $p<0.05$)		£7,728 in the nominal scenario	2.7× increase (0.06 vs. 0.16, $p<0.05$)	5× increase (2.82 % vs. 13.89 %)		£4,663
1ET + 2Cycle	2× increase (0.04 vs. 0.09, $p<0.05$)	9× increase (1.56 vs. 14.23, $p<0.05$)	LBR almost 4× lower for 2-cycle vs. 1-cycle (0.04 vs. 0.15, $p<0.05$)	£5,662 in the nominal scenario	2× increase (0.02 vs. 0.04, $p<0.05$)	Increased (0 % vs. 11.76 %)	LBR almost 3× lower for 2-cycle vs. 1-cycle (0.02 vs. 0.06, $p<0.05$)	£8,001
1ET + ≥3Cycle	Moderate increase (0.04 vs. 0.05, p = NS)	4× increase (2.04 vs. 7.29, $p<0.05$)	MBR 30 % higher than 2-cycle with identical LBR	(–)£6,340 in the nominal scenario	2× very low LBR (0.01–0.02)	Increased	6× lower for 3-cycle vs. 1-cycle (0.01 vs. 0.06, $p<0.05$) and 0.5× 3-cycle vs. 2-cycle (0.01 vs. 0.02, $p<0.05$)	£20,906
2ET + 1Cycle	10 % increase (0.36 vs. 0.40, $p<0.05$)	50 % increase (27.32 % vs. 40.97 %, $p<0.05$)		£45,964 in the nominal scenario	1.4× increase (0.16 vs. 0.23, $p<0.05$)	1.7× increase (13.89 % vs. 23.87 %, $p<0.05$)		£10,045
2ET + 2Cycle	50 % increase (0.09 vs. 0.14, $p<0.05$)	40 % increase (14.23 % vs. 19.91 %, $p<0.05$)	LBR 4× lower for 2-cycle vs 1-cycle (0.09 vs. 0.36, $p<0.05$)	£8,943 in the nominal scenario	2× increase (0.04 vs. 0.08, $p<0.05$)	Slight decrease (11.76 % vs. 10.07 %)	LBR 4× lower for 2-cycle vs. 1-cycle (0.04 vs. 0.16, $p<0.05$)	£3,173
2ET + ≥3Cycle	60 % increase (0.05 vs. 0.08, $p<0.05$)	Decreased (7.29 % vs. 6.44 %)	LBR 7× lower for 3-cycle vs 1-cycle (0.05 vs. 0.36, $p<0.05$) and 0.5× lower for 3-cycle vs. 2-cycle (0.05 vs. 0.09, $p<0.05$)	£14,016 in the nominal scenario	3× increase (0.02 vs. 0.06, $p<0.05$)	4× decrease (14.29 % vs. 3.41 %, $p<0.05$)	LBR 8× lower for 3-cycle vs. 1-cycle (0.02 vs. 0.16, $p<0.05$) and 0.5× lower for 3-cycle vs. 2-cycle (0.02 vs. 0.04, $p<0.05$)	£4,969

(continued)

Table 21.1 (continued)

	<38 years of age				≥38 years of age			
	ΔLBR	ΔMBR	LBR comparisons	ICER	ΔLBR	ΔMBR	LBR comparison	ICER in nominal scenario
3ET + 1Cycle	2.7× increase (0.15 vs. 0.40, $p<0.05$)	15× increase (2.81 % vs. 40.97 %, $p<0.05$).		£13,440 in the nominal scenario	3.8× increase (0.06 vs. 0.23, $p<0.05$)	8× increase (2.82 % vs. 23.87 %, $p<0.05$)		£6,864
3ET + 2Cycle	3.5× increase (0.04 vs. 0.14, $p<0.05$)	13× increase (1.56 % vs. 19.91 %)	LBR 3× lower for 2-cycle vs. 1-cycle (0.14 vs. 0.40, $p<0.05$)	£7,223 in the nominal scenario	4× increase (0.02 vs. 0.08, $p<0.05$)	10× increase (0.0 % vs. 10.07 %, $p<0.05$)	LBR 3× lower for 2-cycle vs. 1-cycle (0.08 vs. 0.23, $p<0.05$)	£4,519
3ET + ≥3Cycle	2× increase (0.04 vs. 0.08, $p<0.05$)	3× increase (2.04 % vs. 6.44 %)	LBR 5× lower for 3-cycle vs. 1-cycle (0.08 vs. 0.40, $p<0.05$); and 0.43× lower than the 2-cycle (0.08 vs. 0.14, $p<0.05$)	£7,169 in the nominal scenario	6× increase (0.01 vs. 0.06, $p<0.05$)	3 sets of twins born	LBR 4× lower for 3-cycle vs. 1-cycle (0.06 vs. 0.23, $p<0.05$) and 0.33× lower for 3-cycle vs. 2-cycle (0.06 vs. 0.08, p = N/S)	£9,249

Note: ET = embryo transfer; ΔLBR = change in live birth rate; ΔMBR = change in multiple birth rate; ICER = incremental cost-effectiveness ratio; (–)£ = cost savings

In women aged ≥38 years with two prior cycles, the ICER was approximately one-third the value compared to those who received one IVF cycle and 65 % of the value estimated for those who received three or more IVF cycles.

SET to 3ET ICER Comparison

In moving from SET to 3ET in women aged <38 years, the ICER for women who received only one IVF cycle was twice the ICER values of women who underwent two and three or more IVF cycles (£13,440 vs. £7,223 and £7,169, respectively). Among IVF patients age ≥38, moving from SET to 3ET created an ICER of £6,864 per additional live birth for women who received one IVF cycle. This value declined to £4,519 for women who received two cycles, but then increased to £9,249 for women who received three or more IVF cycles. For women ≥38 who received one IVF cycle, the move from SET to 2ET was more cost-effective than a move from SET to 3ET (£4,663 vs. £6,864, respectively).

Of note, moving from SET to 3ET in ≥38-year-old IVF patients was approximately twice as cost-effective as the move from SET to 2ET in the case of women who received either two or three or more IVF cycles. In the case of women who received two cycles, the ICER for the move from SET to 3ET was £4,519. The corresponding ICER for SET to 2ET was £8,001. In the case of IVF patients who received three or more cycles, the incremental cost to achieve an additional child with a move from SET to 3ET was £9,249. The corresponding ICER for SET to 2ET was £20,906.

Discussion

Trends with Increasing Treatment Cycles

The findings in this investigation are in parallel with those reported earlier by Templeton et al. [5], who analysed the HFEA dataset from August 1991 to April 1994. For IVF patients undergoing SET, we noted that the LBR is observed to decline precipitously with increasing treatment cycles, from 0.15 live births per patient in the <38/SET/1 cycle group to only 0.04 live births per patient in both the <38/SET/2 cycle and <38/SET/≥3 cycle populations (0.15 vs. 0.04; $p < 0.01$). In the case of the 2ET population, as with the SET population, the live birth rate declines precipitously with increasing IVF cycle attempts. However, this live birth rate is higher than in the case of the SET population, at 0.36 births per patient in the <38/2ET/1 cycle group. This rate declines by a factor of 4—from 0.36 to 0.09—when comparing <38 one-cycle to <38 two-cycle women. It declines even further from 0.09 to 0.05 in comparing <38 two-cycle women to the <38 women who receive three or more IVF cycles. This indicates that women who received two IVF cycles were significantly less likely to conceive than women who underwent only one IVF cycle attempt and women who received three or more IVF cycles were significantly less likely to conceive than women after one or two IVF attempts.

Trends with Increasing Age

In the case of IVF patients having 3ET who received only one treatment cycle, those age <38 years compared to those age ≥38 achieved twice the live birth rate (0.40 vs. 0.23; $p<0.001$), although this was accompanied by an essentially doubled rate of multiple births (0.41 vs. 0.24; $p<0.001$). This doubled chance of a live birth for younger women was not observed in the 2- and ≥3-cycle populations of 3ET, however.

For IVF patients undergoing 2ET, those aged <38 years compared to women aged ≥38 were more than twice as likely to deliver a live birth. However, this often resulted in multiple births and in the case of patients undergoing IVF with 2ET, the multiple birth rate ratios between mothers <38 years and those age ≥38 declined as the number of IVF cycles increased. Stated another way, the ratio of multiple births in 2ET patients who are <38 years compared to the ratio of multiple births in 2ET women who are age ≥38 declines with increasing IVF cycle attempts.

Incremental Cost-Effectiveness

This study created a novel way of comparing total cost as a function of optimizing live birth events while minimizing and bringing awareness to the risk of multiples. For women aged ≥38, our analysis suggests that the most cost-effective ICER occurs in the setting of 2ET treatments for patients who received two IVF cycles. Here a policy change from 2ET to 3ET presents an additional cost of £3,173 per additional live birth. For women <38 years of age; the least cost-effective ICER occurred in the first IVF cycle population of women, when changing policy from 2ET to 3ET. For this latter group, a third embryo at transfer yielded an ICER of an additional £45,964 per additional live birth event.

A paradox was observed in that the most cost-effective and least cost-effective scenarios occurred, respectively, with <38-year-old and ≥38-year-old patients who underwent three or more IVF cycles, in the move from SET to 2ET. In the case of patients age <38 with at least three IVF cycles, 2ET in comparison to SET yielded an incremental cost savings of approximately £6,392 per additional live birth. This occurred because, in women aged <38 years who had at least three IVF cycles, the SET group had fractionally fewer IVF cycles compared to the 2ET group (3.88 vs. 3.83 cycles). In contrast, for women aged ≥38 years who underwent ≥3 IVF cycles, a policy move from SET to 2ET yielded an ICER of approximately £20,944 per additional live birth.

Importantly, this analysis shows that moving from 2ET to 3ET is not cost-effective in any cycle group of patients age <38. For women with one or two prior IVF cycles only, allocating 3ET to those who received SET on their last cycle is cost-effective if the willingness to pay is at least £13,600 and £8,160, respectively. For women aged <38 who have undergone three or more IVF cycles, a move from SET to 3ET is cost-effective if the willingness-to-pay is at least £6,800. Further, a

Table 21.2 Recommendations based on cost-effectiveness (arbitrary values used for willingness to pay)

Willingness to pay GBP 20k		Willingness to pay GBP 10k	
<38 years	≥38 years	<38 years	≥38 years
All policies moving to a higher embryo transfer category (2ET or 3ET) would be cost-effective except:	All policies moving to a higher embryo transfer category (2ET or 3ET) would be cost-effective except:	A policy move from 1ET to 2ET would be cost-effective *only* for women undergoing 1ET who have had two or more prior treatment cycles	A policy move to a higher embryo transfer category would be cost-effective especially for those who have undergone two or more prior treatment cycles
(a) Policy move to 3ET for women who are on their first treatment cycle	(a) Policy move to 2ET in a women ≥38 years with 1ET and history of having undergone ≥3 prior treatment cycles		
(b) Policy move to 3ET for women who are on their third or higher treatment cycle that would otherwise receive 2ET			

move from SET to 2ET is cost saving in this patient population. For those age ≥38 with only one prior IVF cycle, a move from 2ET to 3ET is not cost-effective. However, a move from SET to 3ET in this group of women is cost-effective to the extent that the willingness-to-pay is at least £6,800. For patients age ≥38 who have undergone two IVF cycles only, a move from SET to 3ET (compared to the move from 2ET to 3ET) is cost-effective if the willingness-to-pay is within the above range. Compared to the move from SET to 2ET, a shift from 2ET to 3ET is also cost-effective and within a relatively small willingness-to-pay threshold. A move from SET to 3ET is cost-effective if the willingness-to-pay is at least £8,840. However, a move from SET to 2ET is not cost-effective in this group of women.

The cost-effectiveness planes used to generate this cost analysis are based on baseline assumptions of incremental cost-effectiveness. These may be subject to uncertainty introduced by the omission of certain values (such as neonatal mortality costs) or the inclusion of non-homogeneous patients who have intrinsically different clinical profiles. As such, the above statements should serve only as a guide (Table 21.2).

Conclusion

As clinical reproductive medicine practice has become more conservative in the last decade with respect to number of embryos transferred in IVF, the HFEA national dataset is well suited to allow for a timely evaluation of ET cost-effectiveness for

specific populations of patients. Our investigation shows that the live birth rate declines precipitously with increasing IVF attempts, highlighting that for this refractory subgroup of IVF patients the likelihood to achieve pregnancy and deliver is very limited. Similarly, with one notable exception (women aged <38 having SET, undergoing ≥3 IVF cycles), additional embryos for transfer are more costly in facilitating an ever valued increase in the live birth rate.

The population-based findings reported in this chapter show that IVF is more likely to lead to twins and triplets among fertility patients undergoing their first IVF cycle. Since triplets have been shown to have higher mortality rates [6], contribute disproportionately to hospital inpatient costs [4], and require antenatal and NICU services that are higher than the cost of corresponding singletons and twins [7], their incidence must be regarded as a major health risk.

Much of the advocacy for fewer embryo transfers (and especially SET) is based upon the well-known risks of cerebral palsy [8], epilepsy [9], congenital malformations [10], and other neurological sequelae [11] that accompany multiple births, rather than the iatrogenic complications of IVF. Studies on growth and physical outcomes show no differences between children conceived by IVF or by natural conception, at least on the measures of major dysmorphism and organ abnormalities during the first 2 years of life [12]. While IVF may not cause unreasonable harm when successful, a more fundamental problem is that it very seldom yields a live birth for the patients who receive more than three IVF treatments.

During pretreatment counselling, IVF patients are sometimes informed that women experience the same chance of delivering a live birth irrespective of the number of previous cycle attempts. Our analysis gives a starkly different view, indicating that an IVF patient's best outcome is achieved with her first treatment attempt where appropriate embryo transfer policies should be encouraged. Indeed, these data show the refractory nature of infertility encountered over three or more IVF attempts presages a bleak reproductive outcome for these 'repeat' patients.

Nevertheless, the forecast for IVF patients with a failed first cycle who seek a second opinion (and another IVF attempt) need not be dismal. While the treatment data used for our calculations were collected from a large number of IVF cycles, these treatments were completed before molecular testing of embryos was widely available. This means that embryo selection for these cases was based on conventional morphologic criteria, rather than comprehensive chromosomal screening. Incorporating genetic assessment of embryos is one way to individualise patient care during IVF to improve live birth rate and reduce incidence of multiple gestation. Indeed, personalised treatment guidelines for specific populations are urgently needed in order to maximise the effectiveness of IVF with respect to its long-term costs. Patients attending for reproductive endocrinology consultation should have treatments tailored to their specific age and IVF histories which can help estimate their treatment response and reproductive outcome. The data presented here suggest that maternal age and number of prior IVF cycles are highly informative in estimating the cost-effectiveness of IVF.

Can improvements in the live birth rate from transferring additional embryos be justified by the additional cost associated with a higher incidence of multiple gestation? Whether an ET policy is estimated to be cost-effective or not, patients should be

entitled to make informed decisions based on the facts, which include the short- and long-term costs and short- and long-term willingness-to-pay for treatments and outcomes;,views which will change between populations and over time. It is also important to keep in mind that the majority of cost burden due to multiples stems from patients age <38 and, more particularly, from younger women who are on their first IVF cycle. This analysis strengthens the impression that SET would be cost-effective from the vantage point of insurance companies or health authorities which must absorb the additional cost of multiple births.

At present an absolute limit on number of embryos to transfer based on cost-effectiveness theory may miss the mark, however. Analysis of cost-effectiveness is discriminatory by nature. In this investigation, the central question is whether SET is a policy where a threshold will be ignored for societal preferences to help particular patients have children. By not offering more embryos to older patients with poor fertility prognosis, any absolute SET (or 2ET) limit may be viewed as an unacceptable discriminatory practice that unfairly prevents some patients from delivering progeny. Thus, any cost-effectiveness analysis should not be the sole factor for consideration in determining the role for public support for IVF coverage in general, and ET policy in particular.

This analysis had sufficient sample size to arrive at conclusions that are both meaningful and immediately relevant to decision-makers. With the exception of women aged <38/1ET/≥3 cycles, a SET policy appears to be the best value for money across the population. This is particularly the case for younger women who are on their first IVF cycle attempt. Table 21.1 summarises the conclusions made regarding embryo transfer policy with willingness to pay thresholds adjusted for inflation to 2012–2013 based on published annual healthcare inflation for the UK.

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Conflict of Interest Dr. Jones has an ownership interest in a proprietary predictive counselling tool developed from this research, marketed by ForMyOdds LLC.

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Chapter 22
The Quebec Experience—One Plus One Equals Two at Once: Presenting Cumulative Pregnancy Rates as the Ideal Outcome in Elective SET Programmes

Maria P. Vélez, Isaac-Jacques Kadoch, Simon J. Phillips, and Francois Bissonnette

Introduction

Single Embryo Transfer (SET) is the most effective approach to reduce the incidence of multiple pregnancies associated with Assisted Reproductive Technologies [1]. Although the pregnancy rate after one fresh SET is reported to be lower compared with one fresh double embryo transfer (DET), no difference exists when one DET is compared with elective SET followed by one Frozen embryo Transfer (FET) [2]. Elective SET requires the selection of good prognosis patients and the transfer of the best high quality embryo [3]. As noted elsewhere in this volume, sophisticated molecular techniques continue to be refined for evaluation of the chromosomal competencies of embryos, thus permitting an improved selection process to enable SET. Of note, several observational studies comparing elective SET with DET have not found differences in terms of pregnancy rate among both groups [3–5]. The few Randomized Controlled Trials that have compared a single cycle of DET with one cycle of fresh SET followed by one frozen/thawed SET have shown that there is no significant difference in terms of cumulative live birth rates [2]. Moreover, in regard to health expenses, elective SET embryo is substantially cheaper than DET in women younger than 38 years with a good prognosis [5].

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Public financing of assisted reproductive technologies (ART) is intended to increase access to fertility treatments by reducing the financial burden to patients. In counterpart, governments aim to reduce the health expenses associated with multiple pregnancies attributable to the use of ART. Although comprehensive government initiatives are associated with greater utilization of fertility treatments and lower rates of multiple pregnancies [6, 7], critics of public financing argue that public programmes are also associated with lower pregnancy rates [8].

In Canada, health care is the responsibility of the individual provinces. In the Province of Quebec, the cost of all IVF procedures was covered by the patient and partially reimbursed as a 50 % tax rebate before 2010. There was no regulation regarding the number of embryos to transfer or the number of treatment cycles provided to each patient. On 5 August 2010, the Quebec government introduced a public IVF programme, marking the beginning of a new era in the field of ART here. Under this programme, all costs related to IVF are covered by Quebec's universal health insurance plan. This includes the cost of all medical procedures related to IVF for three stimulated cycles, or up to six modified natural cycles (mnIVF) [9, 10].

We previously assessed the clinical outcomes and the economic effect of the IVF cycles performed in Quebec during the first year of provincially funded ART [6, 7]. We reported that the implementation of a public IVF programme favouring elective SET not only sharply decreases the incidence of multiple pregnancy but also reduces the cost per live birth [7]. In addition, we have shown that the cumulative pregnancy rate per initiated cycle (i.e. the proportion of clinical pregnancies after the first fresh IVF cycle, including the resulting first frozen/thawed embryo transfer) was comparable to DET before the public IVF programme in one of the IVF centres offering ART treatment in Quebec [11]. In this chapter, we aim to demonstrate that the pregnancy rate after one fresh elective SET plus one FET is comparable to the pregnancy rate after elective DET using data from the whole province of Quebec. We underscore the importance of presenting cumulative pregnancy rates as the preferred outcome to evaluate the impact of elective SET programmes.

Methods

Study Population

The methodology of this prospective comparative analysis has been previously described [7]. For this specific analysis, period I includes the elective DET performed in the five centres offering IVF treatment in Quebec during 2009, the year prior to the start of the Quebec public IVF programme. Period II comprises the elective SET performed in the same centres during 2011, the first full calendar year of the programme, plus the first FET from embryos created from fresh 2011 cycles. Elective Single Embryo Transfer (eSET) refers to the transfer of only one embryo when at least one more embryo was available for cryopreservation at the time of

transfer. Elective Double Embryo Transfer (eDET) refers to the transfer of two embryos when more than two embryos were available at the time of transfer.

Data were obtained from the Canadian Assisted Reproductive Technologies Register (CARTR). CARTR collects treatment cycle data from Canadian fertility centres that are using ART. Staff at each centre provides information for each IVF cycle initiated. The complete anonymous case records are sent electronically each year to the CARTR coordinating centre, where they are checked for accuracy and completeness [12].

Clinical and Laboratory Procedures

Ovarian stimulation protocols, including long gonadotropin-releasing hormone (GnRH) agonist, short GnRH agonist, and GnRH antagonist, were selected based on physician preference and patient characteristics. Oocyte retrieval was performed 36 h after the administration of human chorionic gonadotropin. Insemination was performed using standard IVF or intracytoplasmic sperm injection when indicated. Embryo culture was performed using standardized procedures. Embryo transfer was uniformly performed under ultrasound guidance on Day 2, Day 3, or at the blastocyst stage, depending on cycle-specific characteristics. Each clinic applied its own internal policies with respect to embryo quality and selection and regarding the pertinence of transferring more than one embryo. In general, the woman's age, her IVF history, and the quality of the embryos were primary factors in a decision to transfer multiple embryos. Embryo quality characteristics were applied when selecting suitable embryos for cryopreservation, based on the clinic's internal protocols. Oocyte and embryo development parameters were strictly applied to eliminate embryos with very low implantation potential.

Outcomes

Pregnancy was assessed by serum human chorionic gonadotropin concentration 15 days after egg retrieval ($\geq$25 IU); and clinical pregnancy was determined by ultrasonographic evidence of intrauterine fetal heartbeat between 7 and 8 weeks of gestation. For this tabulation, clinical pregnancies excluded ectopic pregnancies. Multiple gestation was defined according to the number of embryos with positive cardiac action. Frozen embryos were defined as the number of surplus (non-transferred) embryos available for cryopreservation.

We estimated the time-limited cumulative pregnancy rate among patients undergoing a fresh eSET during 2011, plus the resulting first FET performed during the same study period. The 2011 cumulative pregnancy rate was then compared with the pregnancy rate after eDET performed in 2009. Time-limited analyses using proportions provide information on the likelihood of pregnancy per woman with a predetermined number of IVF cycles in the time period defined. This method is an

alternative to life table analysis, which although frequently used may overestimate treatment effect [13]. We then conducted a sensitivity analysis to extrapolate the cumulative pregnancy rate for those women who following a negative pregnancy test, and having cryopreserved embryos, were unable to have their first FET during 2011.

Statistical Analysis

Proportion comparisons were performed by chi-squared test or Fisher's Exact test, as appropriate. Student's *t*-test and ANOVA were used to compare means. A *p*-value <0.05 was considered to be statistically significant. Statistical analysis was performed using STATA version 10.0 (Stata Corporation, College Station, TX, USA). Because the data analysed for this investigation are publicly available through the Canadian Fertility and Andrology Society website and do not include patient-specific information, this study did not require Institutional Review Board approval.

Results

The mean age was similar in both groups [32.54 years (SD 3.69) in the eDET group versus 32.52 (SD 3.62) in the eSET group, $p=0.92$]. Additional characteristics are presented in Table 22.1. The two groups were similar in terms of the distribution of age categories, number of prior pregnancies, and number of previous IVF cycles. There are some differences in the diagnosis of infertility, with some indication that the eSET group is at a disadvantage for some prognostic factors. As Table 22.2 reflects, the mean number of oocytes retrieved, cleaved embryos, and embryos cryopreserved was lower in the eSET group compared with the eDET (2009).

Table 22.3 presents the outcomes of IVF cycles performed during both study periods according to the two modalities of embryo transfer. There were 514 eDET during 2009. The eDET pregnancy rate was 47.1 % in 2009, and the multiple pregnancy rate was 35.1 %. There were 1,375 eSET cycles in 2011, of which 466 (33.9 %) resulted in a pregnancy, leaving 909 women to have transfer of cryopreserved embryos (by definition, all women having eSET have at least one embryo for cryopreservation). Of these, 378 women had a first FET during 2011 with a pregnancy rate of 21.2 %, which leads to a clinical pregnancy after one fresh eSET plus one FET of 39.7 % (Table 22.3).

Next, we conducted a sensitivity analysis to extrapolate the cumulative pregnancy rate. Applying a conservative survival rate of 90 % to the remaining cryopreserved embryos, 478 women still waiting for their first FET after eSET will be able to undergo an FET. Extrapolating the 2011 FET pregnancy rate of 21.2 % to the remaining women, 101 more pregnancies would be expected. So the pregnancy rate after one fresh eSET plus one FET would be 47.1 % (647/1,375). This is equal to the 2009 eDET pregnancy rate, showing equivalence between the two approaches ($p=0.8$).

Table 22.1 Characteristics of the Quebec study population

	2009 (eDET), $n=514$		2011 (eSET), $n=1,375$		
	n	%	*n*	%	*p*
Age					0.15
<35	367	71.4	943	68.6	
35–39	132	25.7	405	29.5	
≥40	15	2.9	27	2.0	
Prior pregnancy					0.99
No	266	51.7	620	45.1	
Yes	134	26.1	313	22.8	
Missing	114	22.2	442	32.1	
Prior ART cycles					0.50
0	317	61.7	1,053	76.6	
1	59	11.5	240	17.5	
2	14	2.7	62	4.5	
≥3	6	1.1	20	1.4	
Missing	118	23.0	0	0	
Diagnosis category					0.01
Unexplained	90	17.5	247	18.0	
Male factor only	224	43.6	544	39.6	
>1 female factor	12	2.3	26	1.9	
Male + female factor	71	13.8	133	9.7	
Endometriosis only	37	7.2	96	7.0	
Tubal only	41	8.0	122	8.9	
Ovulatory only	27	5.3	107	7.8	
DOR only	6	1.2	47	3.4	
Other female only	4	0.8	22	1.6	
Missing	2	0.4	31	2.3	

Table 22.2 IVF outcomes in Quebec, as a function of ET strategy

	2009 (eDET), $n=514$		2011 (eSET), $n=1,375$		
	Mean	SD	Mean	SD	*p*
Oocytes retrieved	14.7	6.5	13.3	6.3	<0.001
Cleaved embryos	8.9	4.1	7.80	4.2	<0.001
Embryos cryopreserved	4.2	2.9	3.18	2.5	<0.001

Table 22.3 Clinical pregnancies following elective DET (2009) vs. elective SET + one FET (2011) in Quebec

	2009	2011			
	Fresh eDET cycles	Fresh eSET cycles	First FET after eSET	Cumulative	*p*
Transfers	514	1,375	378		
Clinical pregnancies	242 (47.1)	466 (33.9)	80 (21.2)	546/1,375 (39.7)	0.004
Single pregnancies	157 (64.9)	458 (98.3)	70 (87.5)	528 (96.7)	<0.001
Multiple pregnancies	85 (35.1)	8 (1.7)	10 (12.5)	18 (3.3)	

Note: All data presented as *n* (%)

Conclusion

Our goal was to present data regarding the implementation of a predominant eSET treatment strategy through public funding in the province of Quebec. One concept that was alluded to in the presentation of the initial data from this programme was that the transfer of two embryos, one at a time, would result in pregnancy rates similar to those seen when eDET was used prior to the programme [6]. This concept was recently supported with data from one of the five centres offering IVF treatment in Quebec [11]. The present analysis, which includes data from the entire Quebec province, goes towards the same direction. The extrapolated pregnancy rate after one fresh eSET plus one first FET in 2011 was not different from that of fresh eDEt alone in 2009. Moreover, it is similar to the pregnancy rate of 47.7 % reported in a large randomized controlled trial comparing these two treatment strategies [14]. We recognize the limitations of extrapolating clinical pregnancies, but this is our best estimate since the Canadian ART Register collects data per individual cycle, not longitudinally per patient. We consider, however, that our results are valid and conservative. Indeed, some patients who won't be pregnant after their first FET will have cryopreserved embryos still available, increasing even more the probability of pregnancy. Our data support, therefore, that cumulative pregnancy rates should be presented as the ideal outcome in the evaluation of the effectiveness of eSET programmes.

Conflict of Interest The authors declare no conflict.

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Chapter 23
Regulatory Aspects of Embryo Transfer: An Israeli View

Zeev Blumenfeld and Foad Azem

Introduction

The Israeli population of approximately eight million citizens consists of about 75 % Jews, 20 % Muslims, and the rest mostly Christians and Druze. The social importance of fertility is of the highest possible level, since many Jewish religious Orthodox families aspire many children, similarly in the case of many Muslim families. Furthermore, many families are interested in male children, in addition to female (in the case of Orthodox Jewish families) and mainly male heirs in the case of Arab families. Furthermore, many young couples apply for medical consultation and assistance long before 1 year of unprotected intercourse without conception, sometimes due to the social impact and "pressure" from family and neighbors. The social impact of "infertility" in the Israeli population is probably much more intense compared to the other developed countries and Western society. Due to these and other reasons, the Israeli health system subsidizes infertility treatment to every Israeli couple up to the first two children, almost at no cost. Even unemployed citizens are eligible for free infertility treatment, up to the successful birth of two children. For citizens who are insured by additional insurance (paid by themselves), they can get subsidized treatment (by 50 % of cost) even for the third and fourth child. Moreover, every infertile woman is eligible until the age of 45, according to the decision and regulations of the Ministry of Health. A couple may have several children from previous marriage(s) and still be eligible for almost free treatment up to the

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delivery of two children from their current marriage/connection. The indications for IVF/ART/ICSI are compatible with the concurrent accepted indications of ASRM and ESHRE, and the financial cover by the health insurance companies and suppliers depends on the female age. In the young age, failure to achieve a successful pregnancy by at least six cycles of COH using FSH/hMG/hCG with or without intrauterine insemination (IUI), is an indication for IVF. However, in the older age (> 40), it may be after two to three cycles, or even less than that, in cases of severe oligo-terato-asthenozoospermia (OTA), or concurrent incomplete mechanical factor, and/or endometriosis.

In case pregnancy was not achieved despite six successful ET cycles of viable embryos, a committee is to decide whether it is recommended to continue IVF attempts or not. In case no eggs are retrieved, or no available embryos for transfer were generated, the committee is to decide whether it is recommended to continue IVF attempts or not.

Embryo transfer of multiple embryos in IVF program may increase the multifetal pregnancy rate associated with premature delivery and maternal and perinatal morbidity [1].

Most Israeli fertility specialists consider single embryo transfer as a means of minimizing the risk of multiple pregnancy. On the other hand, patients and many physicians are considering the possible risk of significantly decreasing the overall live birth rate.

Israeli Regulations

The Israeli recommendations, based on a committee of the Israel Fertility Association (IFA) and the Ministry of Health, are aimed to decrease the multifetal pregnancy rate and premature deliveries [2]. Therefore, their recommendations aiming at decreasing premature deliveries and multiple gestations, regarding embryo transfer (ET), are:

1. In the first IVF/ET cycle in a woman younger than 30 years, it is recommended to transfer only one embryo.
2. In case pregnancy was not achieved, it is possible to ET up to two embryos in the next two cycles.
3. No more than two embryos/cycles should be transferred in the first three IVF cycles.
4. In cases of repeated IVF/ET failure, it is possible to transfer more than two embryos, in the next cycles (fourth or beyond).
5. In women older than 35, it is possible to transfer up to three embryos after two failed cycles where two embryos were transferred. In the next cycles (fourth or more), it is possible to ET more than two embryos, if the patient did not conceive.
6. In women older than 40, it is possible to transfer up to three embryos from the first cycle.
7. In any case, no more than four embryos should be transferred.
8. The increase in the number of ET/cycle should be gradual, after ET of two/cycle, where no pregnancy resulted, and later three/cycle achieved no pregnancy.

9. In cycles of egg donation, the number of transferred embryos is determined according to the donor's age.
10. In the case of ET of cryopreserved-thawed embryo, the number of transferred embryos should be as in the fresh ET + one.
11. In the calculation of number of previous cycles, the count should include both fresh ET and thawed ET cycles.
12. Following successful delivery of a neonate, the number of transferred embryos should not exceed the number of embryos that ended up in the successful pregnancy.

Despite these regulations, many physicians are frequently facing pressure from the patients and, not infrequently, even from the embryologists, to ET more embryos than recommended. Many physicians, after explaining the risks of multiple gestations, prematurity, ovarian hyperstimulation syndrome, and miscarriages, are still confronting pressure by the patients who are eager to conceive "no matter what…." In cases where there is a deviation from the recommended regulations for ET, the patients are signing an informed consent explaining that the ET was due to their demand, not according to the medical recommendation.

The Israeli IVF registry is still ongoing, regarding the rate of single ET. It is still not recorded what the exact nationwide rate of single ET is. However, the undocumented preliminary impression from a pilot on only one tenth of the cycles, is that only about 18 % of the ETs were single ET, in cycles where more than one egg has been retrieved.

Discussion

The number of IVF/ET cycles in Israel, relative to population size, is probably the highest in the world. A recent world report on ART of the International Committee for Monitoring Assisted Reproductive Technology [3] has found that overall, 1,052,363 ART procedures resulted in an estimated 237,315 babies born. The availability of ART varied by country from 15 to 3,982 cycles per million of population [3]. The rate in Israel is almost 40,000 ART/IVF cycles/year, for a population of eight million, which is about 5,000 cycles/million citizens. Worldwide, with wide regional variations, single ET represented about 17.5 % of cycles [3]. Unlike Scandinavian countries where single ET is the rule, the Israeli experience is quite different.

It is surprising that even in a country where the public system affords almost unlimited free access to IVF for the first two children, in the current marriage (regardless of number of children in previous marriages), the rate of single ET is very low. One would expect that such a liberal availability of IVF access would encourage patients, embryologists, and physicians to experience single ET in the majority of cases. However, this is not the case.

In a fresh IVF cycle, single ET usually generates a lower live birth rate than double embryo transfer. However, there is no evidence of a significant difference in the cumulative live birth rate when a single cycle of double embryo transfer is compared with repeated single ET (either two cycles of fresh single ET or one cycle

of fresh single ET followed by one frozen single ET in a natural or hormone-stimulated cycle) [3]. The advantage of single ET is the significant decrease in the rate of multiple pregnancies. A policy of repeated single ET may minimize the risk of multiple pregnancies in couples undergoing ART without substantially reducing the likelihood of achieving a live birth [3].

Another possible bias, leading to the relatively low rate of single ET in Israel, is the relatively high percentage of older infertile women (above 40 and even above 44) in our IVF population. Due to the liberal and almost free access to IVF up to the age of 45, many old patients refuse to give up their attempt of egg retrieval even when the chance of successful delivery is negligible. In this population of older women, the single ET is almost never accomplished.

Despite all the hopes, some physicians do not believe in single ET. Indeed, a recent publication [4] concluded that elective single embryo transfer does not reduce the risk of preterm delivery associated with IVF. These investigators [4] have found that the overall preterm delivery rate at 20–37 weeks gestation following elective single ET was 17.6 % (269/1,527), significantly greater than the preterm birth rate for all their patients undergoing IVF over the same time period (12 %, $P<0.001$). Furthermore, the 3,125 elective single ET cycles in their study resulted in 1,507 live births (live birth rate 48.2 %). Among their generated deliveries were 27 twins (1.8 %) and a set of triplets (0.07 %), suggesting that elective single ET cannot eliminate the risk of multiple gestations. However, most investigators do not oppose elective single ET, at least in young patients.

It is expected and hoped that the increasing use and popularity of real-time assessment of the embryo's development, using the EmbryoScope system, and at the same time the increasing use of blastocyst transfer instead of day 2–3 transfer may also increase the rate of single ET.

Conflict of Interest The authors have declared no conflicts of interest.

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Chapter 24
Single Embryo Transfer: The Québec Experience

Hélène S. Weibel and William Buckett

Introduction

In Québec, as with the rest of Canada, the population benefits from a universal state-funded health coverage that is administered by the Régie de l'assurance maladie du Québec (RAMQ). Assisted reproductive treatments (ARTs) were not fully covered prior to 2010. These were only available privately, at the patient's personal expense. During this time, embryo transfer practices were similar to those in the rest of Canada and the United States. ART-conceived twin pregnancy delivery rates were 28 %, and high-order multiple pregnancy delivery rates were 1 %. The elective single embryo transfer (eSET) rate was 5.6 %, and 70 % of fresh cycles had double embryo transfer (DET) [1].

The concept that infertility can be considered as an illness raised the idea that it may warrant public coverage. Consequently, in 2000–2001, some financial help for assisted reproduction was initially put in place by the provincial government in the form of tax credits. After the Supreme Court ruled in 2010 that the access to fertility treatments would be under provincial jurisdiction, it allowed Québec to create a fully publicly funded assisted reproduction program [2].

On August 5, 2010, the Québec ministry of health and social services launched the assisted reproduction program, under which treatments offered to patients would be covered by the RAMQ. The three main stated objectives of the program were:

1. To allow infertile couples to have children
2. To increase the birthrate by 1,000 to 1,500 births annually
3. To diminish the proportion of multiple pregnancies issued from IVF from 25–30 % down to 5–10 %

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The establishment of this program was covered extensively in the media and much criticized due to the anticipated high costs and whether or not it could be afforded by the population. A preemptive calculation suggested that with widespread use of eSET, this would reduce the proportion of multiple pregnancies and thus lower the rate of prematurity, neonatal intensive care unit admission, and pregnancy complications. Such savings would be sufficient to cover the costs of the assisted reproductive program.

The Québec Experience

The treatments covered by the public program in Québec include ovarian stimulation or induction and insemination, as well as all aspects of ART, such as IVF, ICSI, cryopreservation, sperm retrieval procedures, and oocyte donation. Fertility preservation for cancer patients as well as preimplantation genetic diagnosis (PGD) for severe genetic diseases is also included in the coverage. A maximum of three stimulated IVF cycles or up to six unstimulated in vitro maturation (IVM) or natural cycle IVF (nIVF) cycles are covered. Supernumerary embryos obtained during these cycles should be cryopreserved, and all good quality embryos must be transferred one by one before starting a subsequent fresh IVF cycle. Single embryo transfer is mandatory for most patients; however, up to two cleavage stage embryos may be transferred in patients younger than 36 years old in exceptional cases or up to three embryos (two if blastocysts) in patients older than 37 years old in exceptional cases. Nevertheless, the decision to transfer more than one embryo has to be justified by the treating physician. Once a couple has a live birth, the number of cycles allowed is restored to the baseline again.

The payment for medications used in ovarian stimulation and assisted reproduction is similar to the standard coverage offered for prescription drugs in Québec. Patients either have their own private insurance or are registered under the RAMQ medication plan which covers a substantial percentage of the cost of medication. The patients are responsible for the remaining balance of the cost of medications.

There are no specific selection criteria defined by the law for accessibility to the program, such as age limits, maximal body mass indexes (BMIs), or comorbidities. Hence, assisted reproductive treatments are available to patients of any age and marital status, as well as to same-sex couples.

Outcomes of the Program

All fertility clinics in Canada, including Québec, submit their data on a voluntary basis to the Canadian Assisted Reproductive Technique Register (CARTR) which enables the Canadian Fertility and Andrology Society (CFAS) to monitor practices and outcomes of ART in Canada. An analysis of these numbers was performed 6

Table 24.1 Summary of the first 6 months of public ART funding in Québec

	Before funding (%)	After funding (%)
# eSETs	1.6	49
% clinical pregnancy per embryo transfer	42.7	31
Multiple pregnancy rate	27.2	5.2

months after the introduction of the public funding program in Québec, and the results were quite encouraging.

In 2009, prior to the introduction of the program, elective single embryo transfer (eSET) was performed in 1.6 % of IVF cycles in Québec and 5.6 % of Canada overall. The multiple pregnancy rate was 27.2 % provincially compared to 30.9 % nationally. Six months after the introduction of the program, eSET was performed in 49 % of Québec IVF cycles overall and 78 % of IVF cycles in patients younger than 35 years old. The clinical pregnancy rate was 31 % compared to 42.7 % nationally, but the most significant effect was the marked decrease in the rates of multiple pregnancies, from 27.2 % to 5.2 % (Table 24.1) [3].

As these data suggest, an increased proportion of elective single embryo transfer is clearly associated with a major decrease in the number of multiple pregnancies. However, this may be at the cost of a lower clinical pregnancy rate per embryo transfer. Nevertheless, there is some evidence that an equivalent clinical pregnancy rate per cycle is obtained after serial frozen/thawed eSET is performed [4, 5]. A study looking at cumulative pregnancy rates in a single center in Québec after the first year of implementation of the program reported pregnancy rates before coverage in patients of all ages after a fresh cycle to be the same as the cumulative pregnancy rate per cycle initiated after public coverage, without any statistically significant difference [4].

A randomized controlled trial was also recently published to study eSET vs. DET in good prognosis patients <38 years old. The results showed pregnancy rates similar after DET (46.9 %) and cumulative eSET (49.1 %). The live birthrates were also similar in the two groups: 42.2 % vs. 38.6 %, respectively. Interestingly, there were no multiple pregnancies in the eSET group, whereas the DET group had a multiple pregnancy rate of 27.6 % [5]. The higher pregnancy rate quoted in the second study is likely due to the fact that their study population was younger and had a good prognosis.

Over a longer term, it has been interesting to see if that effect of IVF public funding and mandatory eSET on treatment outcomes has been maintained. In 2013, the health ministry requested an analysis of the current situation by the Québec health and welfare commissioner. His report was published in 2014 [6] and offers a wealth of information on the program itself and its outcomes on population statistics. The data used in the analysis was mainly obtained from three sources: the Canadian ART Register of the CFAS, from billings of medical acts to the RAMQ, and lastly from the ministry-run MED-ECHO database, which collects statistics about hospitalization of patients. The numbers are extracted from MED-ECHO using mainly coding for admission diagnosis.

Proportions of eSET and Pregnancy Rates

First, looking at assisted reproduction techniques themselves, there has been a continued increase in the practice of eSET throughout the period of public coverage as well as a sustained decrease in multiple pregnancy rates. As demonstrated in Fig. 24.1, the rate of elective single embryo transfer dramatically increased after the implementation of public funding, from 1.6 % of all transfers to 49 % after 6 months, 62 % after 12 months, and 71 % of all transfers after 2 years. This change in practice remains the main explanation for the subsequent decrease in multiple gestations, from 27.8 % to 6.8 %. However, this success was at the cost of a decrease in the clinical pregnancy rates per single fresh cycle from 37.6 % to 22.4 % over the 3 years [6]. A clinical pregnancy was defined as intrauterine gestation (presence of a gestational sac on ultrasonography), ectopic pregnancy, or miscarriage diagnosed by histology. Cycles with only a positive pregnancy test (biochemical pregnancy) were not considered to have a clinical pregnancy [7].

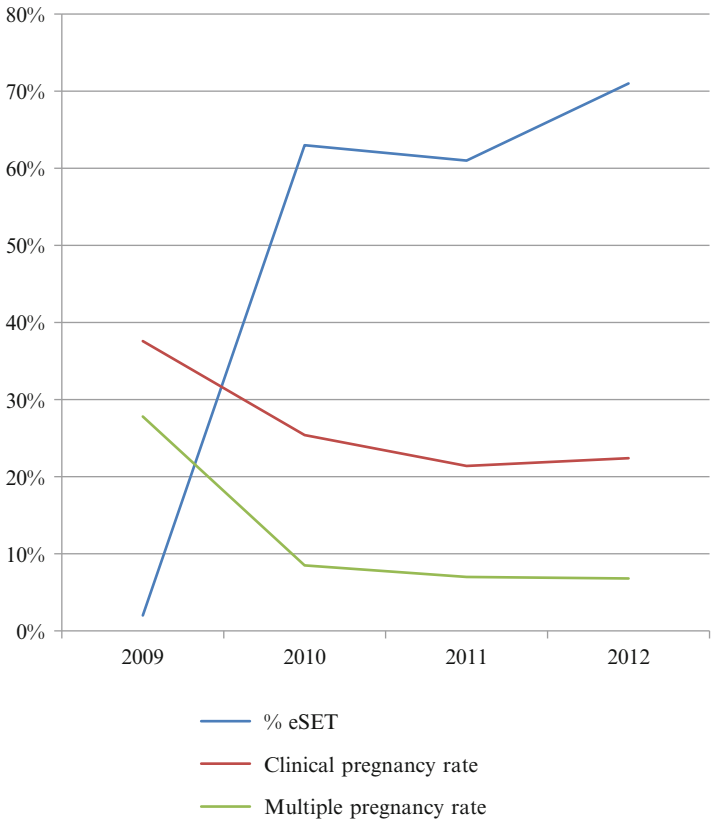

Fig. 24.1 Single embryo transfers, pregnancy rates, and multiple gestation rates, 2009–2012 [6]

Neonatal Outcomes

It is intriguing to explore whether this decrease in multiple pregnancy is translated into a clinically significant difference in neonatal outcomes. The hypothesis is that less multiple pregnancies should result in a diminution in premature births and consequently less neonatal intensive care admission. Data available from hospitalization of patients and admission codes was published in the commissioner's report and is presented in Fig. 24.2. Overall, in the assisted reproduction population, the rate of preterm birth at less than 32 weeks and less than 37 weeks has diminished over the course of the program: from 4.9 % to 3.8 % and from 29.6 % to 19.1 %, respectively. Interestingly, NICU admissions were also decreased, from 18.8 % down to 11.8 % of all the births following assisted reproduction. However, average hospital stay was increased, especially for neonates born under 36 weeks of gestation. Neonatal death rate remained unchanged. Whereas the diminution in preterm birth and NICU admission is significant when analyzing the population of neonates born after ART, the effect is not seen when the whole population of newborns is taken into account. This is likely due to the fact that the ART-issued babies account for only 2 % of the newborn cohorts in Québec and therefore not in a proportion large enough to affect the overall population outcomes. The main limitation of this data however is the fact that it was obtained retrospectively, from charts review. Also the MED-ECHO databank is not linked with the RAMQ billing figures; hence, not all IVF cycles are necessarily accounted for.

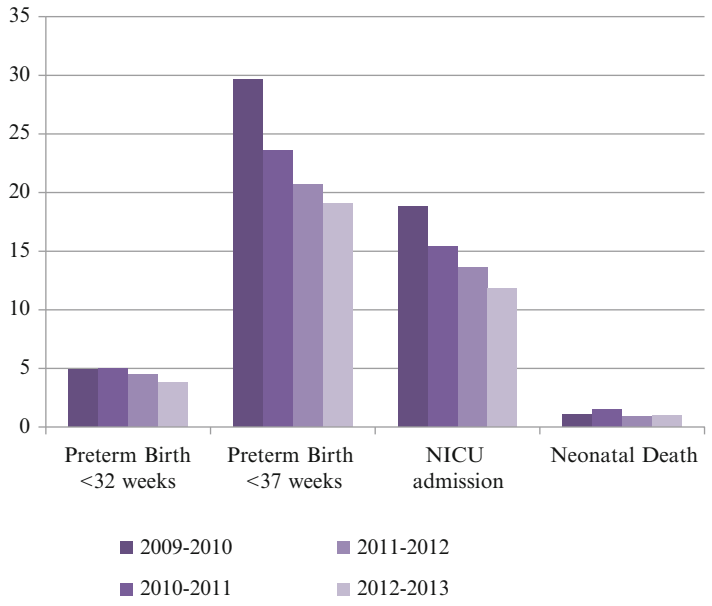

Fig. 24.2 Neonatal outcomes of ART-issued pregnancies after IVF funding in Québec [6]

Obstetrical Outcomes

It is well known that ART pregnancies and especially multiple gestations are associated with adverse obstetrical outcomes. Cesarean section rate, maternal hospitalization frequency, and stillbirth rate were analyzed in the commissioner's report. Interestingly, cesarean delivery rate in the assisted reproduction patients increased, mainly in singleton pregnancies, from an initial rate of 28.0 % to 37.0 %. The reasons for this are unclear and may reflect increased numbers of patients with concomitant diseases now accessing treatment. For the multiple pregnancies, there was a decrease in the rate of cesarean birth from 74.4 % to 66.0 %. Maternal hospitalization rate also increased, from 32.2 % to 37.4 %. The main reason documented for maternal hospitalization was "increased surveillance for advance maternal age" (Fig. 24.3).

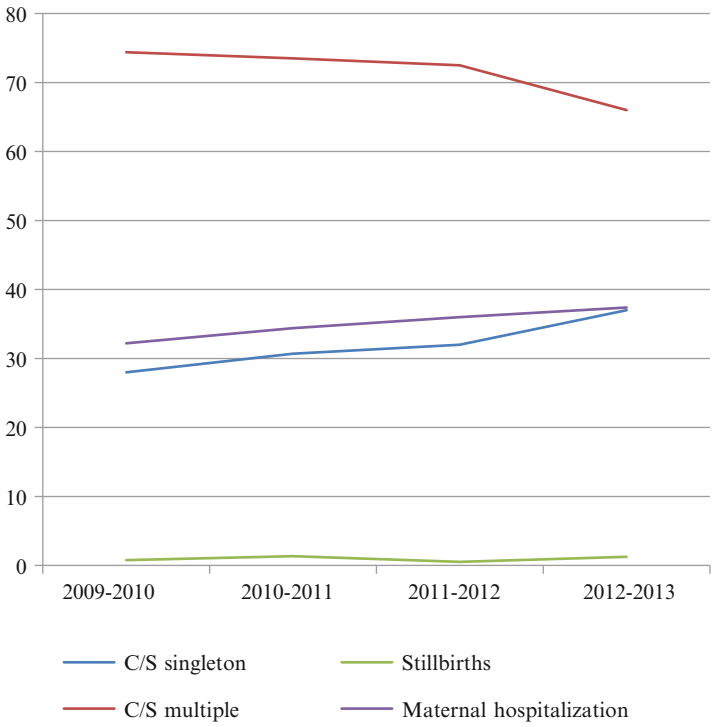

Fig. 24.3 Obstetrical outcomes among assisted reproduction pregnancies since public IVF funding in Québec [6]

Summary of the Québec Outcomes with eSET

In summary, we can see that the implementation of a public program to fund fertility treatments, along with strict rules regarding elective single embryo transfer, has had a significant impact on clinical outcomes of treatments. Obviously, eSET rate has increased tremendously, and this has now become the norm. This is possibly at the expense of a lower pregnancy rate per fresh embryo transfer. However, there seems to be evidence that cumulative pregnancy rates per cycle are similar if transfers of cryopreserved embryos are taken into account. As a result of increased eSET utilization, the number of multiple pregnancies has definitely been greatly reduced, down to less than 10 %. Most importantly, high-order multiple pregnancies have been virtually eliminated. This proportion of ART twin pregnancies is still higher than the spontaneously conceived pregnancy rate measured in the general Québec population, where incidence of twin pregnancy is reported at around 1.3 %. This reduction in multiple pregnancies seems to be reflected clinically in a diminution of preterm births and NICU admissions of neonates conceived with ART. As we know that prematurity is a major cause of mortality and morbidity in newborns, this is undeniably a remarkable effect. In this regard, the public program in Québec can be viewed as an efficient harm-reduction method. It is important to note that the overall preterm birthrate or NICU admission in the general population was unchanged, but this is likely due to the fact that the assisted reproduction babies account for a mere 2 % of the birthrate in Québec.

Québec Versus the Rest of Canada

Obviously, since 2010, there has been an increase in the uptake of eSET everywhere in the world. However, comparing the data from Québec (which had a very similar practice to the rest of Canada before 2010) with the data from the rest of Canada since the introduction of RAMQ-funded ART coverage can determine the impact of universal coverage of the uptake of eSET and the clinical outcomes.

As noted above, uptake of eSET in Québec increased dramatically in the period 2009–2012 [1, 6–9], and although the use of eSET in the rest of Canada has increased, it is still very low. See Fig. 24.4.

Although clinical pregnancy rate per fresh embryo transfer has fallen since the start of funding, when compared with all cycles for all patients throughout the rest of Canada, the rates are similar. As noted above, the clinical pregnancy rates per single cycle fell from 37 % in 2009 to 24 % in 2012. This compares with clinical pregnancy rates in Canada as a whole over the same period which fell from 34 % in 2009 to 28 % in 2012 [7–9].

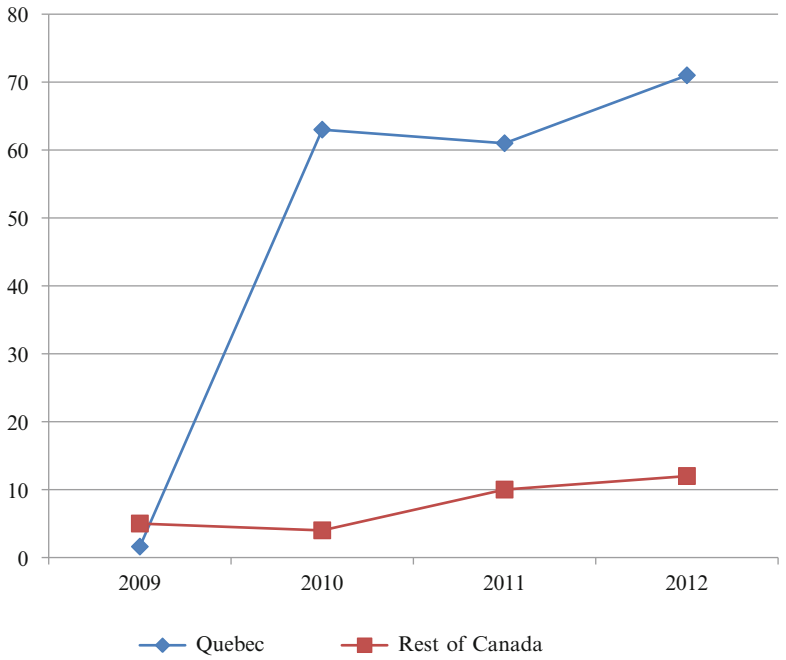

Fig. 24.4 Elective single embryo transfer utilization rates reported in Québec and the rest of Canada, 2009–2012

Finally, although multiple pregnancies have fallen in both Québec and the rest of Canada, the rate of decline has been much faster in Québec. As noted above, the rate of triplet pregnancies, although low in the rest of Canada at 0.5 %, is zero in Québec. In 2012 in the rest of Canada, still 18 pairs of triplets were born. See Fig. 24.5.

Population Perspective on Public Funding of Fertility Treatments

The commissioner's report included a survey of the general Québec population done by an independent firm to sample public opinion regarding this program. Around 1,000 citizens were surveyed and asked various question about the public funding of IVF. The overall consensus was that the population is empathic to the problem of infertility, and they would have a lot of difficulty dealing with such an issue. It was also believed that the program gives a positive image of Québec at the national and international level, namely, the fact that everyone has access to treatments, regardless of marital status or sexual orientation. This projects an accepting

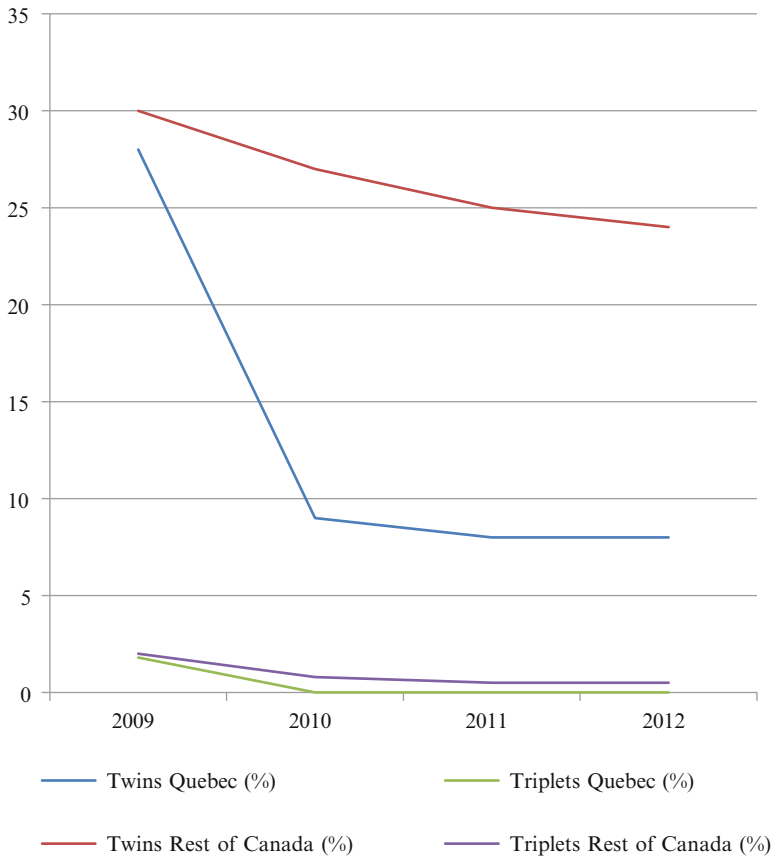

Fig. 24.5 Multiple pregnancies in Québec and the rest of Canada since the start of RAMQ funding (2009)

and tolerant image which is celebrated by many. There is also a positive perception that the funding fertility treatment provides safe healthcare and protects women and their future children by providing reasonable guidelines, avoiding excess and complications.

Patient Perspectives

In general, patients in the fertility clinic are grateful to have access to treatments they could otherwise not afford. A number of Québec patients who needed ART to conceive were waiting for public funding in order to start treatments. This leads to a dramatic increase in the number of IVF cycles performed in Québec annually,

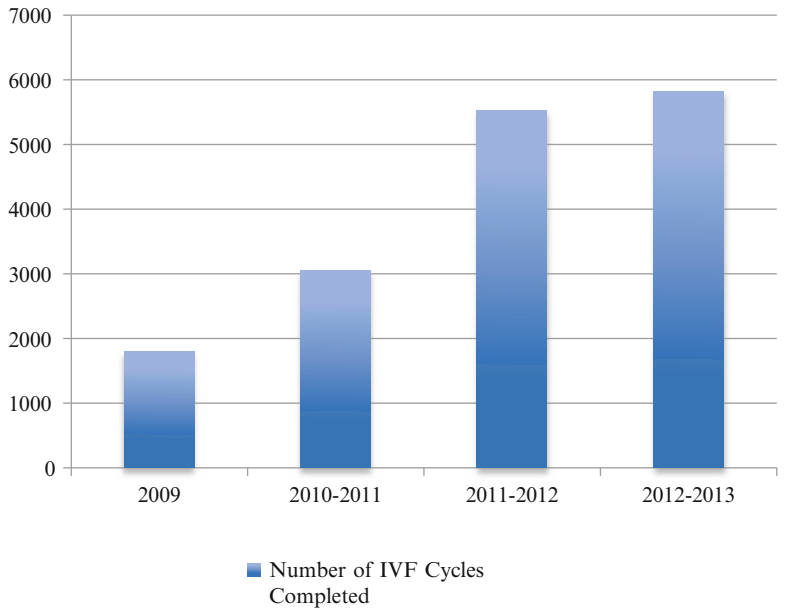

Fig. 24.6 Number of completed IVF cycles performed annually in Québec since public coverage of ART [6]

from an average of 2,000 IVF cycles to over 5,000 cycles in 2013, as illustrated in Fig. 24.6. Often, it means that there will be delays for initiation of treatments. Early after the initiation of the program, some couples requested DET; however, the new recommendations were widely disseminated, and now, there are virtually no concerns from patients regarding eSET. It has become a norm that is universally accepted; the expectation of the patients in Québec now is to have one embryo transferred per cycle.

IVF Practitioner and Staff Perspectives

For staff working in a reproductive center, this increase in activity meant having to deal with a larger volume of patient all of a sudden. According to the staff, there does not seem to be a real change in cumulative pregnancy rates since the implementation of obligatory eSET. However, eSET in every patient does translate into more cycles per patient. Indeed, most IVF patients in Québec will have at least 1 or 2 frozen embryo transfers per fresh IVF cycles, and some will undergo many more. Another counterpart of a widely instituted eSET policy is the increase in number of cryopreserved embryos, causing an increased need for supplies to store such embryos as well as a need for increased clinic storage space.

Perinatology Perspectives

In general, mandatory eSET is applauded by physicians working in the field of high-risk obstetrics. It is a remarkable achievement that ART-conceived high-order multiples have seemed to be completely eliminated. A large diminution in the number of ART-conceived twin pregnancies was also noticed. Obstetricians have consequently noticed a large decline in premature labor and deliveries, especially extreme prematurity cases. These impressions are well reflected in the commissioner's report numbers presented earlier. However, there seems to be an increase in complex obstetrical cases and high-risk pregnancies, such as those in advanced maternal age, in patients with chronic disease, or in patients post-cancer treatment who benefited from fertility preservation before radiation or chemotherapy.

Neonatology Perspectives

When neonatologists were asked whether they noticed a significant change in their patient population since 2010, their answer was noteworthy. Before 2010, most provincial neonatal intensive care units would have at least one set of ART-conceived triplets at any one time and often additional sets of ART-conceived twins or triplets. Now 4 years after Québec's mandatory eSET policy, it is extremely rare to see any triplets or high-order multiple births in any of the neonatal intensive care units. The only cases currently are either spontaneously conceived high-order multiples or from patients who have had treatment outside Québec.

It is important to note that such cross-border reproductive care is likely to be much less than the quoted rates of 5 % in the European Union and of 4 % in the United states, although absolute numbers are unknown [10]. The four main reasons for patients to rely on cross-border reproductive care are accessibility, cost, regulations, and privacy [10]. With full public coverage, and the fact that various other treatments such as PGD and fertility preservation are easily accessible to patients, the issue of cost is eliminated. This also goes along with the concept of harm reduction, as it may help avoid cross-border reproductive care, including its potential harms, such as health and safety concerns, harm to local surrogate and gamete donors, as well as harm to local population [10].

Conclusion

As described, government subsidization of assisted reproduction in Québec with mandatory single embryo transfer has caused this to be the prevailing ART mode of care here. As a result, high-order multiple births have been virtually eliminated, and the rate of ART-conceived twins has been greatly reduced. However, this benefit is associated with a lower clinical pregnancy rate per fresh embryo transfer cycle.

Interestingly, the clinical impact of eSET was reflected into a lower premature birthrate and lower neonatal ICU admissions, both of which are commended by high-risk obstetrics specialists as well as neonatologists. Infertile couples are grateful to have access to such treatment regardless of their financial abilities to pay. Consequently, single embryo transfer has become universally accepted and is now routinely expected by the couples undergoing ART.

Conflict of Interest The authors declare no conflict.

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Chapter 25
Regulatory Aspects of Embryo Testing: An American View

Richard F. Storrow

Introduction

For decades, the high incidence of multiple gestation in the practice of assisted reproductive medicine has been of concern to infertility physicians and regulators alike [1]. The interface between government regulation and medical practice has brought varied responses to bear on this problem. One initiative is the move toward single embryo transfer (SET) in IVF, which is now recommended by physicians' groups and mandated by some governments. Within this initiative, the quest to find the best method of identifying the euploid embryo, the chromosomally normal embryo with the best chance of leading to a healthy pregnancy and healthy offspring, is ongoing [2, 3].

Preimplantation genetic screening (PGS) and preimplantation genetic diagnosis (PGD) are terms often discussed together to designate tests that help clinicians and their patients select the proper embryo to transfer toward the conclusion of an IVF cycle. Although the techniques used are similar, the objectives of PGS and PGD are distinct. Through PGS, doctors aim to identify euploid embryos so that a pregnancy can be achieved and maintained [4]. PGD contemplates screening embryos for specific genetic markers, either to select against embryos possessing anomalies that cause disease in favor of embryos possessing certain nonmedical traits like gender [5]. Thus, whereas PGS is indicated primarily for couples who struggle to become pregnant or who suffer recurrent pregnancy loss, PGD is appropriate for both infertile and fertile couples.

Unlike in other countries where SET is mandated, there has been less movement in this direction in the United States [1]. The high cost of infertility care in this country and the lack of insurance coverage for it create anxiety among patients

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whose motivation toward maximizing the chances of pregnancy conflicts with values inherent in the move to SET [6]. The doctor and the patient may thus find themselves at odds [1]. In the current environment, it behooves medical professionals conducting PGS and PGD to be familiar with the legal and other regulatory dimensions of their practice area.

Methods

This investigation employed a systematic review of published sources on law, bioethics, and reproductive health policy.

Results

The key sources of regulation impacting on the practice of the genetic testing of embryos in infertility clinics include the US Constitution, statutes and administrative regulations, medical malpractice law, and professional norms. Beyond rules that prohibit discrimination, that guarantee the privacy of patient information, and that govern molecular genetic testing in laboratories and the qualifications of genetic counselors, little in the Constitution, statutes, or administrative regulations bears directly on PGS and PGD. This leaves medical malpractice law and the norms of professional societies as the primary regulatory mechanisms that define the standard of care and the requirements of informed consent in embryo testing for IVF. Fertility societies may wish to bring their influence to bear on legislative initiatives to regulate insurance coverage for IVF so that the movement toward SET may be more fully realized.

Discussion

In a classification of the regulation of embryo testing as liberal, prohibitive, or cautious, the United States might well rank as "laissez-faire," a classification reserved for countries with almost no regulation whatsoever [7]. That virtually no federal law or regulation directly addresses embryo testing in infertility care may partly be a function of the fact that legislative competence in the area of medical practice currently lies with the states by virtue of the Tenth Amendment to the United States Constitution. The lack of regulation at the state level as well may have to do with the battle over abortion that continues to rage in this country. Since the regulation of IVF and related practices invariably triggers questions of the status of the embryo, politicians are loathe to grapple with this issue for fear of alienating certain constituencies.

The Constitutional Overlay

The US Constitution acts as a brake on legislation and other state action that impinges on the procreative liberty of individual citizens. Procreative liberty is a negative right guaranteeing against governmental interference in the exercise of procreative aims but not guaranteeing any assistance toward the accomplishment of those aims. Although commentators often posit that restrictions on access to assisted reproduction raise ethical issues of procreative autonomy [8], whether procreative liberty subsumes resort to assisted reproduction as a legal matter remains an academic question. The federal courts have made only a very few discrete pronouncements on the matter [9, 10]. For example, a lower court has held that chorionic villi sampling within the first trimester of pregnancy, falls within the ambit of constitutionally protected procreative freedom, since it is designed to provide information relevant to keeping or terminating a pregnancy [10]. Because PGS and PGD provide information relevant to commencing a pregnancy, they, too, might fall within the protected ambit. Restrictions on them would therefore likely be constitutionally infirm, but a more solid prediction is difficult to make. The issue is, moreover, unlikely to arise with any frequency in a system where so little regulation exists.

For clinics engaged in embryo testing for IVF, the relevance of the Constitution's guarantee of procreative liberty will become clearer if the issue of embryo testing ever reaches the US Supreme Court. The Court could easily draw a distinction between PGS, the aim of which is successful procreation, and PGD, whose aim is the selection of an offspring's traits. The Court might be of the opinion that procreative liberty does not extend to PGD. Moreover, the Court might decide that the line should be drawn between embryo testing and prenatal testing, with the latter on the side of procreative liberty because it implicates a pregnant woman's bodily integrity. A very conservative Court could well determine that neither prenatal testing nor embryo testing are exercises of procreative liberty.

Whether a clinic would ever have to defend itself against a patient's claim of procreative liberty is doubtful. Most infertility clinics in the United States are private entities. Their activities thus do not constitute the state action that is a prerequisite for a valid constitutional claim. Where a clinic is an arm of the state, as where it is a unit within a public university, the Constitution does apply to its actions. But being a public facility in no way means that a clinic is bound to provide any particular service. The most viable constitutional claim in such a context would be one alleging class-based discrimination in the delivery of care. Even then, a clinic may escape liability if the targeted group is not one that receives the highest level of protection in the litigation of individual constitutional rights. At present, regulations that treat individuals differently based on matters of race and ethnicity receive the highest level of judicial scrutiny in constitutional rights cases. Discrimination against other groups, though, may be prohibited by statutes that apply to both public and private facilities (see section "Statutes and Administrative Regulations," below).

For any legislation to be a proper exercise of governmental power, it must promote public health, safety, welfare, or morals and utilize means that are at least rationally related to those goals. To satisfy the Constitution, though, legislation must not be so

vague as to leave unclear what conduct it prohibits. Infertility physicians in Illinois raised vagueness in their challenge of a prohibition on nontherapeutic fetal experimentation [10]. The court agreed that the statute failed to define "experimentation" and "therapeutic" and so left unclear whether it prohibited the physicians' use of evolving prenatal diagnostic techniques. With respect to embryos, statutes in other states contain similar prohibitions on experimentation, but most of these appear to address research on embryos [11]. Since PGS and PGD for IVF are not research experiments and are perhaps routine enough not to be considered experimental [12], these statutes arguably do not apply to these techniques.

Statutes and Administrative Regulations: Privacy, Safety, and Equality

Statutes and administrative regulations are codified rules enacted by legislatures and the agencies to which they delegate rulemaking authority. At the federal level, Congress often delegates rulemaking power when special expertise is required to implement the provisions of a statute. Administrative agencies thus become "arms of Congress" and must act consistently with their statutory mandate.

Very little in either American statutes or administrative regulations bears directly on embryo testing for IVF. Nonetheless, there are several provisions of which clinicians should be aware. These provisions aim to promote privacy and safety in matters of genetic testing and to combat the discrimination that might occur were sensitive genetic information to fall into the wrong hands.

The Centers for Disease Control (CDC) and the Food and Drug Administration (FDA), divisions of the US Department of Health and Human Services, are tasked with protecting the United States from health, safety, and security threats and regulating biological products for human use, respectively. The CDC's regulation of assisted reproduction lies in its implementation of the Fertility Clinic Success Rate and Certification Act. This statute requires clinics that provide IVF services to make annual reports of their success rates to the federal government. These reporting requirements do not include information about the use of or results achieved from PGD [13].

The FDA specifically regulates "human cells or tissues intended for implantation," [14] a category that includes oocytes and semen. The FDA's specific goals are "to ensure that donors do not harbor infections that could be transmitted to recipients" [15] and to minimize the risk of contamination in the handling of human tissues. The governing rules require establishments that handle human cells and tissue to register with the FDA and require screening and testing of tissue donors "for risk factors for, and clinical evidence of, relevant communicable disease agents or diseases" [16]. The necessary screening does not, however, require genetic testing of tissue donors (Anderson H., US FDA 2014, personal communication). The testing requirement also does not extend to "[r]eproductive cells or tissue donated by a sexual intimate partner of the recipient for reproductive use" [17]. The FDA sometimes inspects establishments for compliance with these rules. The FDA also regulates medical devices, such as products used to perform genetic tests [18]. Whether

the FDA is competent to regulate the genetic testing techniques developed by genetics laboratories in-house has been the subject of debate in recent years. The FDA has, however, issued a set of nonbinding recommendations for the regulation of laboratory-developed genetic tests in some cases [19]. Once a medical device is approved by the FDA, the actual use of it by physicians does not fall within the purview of its regulatory authority.

The Clinical Laboratory Improvement Amendments (CLIA) aim to ensure the quality of laboratory testing through a certification program. The program is administered by the Centers for Medicare & Medicaid Services, another division of the US Department of Health and Human Services. The Amendments apply to laboratories that conduct assays on human bodily material in the course of medical treatment. Laboratories that conduct genetic testing must meet the basic criteria for labs performing high complexity tests generally. To enhance this oversight, the CDC has promulgated a set of good laboratory practices in molecular genetic testing for heritable diseases and conditions (good laboratory practices in biochemical genetic testing are the subject of a separate CDC publication). The practices address the qualifications of laboratory personnel, the testing process, and the privacy of patients' information, among other things, but do not explicitly refer to the genetic testing of embryos for transfer [20].

At the state level, there is virtually no direct regulation of PGD, except for laboratory quality assurance programs requiring laboratories performing PGD to acquire a permit [21–23]. Under New York's Clinical Laboratory Evaluation Program, which regulates laboratories performing PGD on specimens originating in New York, a laboratory must "obtain the subject's informed consent and include in their reports a statement of and an interpretation of its findings, the test's technical limitations, suggestions for additional testing, recommendations for referral to a genetic counselor (if applicable), the test methodology, and a list of all variants examined in the assay" [24]. Although a waiver procedure is available, New York's permit requirement has produced anxiety among clinics that the limited number of permitted labs capable of providing specialized assessment of embryos and the tight turnaround time required for IVF will impact negatively on patients [25]. Currently 18 laboratories in New York State and 59 outside of New York have permits to perform molecular genetic testing. Not all of these laboratories, though, offer PGD.

Genetic counseling has also been of interest to state regulators in recent years. Several states require genetic counselors to be licensed, often in conjunction with the certification programs established by the American Board of Genetic Counseling or the American Board of Medical Genetics [26]. These licensing schemes do not in all cases apply to licensed physicians who provide genetic counseling [27] but also may not permit physicians to call themselves genetic counselors without procuring a license [28].

The Health Insurance Portability and Accountability Act (HIPAA) provides minimum standards for ensuring the confidentiality of patients' health-care information. Under HIPAA, laboratories that conduct molecular genetic testing must take steps to "ensure the confidentiality of patient information, including molecular testing information and test results" [29]. Some states have similar privacy laws that explicitly apply to genetic testing and define genetic information as protected health

information [30] or as the property of the individual to whom the genetic information relates [31]. These laws limit the ways in which health professionals may use what they uncover in the course of examining embryos destined for IVF. Civil and criminal liability attaches to the violation of genetic privacy laws [32].

Finally, discrimination in matters of genetic testing is forbidden by statute. The federal Genetic Information Nondiscrimination Act (GINA) [33] and analogous state laws prohibit health insurance carriers and employers from discriminating against individuals based on their genetic information. The drafters of GINA were concerned that discrimination could occur against healthy individuals based solely on their genetic predisposition toward certain diseases. The statute's implementing regulations explicitly include preimplantation genetic diagnosis on embryos created using IVF within the definition of a genetic test [34], and "[g]enetic information" includes "genetic information of any embryo… " [35].

It is difficult to imagine a cognizable claim of discrimination being brought against infertility physicians under this enactment based on the use of information disclosed by PGS or PGD. First of all, to constitute a discriminatory act under GINA, the selection would have to be based on genetic information and not simply on morphology. More importantly, GINA was passed to combat discrimination in the workplace and in the issuance of health insurance. The Act does not implicate differentiating between embryos in the clinic in pursuit of SET, because the selection and de-selection of embryos for this purpose do not relate to employment or to the issuance of health insurance.

Unlike GINA, there are other antidiscrimination provisions that do relate directly to the conduct of clinics. Oklahoma's Freedom of Conscience Act, for example, prohibits employers from discriminating against personnel who refuse for religious reasons to perform a "medical procedure on an in vitro human embryo that is not related to the beneficial treatment of the in vitro human embryo" [36]. Whether this provision relates to the selection and de-selection of embryos via PGS or PGD remains unclear. State statutes prohibiting discrimination in public accommodations also apply to clinics. These statutes do not compel clinics to offer embryo testing services, but the refusal to serve a patient in a specific case because of the patient's sexual orientation or marital status is illegal in some states. A doctor's religious objection would likely be inadequate to defend against a charge of protected class-based discrimination in the provision of care [37]. Any discrimination in the delivery of care would most likely occur well before the point of embryo testing; nonetheless, clinics that offer PGS and PGD will need to be aware of state and local antidiscrimination laws when making determinations about which patients they will allow to receive these services.

Medical Malpractice

Medical malpractice is a type of tort liability applicable where injury to a patient is caused by a physician's failure to discharge a duty of care toward that patient or the physician fails to obtain a patient's informed consent to treatment. Liability for

medical malpractice in the United States is determined by courts deciding individual cases and is remedied by awards of money damages. Without question, with growing scientific understanding of human genetics and the perfection of new diagnostic tools, medical malpractice liability in the genetic screening and testing realm is expanding [38].

Courts have traditionally deferred to professional custom to define physicians' duties of care. Some states have enacted statutes codifying this deferential stance; [39] others have enacted statutes and administrative regulations that define the standard of care for certain practice settings [40] or that specify the elements of informed consent for certain procedures [41]. In the field of reproductive medicine, courts lacking legislative guidance would be likely to take into account standards of care established by physicians' societies like the American Society for Reproductive Medicine (ASRM) (see section "Professional Norms," below). Indeed, some statutes defining the standard of care refer explicitly to the Society's standards [42]. Despite this development, there is no truly uniform standard of care for the practice of infertility medicine in the United States [43].

In the context of preconception or preimplantation screening, medical malpractice liability has been imposed primarily in cases where the harm at issue arose from the negligent screening of gametes or negligent preimplantation counseling [44]. For example, in one case, the clinic knew the egg donor was a carrier of cystic fibrosis but did not undertake to ascertain whether the biological father was also a carrier of the disease [45]. The intended parents alleged that the clinic had been negligent in its preconception and preimplantation counseling and had deprived them of informed consent. Such claims may be dismissed if they are used to disguise what it is essentially a claim of wrongful life brought on behalf of the child. The legal theory of wrongful life is that one may recover damages against a physician if it would have been better not to have been born at all [46]. Whereas courts may reject such claims as better suited to resolution by philosophers or theologians, similar facts have supported claims of wrongful birth, under which parents seek to recover for the cost of raising a disabled child [45].

A recent study documented medical malpractice claims arising from negligently performed PGD [47]. The authors surveyed lawsuits brought against clinics based on theories of negligence as well as those brought based on a failure to obtain informed consent. Within this latter group were allegations that the patients were not told of the particular clinic's inexperience with PGD, to what extent PGD can be error-prone, or even that PGD was an option. Such cases do not specify exactly what physicians should tell patients about PGD. But they do counsel that, at the very least, patients should understand the many uncertainties of PGD, including that the smaller number of embryos available for implantation following PGD makes "pregnancy expectation following PGD somewhat less than for IVF in general" [48]. Likewise, patients agreeing to PGS should know of its limitations, particularly within certain patient populations. For either PGD or PGS, patients should understand that it is unknown whether the biopsy itself might be a source of harm, even though at the present time experts are doubtful [49]. The practice guidelines of the ASRM would be quite useful to clinics interested in developing an informed consent protocol (see section "Professional Norms," below).

The clinician offering PGS and PGD must not only be capable of explaining to patients the goals and techniques of these procedures, but, in the case of PGD, of detecting genetic disorders so as to counsel patients appropriately. This specific duty in the context of PGD is an extension of the general duty of an obstetrician to be "alert to the detection of genetic disorders or other conditions in the patient that could lead to birth defects" [50]. Indeed, the typical factual predicate in cases where liability is imposed for negligently performed PGD is also that a child has been born with a disorder that a properly performed PGD would have disclosed. Negligently performed PGS, however, would normally result in no pregnancy at all, a risk infertility patients already assume given the current state of the technology of IVF. It is thus difficult to see how PGS could result in malpractice liability, unless negligent handling of the embryos resulted in their being rendered unsuitable for transfer at all [51].

Professional Norms

As made clear above, most aspects of infertility clinics' practice are not governmentally regulated in the United States. A majority of clinics oppose governmental regulation but do not resist regulation from within the profession [52]. As such, voluntary professional organizations play an important role in the oversight of PGD [53].

The self-regulation of reproductive medicine physicians consists of a certification offered by the American Board of Obstetrics and Gynecology or the American Board of Urology and membership in the American Society for Reproductive Medicine (ASRM). It is estimated that over 95% of infertility clinics in the United States are members of the Society for Assisted Reproductive Technology (SART). A clinic's membership in SART is made contingent upon its adherence to ASRM's guidelines and minimum standards, the qualifications of its staff, accreditation of its reproductive laboratories, and its reporting of its success rates to the CDC [54, 58]. There are no legal consequences for physicians or clinics that elect not to be members of ASRM or SART, but of course consumers may prefer clinics that are members to those that are not.

The ASRM's practice guidelines relating to PGD are aimed at the treatment of couples at risk for conceiving a child with a genetic disease or other abnormality. They recommend counseling about the risks of extended culture and embryo biopsy and the risk of misdiagnosis in PGD, which may lead to the "transfer of an affected embryo thought to be normal or the discard of a normal embryo thought to be affected" [55]. The opinion recognizes that both PGD and PGS can be used to exclude embryos unsuitable for transfer, but with respect to PGS specifically recommends counseling patients that a false positive result "may lead to the discard of a normal embryo" and that a false negative result "may lead to the transfer of an abnormal embryo." These guidelines would be relevant in a malpractice action (discussed above) to establish the standard of care with respect to the state of the science and to define the scope of the duty to inform. Indeed, they were specifically raised by the plaintiffs in a case that later settled before trial for $1.3 million [45].

Apart from its practice guidelines, ASRM has issued a body of ethical pronouncements intended to advise clinics. As a part of this ethics initiative, the ASRM has issued two guidelines related to the genetic testing of embryos, one addressing sex selection and the other the detection of adult-onset diseases. Although ASRM believes that sex selection for the purposes of disease prevention is ethical, it rejects using PGD for sex selection for nonmedical reasons [56]. As long as sperm-sorting techniques are safe and parents "affirm that they will fully accept children of the opposite sex if the preconception gender selection fails," ASRM does approve of preconception sex selection for family balancing or for first children, because it imposes fewer burdens on embryos and parents [57].

ASRM has recognized IVF with PGD as "a major scientific advance" over post-conception diagnosis and pregnancy termination [55]. Of using PGD to screen for adult-onset diseases, ASRM makes a distinction between serious and less serious adult-onset conditions. It concludes that PGD is ethically justified in cases of serious conditions where interventions for the conditions are nonexistent, ineffective, or burdensome. PGD is also justified in cases of lesser severity as long as PGD is a low-risk procedure [59]. The Committee urges the participation of an experienced genetic counselor to assist patients considering PGD.

Although it is thought that "most practitioners follow [ASRM's ethical] guidelines," the guidelines themselves are in the nature of standards for self-regulation only [52, 54]. A lack of downward pressure on clinics from either the legal system or the primary professional association with regard to these may mean that some IVF clinics do not deliver PGS and PGD in precisely the way ASRM advises. Both the practice guidelines and the ethics pronouncements contain, however, important reminders that clinics, whether or not members of a professional society, must fully inform patients about the risks of any procedures performed so that they may make considered judgments about how to proceed. This advice to clinics, if not heeded, could have legal consequences (see section "Malpractice," above).

Insurance

Financial limitations on the ability of patients to afford PGS or PGD have been identified as barriers to the acceptance of SET as the norm in infertility clinics. At the same time, studies have concluded that IVF with PGD can be highly cost-effective in comparison with prenatal diagnosis and pregnancy termination or the cost of raising a sick child [60, 61]. This research is transferable to the context of PGS for SET, it being well known, for instance, that the high incidence of multiple gestation in assisted reproduction is costly not only for individuals but for society at large [1]. For this reason, ASRM believes that broader insurance coverage of assisted reproduction "could promote the most medically appropriate procedures and reduce the incidence of multiple births with their accompanying risks and costs" [62]. This transformation would occur from two directions. If insured, patients who could otherwise afford fewer rounds of IVF would not be as driven toward the

transfer of multiple embryos; insurers on the other side of the equation would likely require that providers adhere to ASRM's guidelines, as is already true in a handful of states. With patients, physicians, and insurers on the same page, more progress could be made toward establishing SET as a professional norm.

The lack of public insurance for IVF in the United States contrasts sharply with what by comparison in other countries seem to be lavish public subsidies. Public funds, like those available under New York's Infertility Demonstration Program, are rarely available, and most states, unfortunately, do not mandate that private insurers cover or offer to cover infertility care. Of those that do, the statutes vary considerably. Some even exclude IVF, suggesting a lack of coverage for PGS and PGD, which require IVF and may also be considered insufficiently proven therapies. One restriction common to insurance mandates is that coverage extends only to heterosexual couples who have medically diagnosed infertility. Such a mandate would appear to exclude PGD for couples who are not technically infertile. Thus, mandated insurance coverage for PGS and PGD remains largely out of reach [63].

Where insurers do cover IVF, they are likely for some time to come to resist covering PGS and PGD as "experimental" or as not "medically necessary." However, the good news is that some patients holding policies covering expenses related to infertility, genetic counseling, and prenatal testing have challenged such resistance and won coverage for PGD. Although couples who need PGD are not necessarily infertile, the argument that PGD is nonetheless "medically necessary" is particularly compelling in cases where the intended parents are carriers of genes that cause disease, and the insurer will otherwise be responsible for covering the costs of the child's medical care [48]. Furthermore, as the techniques for conducting PGS and PGD become further refined through research and clinical practice, insurers will have less of a basis for objecting to them as experimental. Such a development would bring PGS and PGD further into the mainstream, with salutary effects on the regularization of the use of SET in infertility clinics.

Conclusion

Few regulatory barriers currently stand in the way of clinicians practicing PGS and PGD in the United States. In a 2008 survey of clinics, nearly half of the clinics surveyed strongly agreed that "there will be restrictions on using PGD for nonmedical genetic traits such as sex" [53]. To date, though, there has been no regulatory movement in this direction. Legislative efforts to curb prenatal sex determination and selection have targeted sex-selective abortion in particular [64]. Statutes that circumscribe experimentation on embryos are aimed at research, not at clinical applications. Finally, on the professional side, ASRM has held a firm ethical stance against PGD for sex selection for over 15 years. The concerns expressed in the survey that enforceable restrictions on PGD for sex selection are on the horizon appear to be unfounded.

As Justice Michael Kirby put it in another context, "in the regulation of technology, events rarely, if ever, stand still" [65]. Philosophical positions abound about technological developments in the life sciences, but the translation to regulation must weather the political process. Legislative inaction is often the result, especially where the group that would be most affected by regulation has a powerful enough role in its formulation to advance "self-regulation as a strategy of influencing and possibly preventing future state intervention" [66]. Kirby may as well have been writing about assisted reproduction in the United States, where developments in embryo screening technology have inspired a decidedly minimalist legislative response, but where the profession has been active in promulgating practical and ethical standards for its use in the clinic. Despite this dominance of professional control of embryo testing for IVF, whether SET will become the standard for clinical practice is doubtful in the absence of stronger mandates for funding PGS and PGD. The current state of affairs suggests that medical malpractice law will have the most direct influence on the clinical use of embryo testing for the foreseeable future.

Conflict of Interest The author reports no conflict of interest.

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Chapter 26
The Ethical and Legal Analysis of Embryo Preimplantation Testing Policies in Europe

Judit Sándor

Introduction

In reflecting on regulations for assisted fertility, the law has proven to be a double-edge sword. On one hand, it has repeatedly made attempts to restrict the application of certain contested techniques, and, on the other hand, it has provided a tool to remove existing obstacles to a wider range of other technologies that had been available only to a select few and thus involved some form of discrimination. As a result, new groups of individuals can claim access to assisted reproduction and to the use of preimplantation genetic diagnosis (PGD). So the question emerges: can the law still shape the contours of legitimate uses of this technology? What kind of ethical principles can guide lawmakers and judges to develop grounded responses to the new demands for technology? This chapter will analyze some recent legal debates, the practice of the European Court of Human Rights, and will make an attempt to explore the current legal frontiers of the technology of assisted reproduction.

One of the main questions that have to be raised is what could be the new tool for an ethical and legal assessment of selective reproduction? Should postnatal, prenatal, preimplantation selection be assessed differently? Should the technology—or just the outcome—matter? Can parents simply desire to have children like themselves (even with disabilities) or like a previously born sibling (savior sibling)? Should embryos be screened routinely? And, if yes, should prenatal screening be based on some major serious health conditions, or on all possible testable human traits? In this chapter, I would like to map the contours of this new field by showing what happens if claims referring to the quality of eggs, sperm, and embryos are

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advanced within the preexisting legal framework. My main thesis is that legislators and courts should avoid two traps: First, they should avoid personalizing human body parts and gametes and using simply human rights language uncritically. The other trap would be to accept the property law approach and treating gametes and embryos as commodities.

The advantage of analyzing judicial cases can be found in their limited focus: thus, an otherwise complex theoretical debate is distilled down to one or two questions which specifically concern the parties. These are the questions to which the judge has to apply already agreed-upon legal principles or, in rare cases, to develop new principles to supply the lack of previously available principles. A further element of judicial cases is that judges have to use the apparatus of legal interpretation, including clear and consistent legal categories such as person and body, and to allocate rights, such as the right to privacy or the right to be treated equally. Having said that, we may add that law is one of the most influential contributors to the work of delineating boundaries in the field of biotechnology.

Assisted Reproduction: Disruption of Sexuality and Reproduction

Human reproduction has undergone significant changes since the first successful in vitro fertilization in 1978, and by now it has become a widely spread practice across the world. The other relevant step in biotechnology was the increasingly acknowledged use of genetic testing and screening. These two lines of development in "technoscience" have fundamentally shaped the expectations to human reproduction. Technology blurred the previously clear distinctions between natural and artificial, embryo and fetus, procreation and sexuality, etc. Infertility treatments have been used for two distinct purposes, as a remedy for infertility and also for embryo selection for genetic betterment. A further consequence of these technological advances is that embryos and oocytes can be used for other purposes, such as biomedical research including the production of stem cells. Thus, embryos can be created through fertilization or a process known as somatic cell nuclear transfer (SCNT). In case of assisted reproduction, courts have to face numerous bio-cultural issues and differences which previously they have never faced in the context of unassisted reproduction [1]. In the domain of reproductive rights, the right to privacy (in the United States) and the right to private and family life (in Europe) provide the main pillars of the constitutional framework.

The Oviedo Convention, which has been ratified by 29 European countries already, provides two relevant provisions in the field of preimplantation genetic screening and testing [2]. Article 12 stipulates that "tests which are predictive of genetic diseases or which serve either to identify the subject as a carrier of a gene responsible for a disease or to detect a genetic predisposition or susceptibility to a disease may be performed only for health purposes or for scientific research linked to health purposes, and subject to appropriate genetic counseling." Since preimplantation genetic screening and testing always constitutes a predictive test, this

limitation is applicable as well as the requirement of genetic counseling. Indirectly Article 18 is also relevant especially concerning research use of preimplantation genetic screening. Article 18 provides that "where the law allows research on embryos in vitro, it shall ensure adequate protection of the embryo. The creation of human embryos for research purposes is prohibited" [2]. The Additional Protocol to the Convention on Human Rights and Biomedicine, concerning genetic testing for health purposes [3], specifically mentions that it does not apply to genetic tests carried out on the human embryo or fetus; therefore, in the lack of specific provisions only the Oviedo Convention abovementioned general provisions may provide some guidance. One of the major legal divisions lies on the distinctions for health, for research, and for nonmedical reasons. In some regulatory frameworks, it is assumed that when embryo testing aims to detect conditions that are not medical, then it becomes eugenic selection. The problem with this approach is that it assumes that medical criteria are infallible in assessing what is eugenic and what is not. One may agree that selection based on detection of a minor pathological condition may be regarded also as eugenic, while selecting a specific, nonmedical trait, such as gender, may not have any eugenic motivation at all. In other words, a classification for eugenic does not necessarily follow a medical vs. nonmedical distinction. It is a widely held view in the disability literature that the same condition may be viewed very differently in the medical and in the social model of disability. So this distinction is not only old fashioned, but it is problematic as well. On the other hand, the term *eugenic* has also seen significant change over time. Now it encompasses more individual choice rather than the expectations by society.

The Embryo and the European Court of Human Rights

In Europe, the advanced reproductive technologies are far more regulated than in the United States. Still, at the pan-European level, there is no consensus on the nature and status of the embryo and/or fetus, although these are beginning to receive some protection in the light of scientific progress and the potential consequences of research into genetic engineering, medically assisted procreation, and embryo experimentation. The European Court of Human Rights is convinced that it is "neither desirable, nor even possible as matters stand, to answer in the abstract the question whether the unborn child is a person for the purposes of the right to life provision of the Convention" (*Vo v. France* [4]; see also [5–9]).

Recent cases have addressed questions of access to in vitro fertilization (IVF), wrongful life and birth, and custodial rights over embryos. In these cases, the potentiality of life has to be assessed, but the applicability of abortion case precedents is disputable. For instance, the very same jurisdictions that allow termination of pregnancy during the first trimester based on the request of the pregnant woman may reach an entirely different conclusion when a woman expresses her wish alone to have an in vitro embryo transferred to her.

The moral caution about the status of the human embryo suddenly has become unbearable in cases of disputes concerning embryos from IVF. The European Court

of Human Rights had already confronted this matter in the *Evans v. the United Kingdom* case [8], where the applicant claimed that her privacy rights were infringed by granting the destruction of her embryos based on the partner's request. While access to many forms of in vitro fertilization is accepted as a rule, the issue here was the *conflict between the rights of the prospective mother and the male producer of the embryo*. It is the in vitro procedure and ex utero storage that creates disruption between the phases of human reproduction. The legal contradiction here is while assisted reproduction was developed with the aim of helping to ensure rights of the infertile and to grant them privacy and health service that would eliminate the pain of being childless, the disruption of the procedure created an opportunity to invade privacy and right to family life which would proceed seamlessly in the course of unassisted (natural) reproduction.

As demonstrated in the *Evans* case, procreative liberty was recognized as a negative liberty (so women should not be prevented to carry on their pregnancy), yet this liberty is not applicable in cases of IVF, because the Court recognized that here the fathers' right not to become a parent should prevail over the woman's interest to become a mother. This case may have many different interpretations. The Court took into account the assessment of the new reproductive technologies when it recognized the uncoupling of procreation and pregnancy with IVF. However, what ethical theory the Court employed it is unclear, as the principles of bioethics are not directly transferred into law which relies on traditional forms of rights and interests. Elsewhere the Court stated that moral considerations are not in themselves sufficient reasons for a complete ban on a specific artificial procreation technique such as oocyte donation [9].

The main ethical dilemma in the *Evans* case therefore was whether biological differences in gamete donation could be taken into account in assessing rights of the male and female donors. Furthermore, the court missed the opportunity to recognize the difference between preventing someone to become a parent and the denial of the right to change opinion on biological parenthood.

Embryo Selection: Is There Any Right to Choose a Child with Specific Traits?

There are many examples of selective breeding in humans which reach back to the very origins of civilization. The concept was not alien to Plato's Republic; it manifests in the ancient Spartan practice of terathanasia (i.e., the death of an abnormal infant) as well as in policies of forced sterilization (of the "mentally ill") in the first half of the twentieth century. Now, selecting and screening have taken different forms such as the selection of "super" sperm and egg donors in modern-assisted reproduction. The unspecified desire "to have children" was associated with the woman's wish or—in traditional societies—with the one and only aim of women's lives. Selecting specified characteristics of the child (gender and other desirable features of the offspring) was regarded as a method for establishing public control

over the individual's (mainly the woman's) desire to have children. This distinction between an individual's desire to have children and public expectations to have a child with certain specified characteristics (such as being an only child, a male child, an intelligent child, a physically strong child, a "perfect" child, etc.) has become much less clear. Borrowing the term from Habermas, "liberal eugenics" is based on free and individual choices and not on coercive social expectations. Nevertheless, a preference still exists for the selection of a healthy, strong, and intelligent child, and this preference obviously reflects a commitment to unspoken eugenic purposes.

The first step to screen embryos and fetuses was a derivative effect of ultrasound, which had been developed during World War I to detect submarines. Later, medical doctors used this technology to examine fetuses while still in utero. Although ultrasound can identify some fetal anomalies, IVF clinics now offer genetic testing of embryos before transfer or implantation. Preimplantation genetic testing (i.e., PGD) can be seen as an alternate screening approach for embryos produced by parents with certain genetic predispositions. But now as a result of the development of PGD, soon-to-be parents who long for a "perfect healthy baby, have turned to science, through prenatal testing, to assuage any fears about pending pregnancies" [10]. Carrier testing is one of the more common methods, which involves testing both parents for genetic conditions before they begin trying to conceive to determine the chance they have of passing on any disorders to their children [11].

The genetic tests on the in vitro embryo prior to implantation in the uterus have become the subject of heated debates not only among professionals but also in various social groups. The theoretical possibility of "perfecting humankind" has moved people's imagination, and it often overridden the dispute about the real possibilities offered by PGD. This method has been primarily used worldwide as a screening method for β-thalassemia, sickle cell anemia, cystic fibrosis, spinal muscular atrophy, Huntington's chorea, Duchenne and Becker muscular dystrophy, and fragile X syndrome and hemophilia. So, in these respects, PDG is employed to screen against severe illnesses and not to "create" blue-eyed, athletic-looking children with high IQ scores. The use of embryo selection and the selection criteria themselves have caused significant ethical discussion worldwide. Some of the arguments against PGD include that it relativizes the value of human life, it further marginalizes and discriminates against people with disabilities, and it fashions the mother's body into an even more "clinical object" due to these new interventions. Indeed, the medical literature has now refined the more complex PGD process itself, which involves testing some cells removed from the embryo, and, based on the test results, selecting one embryo for transfer.

Moral Justification

The need for preimplantation genetic tests originates from the desire to avoid abortion following prenatal genetic tests and the resulting physical and emotional suffering by using this technique. It provides help primarily to families where hereditary diseases may be screened before the embryo is implanted in the uterus.

Technology has undergone a number of changes since 1989 when Handyside's team successfully screened an embryo for a genetic disorder related to the X chromosome and subsequently resulted in a successful pregnancy in England [12]. As far as the legal regulatory environment, very little consensus exists in this field. Two of the articles of the 1997 Oviedo Convention contain some reference to the topic [2]: Article 14 prohibits the embryo sex selection and states "the use of techniques of medically assisted procreation shall not be allowed for the purpose of choosing a future child's sex, except where serious hereditary sex-linked disease is to be avoided." In other words, selection of the sex is permitted to screen for serious, sex-linked disorders. But this applies only to a part of preimplantation genetic tests. The other basis is Article 18 of the Convention, which specifies that "where the law allows research on embryos in vitro, it shall ensure adequate protection of the embryo. The creation of human embryos for research purposes is prohibited."

As an international trend, PGD is slowly but steadily gaining ground even in countries traditionally taking a more conservative approach. A good example would be Germany where a 2010 ruling of the federal court acquitted a physician who performed preimplantation tests despite regulatory prohibitions. As a result of the legal debate that erupted, the strictness of the law was finally eased. It was in 2012 when the human rights aspects of PGD were brought before the European Court of Human Rights in a request submitted against Italy [13]. Under a 2004 Italian law, no preimplantation tests are permitted, but abortion may be requested in a later stage of pregnancy even based on the same health condition which could have been screened by PGD. Awareness of the inconsistency of that legal regulatory environment and the resulting controversial human rights situation was raised by an Italian couple who had already had a child suffering from cystic fibrosis, and the mother was forced to request abortion of a later pregnancy for the same reason. As they did not want to go through the ordeal of abortion again yet they longed for another child, they requested PGD although this was not permitted under Italian law. I believe that the court correctly concluded that the right to respect for private and family life (stipulated under Article 8 of the European Convention on Human Rights) was violated when the Italian law subjected a woman to repeated failed pregnancy when this could have been avoided with PGD.

While Austria, Switzerland, and Italy maintain a strict, prohibition-based legal position, Belgium, the Czech Republic, France, Greece, the Netherlands, Sweden, and Slovenia are more permissive in the field of preimplantation genetics. French regulations are more cautious and made changes in a piecemeal way: the bioethics law (amended in 2004) permits preimplantation genetic tests in highly restricted cases when one of the genetic parents carries a genetic mutation that provides a reason for the test. The Norwegian debate in bioethics is characterized by the fear of selection and social isolation, which explains their cautious attitude towards PGD. In 2011, the European Council prepared a comprehensive study on preimplantation and prenatal genetic tests that covered not only the polymerase chain reaction (PCR), fluorescent in situ hybridization (FISH), and comparative genomic hybridization (CGH) but also the whole genome amplification (WGA) method that involves the analysis of the entire genome. If the clinic has knowledge about the

embryo to be implanted carrying a severe disease, it is obligated by law to inform the person(s) requesting the IVF and PGD. However, reproduction is still primarily an element of natural family planning, so the concept of "product liability" is still alien to this field. Hopefully IVF and pre-IVF PID, PGD, or even PGS (WGA) will remain as exceptions and available only in justified cases. Otherwise, we would find ourselves in the world of *Gattaca*, where natural selection is only secondary to carefully planned genetic selection. Due to the lower birth rate in many postindustrial societies, there is a significant incentive towards selective reproduction. And this, of course, puts a greater burden on women as they are the ones who must undergo the physical and emotional consequences of gonadotropin therapy for ovulation induction, embryo transfer, possible spontaneous abortion, embryo selection, reduction, prenatal testing, etc. Therefore, their privacy rights, physical integrity, and reproduction rights must be respected.

In the philosophical debate, a counterargument along the lines of Habermas was presented according to which the prenatal or preimplantation selection of the embryo that meets the parents' wishes may actually affect the personal autonomy of the future child. This element of the debate, however, relates more to the eugenic-type embryo selection rather than the genetic test aimed at preliminary screening of certain disease types.

Preimplantation screening raises more ethical and legal issues than targeted preimplantation genetic testing. The key ethical counterargument is that a full-scale genetic screening would result in eugenic embryo selection instead of the previous approach aimed at avoiding certain diseases. It is also hard to determine whether the danger of a disease that would develop later or with a higher possibility in the future child's life could also justify this procedure or it should be limited to serious diseases to appear early—for example, during childhood. One may live happily for 40–50 years before the disease develops, and during that period of time, there is still a chance for treatment to be found. In such cases, therefore, it is hard to justify embryo selection. A powerful argument for broader genetic screening is that if a specific genetic disorder can be identified, then why should we not make sure that the embryo has no tendency for other serious diseases in addition to the disease the embryo is originally tested for? From the patients' point of view, it is understandable that if they opt for IVF and PGD, they would be deeply disappointed to find out that their baby suffers from another severe genetic disorder that could have been screened. As genetic screening is rapidly evolving, there may be a case for diagnosis when the use of genetic screening would emerge. For this reason, the Human Fertilization and Embryology Authority of England is required to make a separate decision based on a special request before screening for each new genetic disease. The request must specify the so-called OMIM (Online Mendelian Inheritance in Man) number of the specific disease.

With the expansion of the techniques and the range of diseases that can be identified by screening, we come to learn more about the limitations of PGD. For example, it should be noted that there will be embryos whose constituent cells are not all identical (mosaics). As a result, the cell removed for diagnosis may not necessarily provide an accurate picture of the genetic risks of the complete embryo.

In a special type of preimplantation tests, the purpose of embryo selection is to ensure compatibility with an existing person. Usually, parents can use this method to find a suitable donor for an older sibling already born. This application type of preimplantation and HLA tests raises a number of ethical and legal issues. The procedure selects embryos based on some principle of "usefulness," which means that an otherwise healthy embryo is not implanted if it is incompatible—that is, if it does not possess the qualities that could enable the future child to help the ill sibling.

From the perspective of the mother, if IVF and its associated gonadotropin therapy and invasive follicle aspirations occur only to help select one from any number embryos, and if this were an entirely voluntary decision by the mother free of any coercion, it obviously can constitute a violation of her dignity and right to self-determination if this were prohibited. But the so-called "slippery slope" argument in ethics implies that if today we permit embryo selection based on HLA compatibility, tomorrow we may allow selection for other qualities. Obviously, a therapeutic objective is an ethically reasonable and a serious aspect. If the procedure is compared with genetic tests already applied, saving one's life is more acceptable ethically than mere selection based on other criteria. It is a more serious question whether the human dignity of the child produced in this way is violated by the fact that a crucial aspect in his or her creation was to have certain biological properties that can help others at a later point in time. From a different viewpoint, prenatal selection may lead to instrumentalization, which is a decisive danger in terms of human dignity. We need to make several distinctions in terms of ethics. When a mother agrees to a new pregnancy to thereby help her existing sick child, this is different from a scenario where properties relevant in terms of donorship are taken into account in an already planned IVF program.

In addition to developing and enforcing legislation, appropriate information and genetic counseling will also play a key role. Special care must be exercised with regard to the personal rights of the patients and couples as they turn to their physician in this very important private matter. As with all new techniques, a relationship based on honest partnership must be sought with women, men, and couples requesting IVF treatment. Since this is a dynamically changing field, information supply must be adjusted accordingly. For instance, women of reproductive age now can expect to receive information regarding additional options for reproduction. The information provided must be accurate, objective, and personalized and may not be based on prejudices or any nonscientific views on women, disabilities, or age.

Medical and Nonmedical Indications

PGD is usually permitted in special cases to avoid specific and severe genetic diseases. In 2002, the United Kingdom Department of Health issued guidelines for the use of PGD. Nonmedical reasons refer to cases when embryos are selected for gender or specific desired trait. A liberal attitude to PGD can be seen in Belgium, Czech Republic, Portugal, and Spain [14]. In the United Kingdom, PGD has been applied since 1994. More reluctance can be seen in the German-speaking countries.

But even in countries that are labeled as "liberal" in their biomedical law, there are some restrictions on the use of preimplantation genetic screening. In Belgium, the law on assisted reproduction adopted in 2007 prohibits the use of PGD for eugenic choices which is understood as choosing embryos for selection or enhancement of non-pathological genetic characteristics [15]. The other possible approach is to differentiate between various causes of the medical conditions to be tested. Following this line of thought, the Portuguese law [16] does not allow preimplantation genetic screening for multifactorial conditions in which the predictive value of the test is very low.

According to the current Czech law [17], genetic examination of a human embryo or a fetus may be performed with the proviso that a doctor with specialization in the area of medical genetics provides genetic consultation. Laboratory genetic examinations of a human embryo or a fetus shall only be performed after the submission of information and with written consent of the mother. In the Netherlands, a detailed website provides assistance to couples who seek PGD [18]. The website specifies the conditions in which PGD is available, such as Huntington's disease, hereditary breast and ovarian cancer, myotonic dystrophy type I (Steinert's disease), familial adenomatous polyposis coli (FAP), Marfan syndrome, neurofibromatosis type I, cystic fibrosis, spinal muscular atrophy, fragile X syndrome, hemophilia A&B, and Duchenne muscular dystrophy. The British HFEA website also lists conditions in which preimplantation genetic testing can be performed and also on those conditions in which PGD is still contested [19]. The Swiss law [20] seems to be one of the most conservative with regard to preimplantation genetic testing, partly due to the fact that the Swiss Constitution addresses this issue [21].

Cases of Savior Siblings: Is There a Right to Select a Single "Matching" Embryo?

As a result of advances in medical genetics and embryology, it is now possible to examine a set of embryos produced through IVF to choose a healthy embryo that fits to some relevant medical criteria. Thus, preimplantation genetic testing may be used to ensure that the child to be born does not carry a certain genetic disease that has occurred in the family. Similarly, donor compatibility with an already born sibling might also be a reason for selecting a healthy embryo out of a pool of embryos for single transfer. In a rather journalistic and sensationalist way, such a child, once born, is sometimes termed a "savior sibling." The ethical dilemma of whether it is right and acceptable to create a savior sibling has been discussed in relation to a number of well-known cases. The first such case was the birth of Adam Nash in 2000 in the United States [22]. Adam was the first newborn baby who was deliberately selected as an embryo from several IVF embryos to help in curing his ill sibling. Adam was born in Chicago after four unsuccessful attempts at embryo implantation, and the stem cells extracted from his umbilical cord blood was used to cure his sister suffering from Fanconi anemia.

In England, two similar cases raised further ethical questions: shall we limit the use of embryo selection to saving family members suffering from genetic diseases or may we extend the application of this technique to other illnesses as well? In the *Hashmi* case, the family requested the selection of an embryo that does not carry the gene responsible for the development of β-thalassemia, a blood disorder. In 2002, the British Human Fertilization and Embryology Authority (HFEA) accepted this request and approved the embryo selection. In the *Whitaker* case, however, the family asked the authorities for approving embryo selection in the IVF process in order to bring a baby to life who could help in curing their child suffering from Diamond-Blackfan anemia, a rare form of anemia where the bone marrow produces few, or no, red blood cells. The origin and causes of this disease are not completely known, and only a matching bone-marrow donor could help the patient. Such donor could not be found, however. Shortly after approving embryo selection in the *Hashmi* case, the HFEA rejected the Whitakers' request. The panel's decision was based on the consideration of whether the child to be born benefits from the intervention. While in the previous case the couple used preimplantation genetic diagnosis (PGD) in order to prevent the passing of a hereditary disease on the embryo, in the latter case, the embryo itself would not benefit from PGD because the *sole purpose* of conducting PGD was to determine if the embryo is a suitable donor. Since the Whitakers' request was not granted in England, the family traveled to Chicago where IVF and embryo testing was successfully done. A healthy "savior sibling" was born in 2004, which allowed the older brother, Charlie, to undergo stem cell therapy.

In 2004 another British family, the Fletchers, asked the HFEA to approve a similar procedure, and the authority granted the request in this case. The Human Fertilisation and Embryology Act of 1990 (as amended in 2008; see [23]) provides the legal condition for savior siblings: the intended recipient of any donated tissue from a child born following tissue typing must (a) be a sibling of any child born as a result of treatment and (b) suffer from a serious medical condition that could be treated by umbilical cord blood stem cells, bone marrow, or other tissue (excluding whole organs) of any resulting child. The law also permits tissue typing if the embryo will not, in addition to the histocompatibility test, be tested for a particular genetic or mitochondrial abnormality.

Creating a savior sibling from IVF is based on the results of PGD. In France, the National Consultative Ethics Committee for Health and Life Sciences (CCNE) published its Opinion No. 107 on ethical issues related to prenatal and preimplantation diagnosis in 2009 [24]. In this opinion, the Committee pointed out that the creation of a *bébé-médicament* (or "therapeutic baby") should be considered only as a last resort solution, when no other type of treatment would prove to be effective. Instead, the committee encouraged development of the system of community-based umbilical cord blood banks to provide stem cells for as many children suffering from genetic illnesses as possible. If a child is already born with a genetic disease and the selection of a donor embryo is the only viable solution, then the CCNE proposes that the couple making the decision is provided with medical and psychological assistance.

The most common argument against selecting a savior sibling is that the birth of the child is not an end itself but rather is designed with the definite purpose of saving the life of another person—and this contradicts the principle of human dignity. This is indeed a significant argument that needs to be taken into consideration, and any legal regulation should be based on the foundation of protecting this principle. But we also have to bear in mind that the birth of a savior sibling is preceded by the same nine months of childbearing and laborious childbirth as in the case of any other fetus, and thus the whole process rests a much heavier burden on the mother than a simple act of embryo selection. No woman could undergo such an arduous procedure without a strong emotional bond with the future offspring and feeling of responsibility.

If we compare PGD and single embryo transfer for a savior sibling to other genetic examinations, then saving the life of another person is certainly a more ethically acceptable reason for selecting an embryo for any other consideration. It is a more complicated issue to consider if the right to human dignity of a child is violated, if it was a crucial aspect in deciding over his/her birth to life that he/she has the biological traits making him/her suitable to help others. In other words, can we claim that prenatal selection in itself leads to the danger of instrumentalization so decisive in relation to human dignity?

If we continue the analogy of a living donor, then it might be argued that for the "savior sibling," it remains a life-long moral and psychological gain that he or she has already saved someone else's life. Follow-up studies on cases of transplantation involving living donors has shown an interesting outcome: among living donors, they are likely to live longer than age-matched individuals who did not donate organs.

The first savior sibling in France was born on January 26, 2011, to parents of Turkish origin, and he was named Umut Talha ("our hope" in Turkish) [25]. As an embryo, he was selected through IVF and PGD to cure his siblings of β-thalassemia, a genetic disease that causes severe anemia. Based on the results of the initial tests, the stem cells extracted from the umbilical cord of the newborn baby can be used first to treat Umut Talha's sister. The family plans to use the same technique to help his brother as well. Of note, the public comments on ethical scruples were largely focused on how Umut was "objectified," how he had been used as a mere means. In contrast, the ethical issues related to the mother who sacrificed the most in the IVF process—with the numerous injections required to produce eggs and the surgical harvesting of oocytes—was regarded as less important. It was the mother who herself underwent the savior embryo transfer (selected out of 27 embryos) and stands ready to bear another savior sibling to help Umut's ill brother, if necessary. From the mother's perspective, her right to dignity and self-determination may well have been violated if it was not her fully autonomous, unenforced decision to undergo the IVF sequence.

In the bioethics literature by applying a slippery slope argument, it is often stated that if today we select according to HLA compatibility, then there will be other characteristics tomorrow to base our selections on. It is evident, though, that therapy remains an ethically respectable and serious goal of any such intervention.

Also from a bioethical perspective, it must be made clear that the single embryo selected in the IVF process is not simply a therapeutic tool, not just a method to

perform a surgery, not merely a type of medication, but an intervention which will likely culminate in the birth of an autonomous human being—a new individual. It is doubtful that any parent would endeavor to undergo such processes, especially since they are exhausting and psychologically draining for the mother, only to produce a child compatible with an already living sibling. While such a calculative decision is possible, it seems more likely for a couple to genuinely want a new baby anyway; if it should be an added benefit that the umbilical cord blood of the newborn baby (produced from IVF) can help cure an older sibling suffering from some debilitating disease, then this would simply be a double positive outcome.

Since its early applications in treating infertility, IVF has been used in a growing range of other cases including those for various therapeutic purposes. It seems that preimplantation instrumentalization as such may not lead to commodification, because after the selection of an embryo, an independent human being will exist. The unlikely circumstances which prompted Habermas' concerns in his work on *The Future of Human Nature* [26] seem unlikely to infringe upon the relation between generations, as the savior sibling is an autonomous being whose umbilical cord could save life.

IVF and Wrongful Life Cases: Is There a Right to Have a Healthy Child?

While IVF was developed to "cure" infertility, very soon after its first clinical application concerns towards the quality of gametes used in the procedure were addressed. If infertile couples (or persons) pay for reproduction services, could they claim higher standards of therapy or at least the prescreening of certain serious medical conditions of the gamete donors? Would it change the transaction from a type of personal donation to something akin to product liability?

Naturally born children do not have a fundamental right to be born free of genetic defects. Egg donation does not make a difference in this regard. Similarly, plaintiffs cannot recover damages for the emotional distress they experienced as a result of having a child with a genetic disease. The emotional distress suffered by parents as a result of the birth of a genetically diseased child after IVF cannot be treated any differently from that sustained by any other parents who conceived without medical assistance. However, plaintiffs may state a cause of action for the pecuniary expense arising from the heightened care and treatment of their sick child, including claims for compensation related to the mother's decision to leave her job so that she could care for her child on a full-time basis. Furthermore, plaintiffs can state a cause of action for punitive damages based on allegations of defendants' grossly negligent or reckless conduct.

In 2011, the case of *R.R. v. Poland* [27], the European Court of Human Rights dealt with the complaint of a young Polish mother of several children who, for a month, had to travel from one medical institution to another between Łódź and Kraków to confirm a severe fetal disorder (suspected during ultrasound exam). That information was critical in helping her decide to request an abortion. Her request was denied because genetic exams required a specialist doctor's referral. After long

delays, genetic tests in April 2002 confirmed that her unborn baby did have Turner syndrome. In accordance with a 1993 Polish law, her request for abortion on this basis could be granted. However, fulfillment of her request to terminate the pregnancy was denied on the grounds that the gestational age was too advanced.

Thus, on July 11, 2002, the plaintiff gave birth to a girl with Turner syndrome, as predicted by the prenatal tests. The young woman went to several Polish courts, and in her claim she wanted recognition that her doctors prevented her from the timely completion of the genetic test and an application for abortion based on Polish laws. One peculiarity of the case is that the Strasbourg Court not only found the violation of privacy rights based on information restraint, involuntary pregnancy, and living in fear but also ruled that the inhuman and degrading treatment shown towards the complainant was in violation of Article 3 of the European Convention on Human Rights (ECHR) [28].

Conclusions

As we have seen in the examination of legal cases, contemporary legal discourse in the field of biomedicine with respect to human dignity and the right to privacy may be used to interpret decisions on human reproduction, in general, and PGD, in particular. In this domain, the conceptual problem is how to distinguish between the core scientific and related socials norms. The delineation between science and its application in reproductive biotechnology is often hard to make. Furthermore, interpretation of scientific results in a broader social scope is often problematic. If law simply codifies or acknowledges the science of today, it often contributes to inevitable errors in ad hoc interpretations of current scientific paradigms.

Contemporary judicial interpretation must face scientific questions and terms in a complex way. The mission of the law is to separate scientific advances from commercial interests, to peel off the legacy of an older, paternalistic professional tradition, and to deflect eugenic and reductionist thinking. In the future, it seems that the mere reference to the term “eugenic” will become insufficient for deciding about the legitimacy of PGD. It seems that there is a huge gap between the historical and philosophical expressions in the sporadic regulations on PG, and the highly technical and changing abbreviation used by the clinicians.

Conflict of Interest The author declares no conflict of interest.

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Chapter 27
Preimplantation Genetic Screening for the Single Embryo: Aims and Responsibilities

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Introduction

Screening embryos to enhance the success rate of IVF is not new at all. Almost from the start, a check of the number of pronuclei in order to exclude haploid or triploid embryos was routine during IVF. Such embryos cannot result in a viable baby. Secondly, under the assumption that the morphologically best embryo has the best chance to survive and to yield a viable pregnancy, embryologists have always microscopically assessed embryos before transfer. However, some embryos which appear to demonstrate poor morphology under the microscope have been reported to develop into healthy children. On the other hand, the morphologically best embryo can still carry serious (and sometimes lethal) chromosomal abnormalities, meaning that there is still a level of uncertainty involved.

About 15 years ago, preimplantation genetic screening (PGS) for chromosomal aneuploidies was proposed as an add-on technology to IVF+ICSI, as almost all aneuploid embryos will give rise to implantation failure or pregnancy loss. PGS for

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aneuploidy can also be applied in the context of preimplantation genetic diagnosis (PGD), where patients are mostly fertile, but undergo IVF and PGD because they are at risk of transferring a severe genetic condition to their offspring. This approach also can be beneficial where patients carry a chromosomal translocation, which would make it hard for them to conceive or which would put them at risk of having severely handicapped offspring. Adding PGS to PGD entails combining the selection aimed at choosing an embryo without a specific condition for which PGD is performed, with the aim of selecting a single embryo that is most likely to develop into a successful pregnancy.

In this chapter, we will first briefly summarize the state of the debate about PGS for aneuploidies and highlight its ethical dimensions. Secondly, we will address the fact that PGS may serve different aims that require independent justification. As we will show, while these aims may overlap, they can also conflict, thus challenging the ethical basis for responsible embryo transfer decisions in IVF.

PGS for Aneuploidy

PGS and PGD make use of the same biopsy methods to obtain the cellular material for molecular analysis. At the time, when this screening was first proposed, this meant that one or two cells were taken from the 3-day-old embryo and analyzed using fluorescent in situ hybridization (FISH). Aneuploid embryos are neither transferred in utero nor cryopreserved, and they are also not donated to others for clinical use—they are discarded. A major challenge of PGS is that day 3 embryos are often mosaic and that cells taken from the embryo may not be representative of the entire embryo. An individual cell that is diagnosed as aneuploid may be the only abnormal constituent cell in the eight cell embryo, and the embryo may be able to overcome this abnormality and develop normally. There is also some evidence that IVF embryos are more prone to mosaicism [1–3]. Since FISH allows for the screening of a limited number of chromosomes, quite a few aneuploidies will slip through the net. A meta-analysis has shown that PGS using FISH to screen embryos biopsied at day 3 does not increase but actually *decreases* pregnancy rates [4]. This has led to position statements from international professional bodies stressing that PGS is experimental and should not be routinely offered to IVF patients [5]. Recently, it has been shown that biopsy at the cleavage stage might be responsible for this, since the process is invasive and appears to reduce implantation rate by about 4 % [6]. Therefore, alternative biopsy methods, such as polar body screening of the oocyte or screening of cells obtained from the trophectoderm (biopsy of the embryo at day 5), have since been developed [5, 7–11]. The drawback of polar body screening is that it only allows for the checking of *maternal* meiotic aneuploidies and will not identify paternal or postzygotic mitotic error. With the techniques of polar body and trophectoderm screening, some of the concerns regarding mosaicism may perhaps be lifted. For example, polar body biopsy occurs at a developmental stage when there is not yet any cell division, and the polar body is deemed to be representative of the maternal contribution to the future embryo. With trophectoderm PGS, more

cells are available for analysis, yielding a more representative sample. However, some embryos that may not make it to day 5 in vitro are viable when transferred at day 3, so potentially viable embryos are lost during the extended culture period required to obtain blastocysts. New freezing techniques such as vitrification may overcome the limited time frame for genetic testing.

A further development is that many new methods for analysis, such as Array-Comparative Genomic Hybridization (Array-CGH), genome-wide SNP analysis, qPCR-based detection, and next-generation sequencing (NGS), enable comprehensive screening of all chromosomes in a cell, hence giving a more complete picture of its status [12–18]. Although some believe that the introduction of these new biopsy and analysis methods in combination with vitrification will eventually be vindicated as a worthwhile addition to IVF and IVF/PGD, the technology is still highly contested [19, 20]. Taking into account the possibility to freeze all embryos for subsequent use, it has been pointed out that the benefit of PGS is to be sought in a shorter "time to pregnancy." Additional benefits are the reduced rate of failed implantation and spontaneous abortions, the psychological burden of which should not be underestimated [21]. Whether these benefits are important enough to make adding PGS for aneuploidy proportional depends on how much time pressure the patient is under, on the balance of the costs of PGS and related procedures on the one hand and the savings that may result from better "time to pregnancy" on the other. Moreover, in the context of IVF only, this gain would need to outweigh any possible adverse effects of the embryo biopsy and extended in vitro culture [22, 23]. If effective, it may be that PGS is proportionally beneficial only for certain subgroups such as patients with repeated implantation failure or for women of advanced maternal age. Furthermore, the screening of polar bodies or embryos might help to identify those patients who are likely to have abnormal embryos only [8]. Clearly, as long as it is not sufficiently established that PGS for aneuploidy does indeed work, the tendency to offer PGS as a routine component of fertility treatment defies the still valid position statements of ASRM and ESHRE is ethically problematic. Offering routine PGS may lead to disadvantaging patients undergoing fertility treatment by raising the cost of treatment for no good reason or even by effectively reducing their chances of having a child through IVF.

The Widening Aims of PGS

PGS traditionally refers to aneuploidy screening as a means to increase the chances of a successful pregnancy after IVF or IVF/PGD. As indicated, this should be qualified as improving the chances of having a successful pregnancy earlier rather than later. If done in the context of IVF, this aligns with the aim for which IVF is offered in the first place. If done in the context of IVF/PGD, things are more complex, at least when patients are fertile and opt for PGD not so much in order to have child but to have a child without the condition that they are at risk to transmit. However, if adding PGS means accelerating the time to a healthy (unaffected) pregnancy, then this can also be seen as serving the original aim of the treatment.

But already with PGS for aneuploidies, the aim may widen beyond the success of IVF or IVF/PGD treatment to also include avoiding the birth of a child with a chromosomal abnormality (unrelated to a possible PGD indication). Whereas most aneuploidies are lethal, some of them may lead to a viable pregnancy. Examples here are trisomy 13, trisomy 18, trisomy 21, and the sex chromosome aneuploidies. Although the chances that such embryos develop into a viable pregnancy vary from extremely low to decreased, there remains a possibility that transfer will result in a live birth. So when PGS leads to discarding embryos thought to be aneuploid, this may serve the overlapping aims of enhancing treatment success by improving time to pregnancy and avoiding the birth of a child with a handicap related to aneuploidy [24]. Of course, whether these objectives can be achieved depends on whether and to what extent PGS can overcome the problem of mosaicism, which is still a contested issue.

With the increasing resolution of microarray technology, the scope for testing embryos for conditions relevant to the health prospects of the future child will only enlarge. For instance, submicroscopic chromosomal abnormalities, including larger copy number variations (CNVs), are associated with an increased risk for conditions such as mental disabilities or autism, although as of yet, the relation between the abnormality and the condition seems one of susceptibility rather than causality. SNP arrays enable the detection of (a subset of) potentially disease-causing mutations at the DNA level, in addition to chromosome abnormalities.

With the advent of next-generation sequencing and single-cell whole genome sequencing, even more information about the genome of the embryo is expected to become available in the future. One might think here of PGS to test for a panel of genetic mutations that include the most common severe congenital disorders or for all genetic conditions that are accepted indications for PGD. Not transferring embryos carrying such mutations would help contribute to healthy offspring after IVF with PGD. Another idea is that health profiles of embryos could be established to determine transfer eligibility ranking. This would also include susceptibility genes or carrier status and may be appealing to clinicians and prospective parents alike. Whether this will indeed become feasible is still very much an open question. Recent research has suggested that these ideas about possible broad scope PGS may be naïve or at least premature. Indeed, some healthy adults have genetic mutations that are annotated as severe and disease causing and that if detected in an embryo would lead to negative transfer decisions. Several factors may explain this. Current tests may not be sufficiently sensitive, or the information in genetic databases may be incorrect. However, it may also be that our knowledge of epigenetics and protective genes remains rudimentary and that a simple extrapolation from genotype to phenotype is at least for the time being not fully feasible [25]. If this is the case, then the same goes for broad scope PGS to avoid health problems or select embryos with the best health profile.

Notwithstanding the feasibility of broad scope genomic embryo screening, it is important to note that PGS may serve two aims that are ethically quite different: treatment success and healthy children. Given the widely endorsed acceptability of IVF and IVF/PGD, and assuming for the sake of debate, cost-effectiveness of PGS for aneuploidies, the first aim (treatment success) is unproblematic from an ethical point

of view. However, things are less clear with regard to PGS to yield healthy offspring. Some would argue that also the justification of this second aim is already implied for that of IVF treatment, as all women or couples would rather have a healthy than an unhealthy child. This would even be more evidently the case in IVF/PGD treatment, which is already done to avoid the birth of a child with health problems. However, this is too simple, given that couples may prefer a child with certain health problems over having no (genetically own) child at all. The difficulty is that, when it comes to transfer decisions, the two aims of (a) successful treatment and (b) a healthy child do not always coincide and compromise may be necessary. This is clearly the case in the scenario of broad scope PGS, where testing would also include all kinds of genetic factors that are independent of the chances of a successful pregnancy.

Because only a limited number of embryos are typically available for transfer, testing for health may in fact lead to lowering the chances of a successful pregnancy. But the need for making trade-offs already emerges with the more limited scenario of PGS for aneuploidies only, given that some milder aneuploidies are only weakly related with lower chances of a successful pregnancy. From an ethical point of view, this is important because, given the different nature of the two aims, the question arises which trade-offs this should be and by whom.

Reproductive Autonomy and Professional Responsibility

In the context of prenatal screening and prenatal diagnosis, the overriding principle is that of reproductive autonomy of the pregnant woman. Because abortion decisions should remain personal, genetic counseling in this context should be nondirective. One might think that the same ethical framework with its emphasis on reproductive autonomy and nondirectiveness would then also apply to the context of assisted reproduction in general and "embryo selection" in particular. But things are more complex than that.

Of course, as long as PGS for aneuploidy is a costly accessory to IVF, couples should be free to make an informed decision not to have PGS. And clearly, as long as PGS for aneuploidy is experimental, no one should be offered this test without being made aware of its contested status. However, assuming that PGS for aneuploidy works and the couple has consented to this extra test, whenever this leads to transfer choices clearly relevant to improving treatment success, it can be argued that these are medical decisions that as such belong to the remit and responsibility of the IVF team. This would be very much the same as with regard to triploidy and morphology testing, which is also done in view of selecting out embryos that are nonviable or have lower chances of successful development. As there is no reason for discussing transfer policy based on the outcomes of those tests with the applicants, neither would there be a need to do so with outcomes of PGS aimed at improving treatment success.

There can be no debate about this when conditions are revealed that render the embryo nonviable or that would at best lead to a child facing early death from a

lethal condition like trisomy 13 or 18. In the latter cases, the two aims of PGS can be said to overlap and point in the same direction, given the low chances of a live birth and the severe health problems and short life span these children have. This means that if embryos with trisomies 13 or 18 are the only ones available, then transferring them would still be unacceptable. But what about an embryo with a sex chromosome aneuploidy? Embryos with 45,X are almost invariably lost during pregnancy. On the other hand, there does not appear to be selective loss of either 47,XYY and 47,XXX fetuses in spontaneous abortions, and about 50 % of all 47,XXY conceptions seem to be lost during early gestation. This is surprising in view of the usual lack of any severe anomalies among XXY live births [26, 27]. These Klinefelter males and their parents may be unaware of the extra chromosome until after puberty when infertility problems become manifest. The question is whether the existence of the extra X-chromosome is a sufficient reason to discard such an embryo, even when it is the only single embryo available for transfer and therefore may represent the couples' last chance of having a child that is genetically their own. Should priority be given to treatment success or to avoiding the birth of a child with (mild) health problems? And indeed whose decision should this be?

The second aim (healthy child) obtains a separate status when PGS is broadened to include conditions that may affect the future child's health, but are unrelated, or only weakly related, to treatment success. Here the question is, why to offer this wider testing in the first place? Is this to allow the intended parents to make autonomous reproductive decisions? Against the view that reproductive autonomy should be the guiding principle with regard to choosing between possible children [28], some have argued that, if doing so is reasonably possible in the circumstances, reproducers have a responsibility to choose the child whose life is expected to go best (reproductive beneficence) [29] or to make a decision that would not negatively affect others or society [30]. Following this line of reasoning, intended parents who make use of IVF/PGD may, under conditions of proportionality, have a responsibility to accept an offer of broad scope PGS and act upon its findings. Of course this would also depend on whether such testing leads to accurate predictions of the future child's phenotype, something that, as we have seen, is not always obvious.

But apart from whether reproducers do indeed have this responsibility to choose the best possible child or to make reproductive choices that avoid harm to others, the main reason why the idea of PGS to facilitate autonomous reproductive decisions cannot unconditionally be maintained is that it is at odds with acknowledging the co-responsibility that fertility professionals have for the welfare of the children in whose conception they are actively and causally involved. In comparison to the context of prenatal screening, this entails a shift of decision-making authority, requiring professionals to take their own responsibility rather than nondirectively accepting whatever decisions are made by prospective parents [31]. This is why fertility professionals may, for instance, refuse requests for assistance by applicants with a history of child abuse or otherwise lacking basic parental capacities. Debates about this issue have centered on the definition of the standard to be used for determining when professionals can be expected to refrain from collaborating with the reproductive project of the applicants [32]. The standard defended by the European

Society of Human Reproduction (ESHRE) is that professionals should refuse treatment if there is a high risk that the child will have a seriously diminished quality of life [33]. Clearly this is not a sufficient reason for making PGS a coercive offer for those wanting to have IVF or IVF/PGD. However, it does mean that if PGS outcomes allow transfer choices that are relevant for the health prospects of the future child, professionals should not go ahead with parental transfer requests that would entail "a high risk of serious harm." Of course, the application of this criterion to concrete cases may be a matter of debate, except for very serious or only mild conditions at both ends of the spectrum.

For example, on the basis of this criterion, it is obvious why transferring a trisomy 13 or 18 embryo should be out of the question even apart from considerations about treatment success, whereas on the other hand, there would not seem to be sufficient reason for rejecting a parental demand for transferring an embryo with a 47,XXY karyotype (Klinefelter syndrome) [31].

A more difficult case concerns trisomy 21 (Down's syndrome). Clearly, if PGS for aneuploidy works, there are good reasons based on the aim "treatment success" for preferentially not selecting any trisomy 21 embryos, because their viability is substantially restricted. But what if the only embryo left is a trisomy 21 embryo, and the intended parents ask for transfer of that single embryo, insisting that they would also be happy with a Down's syndrome child? Would proceeding with this request amount to a violation of the "high risk of serious harm" criterion? If so, then professionals should insist that no trisomy 21 embryos are to be transferred, even if they represent the couples' last chance of a (genetically related) child. For this position, one may refer to the often high comorbidity and related health needs of Down's syndrome children. Some would argue that also the high societal costs of caring for children with Down's syndrome should be considered in this context. Conversely, others may argue that Down's syndrome is a variable condition, that many persons with this condition live happy and rewarding lives, and that allowing societal costs to enter the equation is a first step on the path toward a morally problematic form of eugenics [24]. Following this line of reasoning, it may be argued that there is no "high risk of serious harm" and that professionals should leave the decision to the (well informed) parents. Professionals who would go ahead with a parental request to transfer a trisomy 21 embryo (if no other options are left) cannot be said, then, to act irresponsibly. Obviously, it is important that an institution's policy in these matters is clearly communicated to patients as part of the pretreatment informed consent.

Conclusion

The introduction of PGS in the context of IVF and single embryo transfer raises many difficult questions. First and foremost, it is still not clear whether the new biopsy and analysis approaches will make PGS for aneuploidy more successful in terms of improving treatment outcomes—and if it does, which specific subgroups of IVF patients will benefit? Given the possibility of freezing and subsequently

transferring single embryos obtained from a given follicular recruitment cycle, this improvement, if PGS works, would result in improving time to pregnancy by reducing the number of frozen embryo transfers needed and avoiding the related burden of implantation failures and spontaneous abortions. As long as the value of PGS for treatment success has not been proven, the screening should only be offered in the context of research.

Whereas improving treatment outcomes is a justified aim for adding PGS to IVF or IVF/PGD, the wider aim of routine testing for a healthy child requires separate justification. If this decision is conducted in the setting of single embryo transfer, then it becomes a particularly high-stakes choice. Where both aims overlap and point in the same direction, the second aim so to speak rides along with the first. But where wider testing leads to findings unrelated or only weakly related to treatment success, the question arises why such a test should even be added to IVF or IVF/PGD in the first place. This might be argued in terms of either the reproductive autonomy or the reproductive responsibility of the prospective parents. We have not discussed whether the latter line of argument is convincing, but stressed that the appeal to reproductive autonomy is at odds with acknowledging that, in the context of assisted reproduction, this principle is limited by professional co-responsibility for the welfare of the child. Fertility professionals may reject requests for transfer that, on the basis of PGS outcomes, they consider have a high risk of leading to a child with a seriously diminished quality of life, even if the embryo represents the couples' last chance of having a (genetically related) child. However, it does not follow that a coercive offer of broad scope PGS to all IVF or IVF/PGD patients can be justified by appeal to this professional responsibility for the welfare of the child. After all, there is no "high risk of serious harm" involved in transferring unscreened embryos—while (even voluntary) broad scope embryo screening would raise many issues that should be resolved first, related to both the suboptimal quality of current single-cell whole genome tests and to the adequate protection of the interests of all parties involved, including future children's right not to know [31, 34, 35].

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Chapter 28
Crossing the Rubicon: Assisted Reproductive Technologies and Remaining Human

Lee S. Rayfield

Introduction

Since Julius Caesar passed over a minor river dividing ancient Italy from Cisalpine Gaul, 'crossing the Rubicon' has become synonymous with taking an irrevocable step [1]. The conception and birth of Louise Brown in 1978 was certainly that [2]; *in vitro fertilisation* (IVF) not only transformed treatment for infertility but became the foundation for a succession of assisted reproductive technologies (ARTs) which have gone far beyond. The use of embryos for research, preimplantation genetic diagnosis (PGD), and tissue typing (PTT) and the creation of embryos from human and animal cells have each posed fresh moral and ethical dilemmas and themselves represented potential Rubicons [3–6]. The speed of advances in molecular biology, genetics and cellular manipulation has recently brought us to another Rubicon, mitochondrial replacement (or mitochondrial donation) [7, 8], and this has been the focus of national consultation in the UK.

In what follows the background to this, the processes used, ethical dimensions raised and the recommendations reached following the consultation will be described and reflected upon. It is hoped that learning from the UK experience will be valuable for other legislative domains and for approaching potential future Rubicons. It has been written by a member of the Oversight Group appointed by the Human Fertilisation and Embryology Authority (HFEA) but in a personal capacity and with permission.

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Mitochondria and Disease

Mitochondria have been dubbed the 'battery packs' or, more correctly, the 'powerhouse' of cells because of their relationship to energy production. These cellular organelles have a critical role in the production of ATP via the oxidative phosphorylation pathway; mitochondria contain their own DNA (mtDNA) coding for products required in the process [9]. The DNA of the nucleus (nDNA) also contains genes required for normal mitochondrial function, so mutations in nDNA or mtDNA can both cause disease.

Disorders of mitochondria can be difficult to diagnose because they affect a variety of organs, not necessarily at the same time, while symptoms and age of onset can vary considerably between patients. Organs most likely to be affected are those which require high levels of energy such as the brain, heart, kidney and major muscle groups. Symptoms can include learning difficulties; loss of hearing and/or vision; disorientation or confusion; kidney, liver, respiratory or heart disease; muscle weakness; gastrointestinal complications; loss of immune function; and a range of other problems. In the most serious cases, mitochondrial disease is fatal [10].

Mitochondrial DNA contains 13 protein-encoding genes plus others for RNA molecules involved in the assembly of the proteins [11]; by contrast nDNA contains between 20 and 30,000 genes, some 99.9 % of the total. The incidence of mitochondrial disease, whether due to nuclear or mitochondrial mutations, is relatively low, but this may be due to the difficulties in diagnosis. It has been estimated at 1 in 5,000 with around 1 in 10,000 adults in the UK being severely affected [12, 13]. It should also be noted that the nuclear genome is amazingly rich in functional, non-coding elements and transcribed non-coding RNAs (most of unknown function) and is packaged in highly regulatory chromatin. Variation in nuclear DNA might also affect the expression of mutant mtDNA in different individuals.

Inheritance and Future Generations

Mitochondria are entirely inherited through the maternal line; sperm are virtually devoid of these organelles, whereas the unfertilised egg contains over 100,000 mitochondria [14]. There are a restricted number of 'family types' of mtDNA (haplogroups) which are associated with broad population groups [15]. The mutation rate for mtDNA is about 10 times that of nDNA [16], and with so many gene copies in each cell, there can be a mix of functional and non-functional mitochondria carried in the egg. A mixture of variants is termed heteroplasmy, whereas a uniform population is known as homoplasmy [17]; the former is thought to be a contributor to variation in disease expression, however, individuals manifest different abilities to tolerate a given mutant load [13]. Homoplasmy for mutant mtDNA may prevent implantation, cause miscarriage or lead to symptoms in infancy. All these factors mean that an apparently healthy mother can give birth to a child who develops serious mitochondrial disease.

At present in the UK, those at risk of passing on mitochondrial disease have a number of potential options: adoption, egg donation, PGD and prenatal diagnosis. However, women with homoplasmy cannot benefit from PGD, and the non-Mendelian inheritance pattern of mtDNA creates potential uncertainties in heteroplasmy [18]. An embryo has a high probability of becoming a healthy child with a mutant mtDNA load of 18 % or less, but even slight increases in load significantly affect disease risk.

Mitochondrial Replacement

This is the context from which a novel ART has emerged—a serious and potentially lethal disease of variable phenotype and penetrance resulting from mutations in the DNA of mitochondria. Mitochondrial replacement offers a potential means of replacing defective organelles with functional mitochondria from a healthy donor. In the UK all treatment and research associated with fertility treatment and assisted reproduction is covered by the HFE Act (1990, as amended in 2008) and is closely regulated by the HFEA. In 2012 the Secretary of State for Health, together with the Secretary of State for Business, Innovation and Skills, jointly asked the HFEA to seek public views on emerging techniques designed to prevent mitochondrial disease.

At that time there were two such techniques. The first, maternal spindle transfer (MST, Fig. 28.1), involves unfertilised eggs (oocytes); the second, pronuclear transfer (PNT, Fig. 28.2), involves fertilised eggs (early embryos, Fig. 28.2). In MST, donor nDNA is removed from the cytoplasm of a donor egg containing normal mitochondria at an early stage of meiotic cell division when the DNA 'lines up' in a spindle formation. This donor nuclear material is discarded. In a similar fashion, the mother's nDNA is also removed from her egg, but this is injected into the enucleated donor egg. The mother's enucleated egg, containing abnormal mitochondria, is discarded. The reconstructed egg thus contains the functioning mitochondria from the donor together with the nuclear DNA of the mother.

PNT is a similar process, but this time two embryos are initially created. A donor egg is first fertilised by the father or another donor's sperm to create an embryo. Towards the end of meiosis, the nuclear DNA of the embryo, from the sperm and the egg, form pronuclei. These are removed and discarded leaving an enucleated embryo with normal mitochondria. A second embryo is created through the fertilisation of the mother's egg by the father's sperm. The pronuclei are again removed, but this time they are transferred into the enucleated embryo. This reconstructed embryo contains the nDNA of the mother and father together with healthy mitochondria from the donor egg. The enucleated embryo containing the abnormal mitochondria is discarded.

MST and PNT were the only techniques considered in the consultation process with the public, but recently, a third technique, polar body transfer, has been described in the literature [19]. At this point in time, the potential efficacy and safety of PBT is being monitored by an expert group appointed by the HFEA [20].

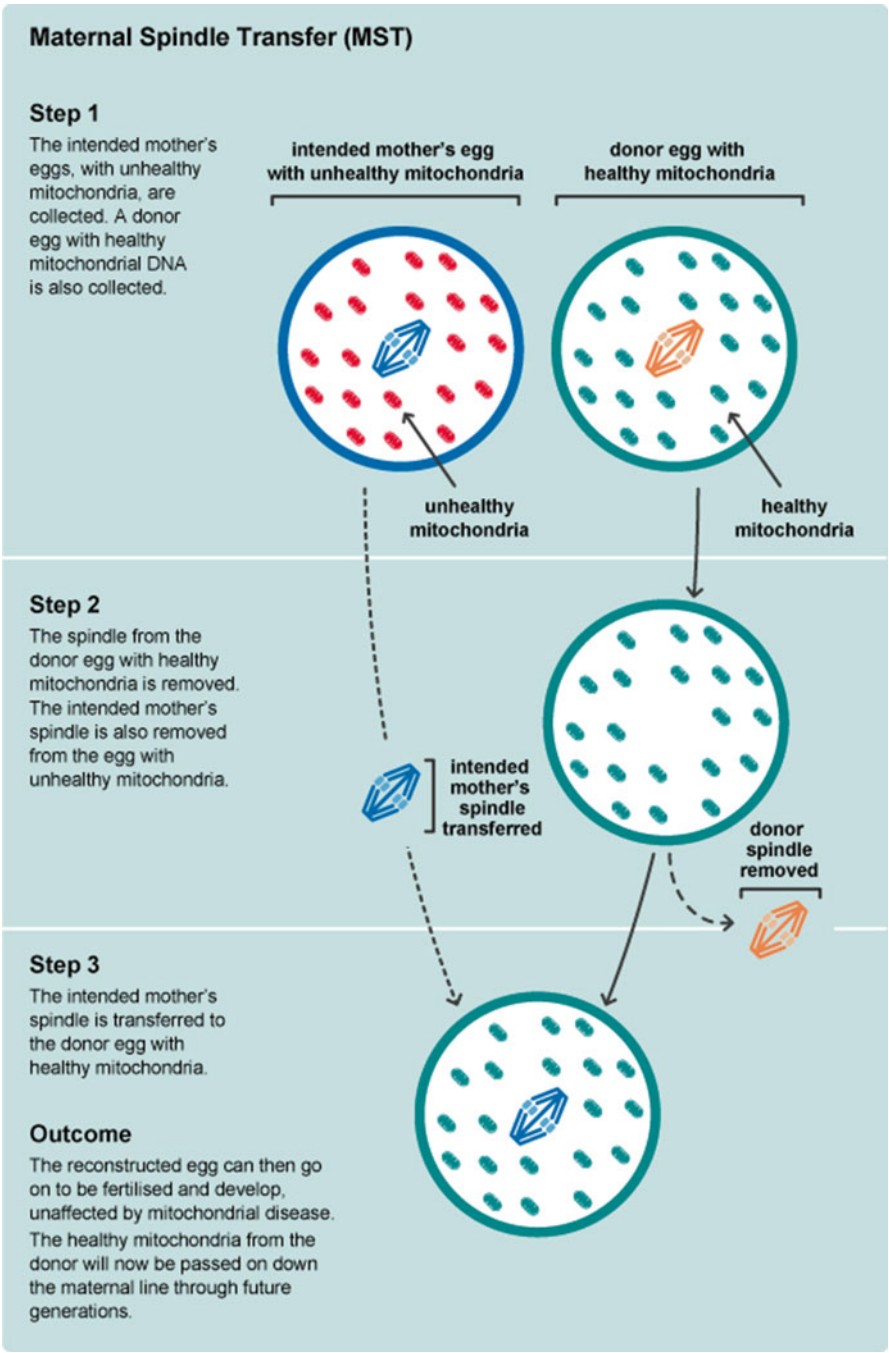

Fig. 28.1 Maternal spindle transfer

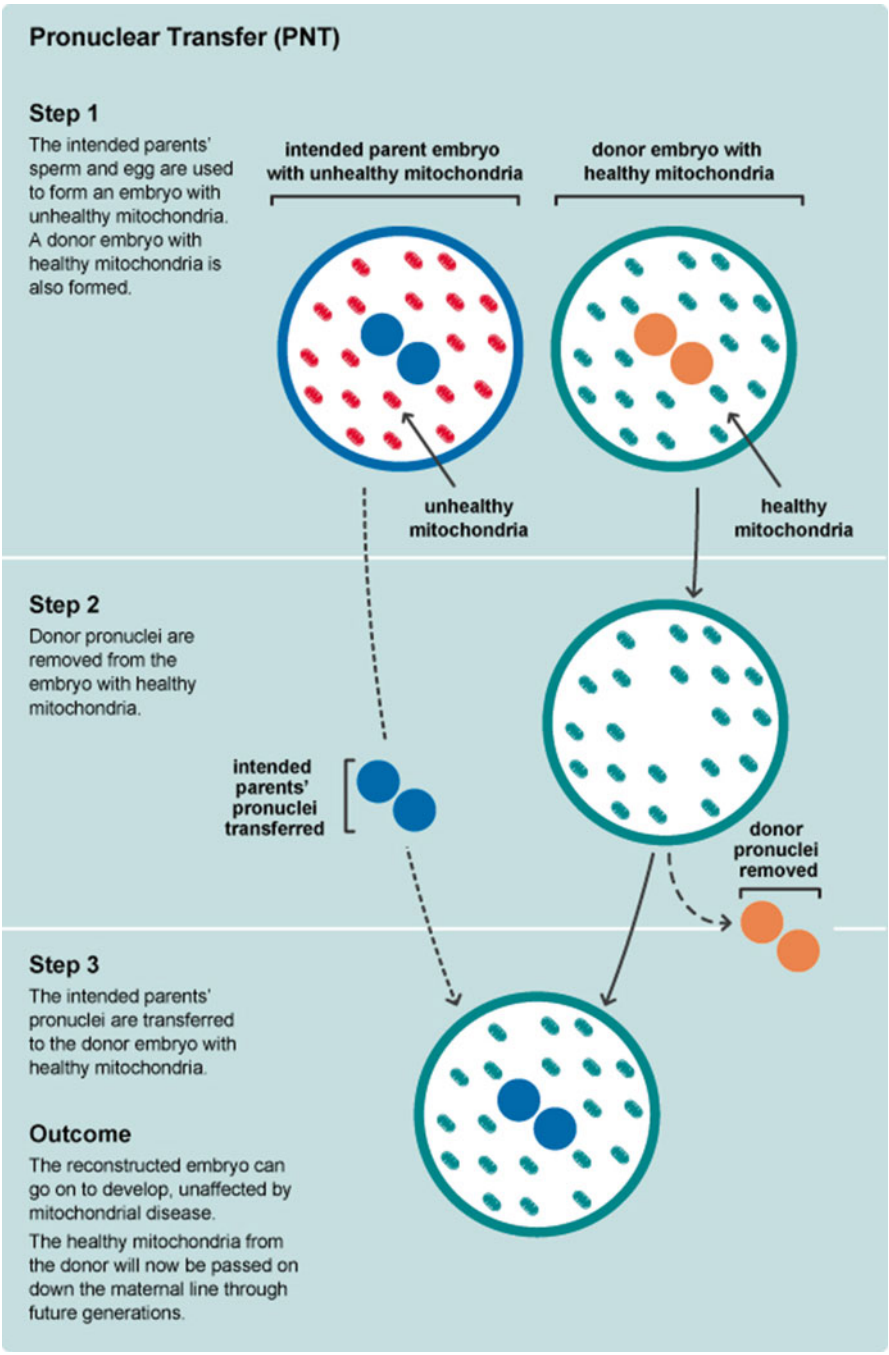

Fig. 28.2 Pronuclear transfer

Engaging the Public

Given the above descriptions of mitochondrial disorders, their inheritance and potential treatments, it is evident that the issues on which the HFEA had to test public reaction were exceptionally complex and controversial; communicating and engaging effectively with the general public would be a demanding task. Following consultation with a small group of stakeholders about its composition and remit, an Oversight Group was appointed. Key elements of the terms of reference were to explore (a) the ethical aspects and issues involved in mitochondrial transfer and (b) the practical implications of allowing mitochondrial transfer within regulation [21]. Members of the Group, who were acting as individuals rather than formally representing an organisation, were drawn from a range of backgrounds: public dialogue/science communication, science/research, bioethics, patient interests, religious interests and welfare of the child. A notable feature of the body that was appointed was its inclusivity [22]; the Oversight Group included members who were in principle opposed to ARTs as well as those committed to alternative family structures.

The role of the Oversight Group was advisory; the HFEA carried responsibility for making decisions on the consultation process, materials and the report to the Department of Health. The Oversight Group and HFEA worked with providers with experience and expertise in public dialogue (Sciencewise-ERC, The Office for Public Management, Forster and Dialogue by Design), and academic social scientists were contracted to monitor the whole process and prepare a report at its completion [23].

In order to understand how people comprehend the ethical and regulatory issues involved in mitochondrial replacement, the difference between informed and uninformed views and the deliberative process people go through to form those views, five different methodologies were agreed (Table 28.1) [24]. Two of these involved members of the public who could not be said to be interested parties: a face-to-face poll of around 1,000 people who formed a random quota sample and three workshops each run over two separate days in three major cities (London, Newcastle and Cardiff) for people who had been recruited to reflect a range of ages, gender and ethnicity. Open consultation meetings were held in two cities (London and Manchester), at which a panel of speakers with differing expertise and perspectives introduced the issues, participants worked together in mixed groups and there was public debate. A focus group for those affected by mitochondrial disease met once to explore the issues. Additionally there was an online open consultation questionnaire consisting of seven questions, though participants were able to send in written copies or respond by e-mail. A variety of different means were used to raise awareness of the 'open' elements (the consultation meetings and the questionnaire), including the use of websites, Twitter, the press, e-mailing stakeholder groups and encouraging networking. Members of the Oversight Group helped shape the material (including video material and diagrams) and frame questions to best enable lay people to understand and respond to the scientific, regulatory and ethical dimensions involved in mitochondrial replacement.

Table 28.1 Strands in the design of the consultation

	Participants	Selection method	Knowledge level of consultation issues	Number of participants
Deliberative public workshops	Members of the public	Recruited to a quota sample	Low at start of the workshops, much higher by the end	Approximately 30 participants at each workshop
Public representative survey	Members of the public	Random quota sample	Most people likely to have had low knowledge of the consultation issues	979 participants
Open consultation meetings	Interested stakeholders and members of the public	Self-selected sample through open invitation	Interested and knowledgeable about the consultation issues, but levels of knowledge were likely to be variable	53 participants [London meeting] and 39 participants [Manchester meeting]
Patient focus group	People directly or indirectly affected by mitochondrial disease	Invited to attend through patient contacts and patient groups	Interested and knowledgeable about the consultation issues, but levels of knowledge were variable	7 participants [including 1 telephone interview]
Open consultation questionnaire	Interested stakeholder and members of the public	Self-selected sample	Varied—relevant information was available via the consultation website which respondents were encouraged but not obliged to consult	1,836 participants responded to the consultation

Gathering and Interpreting the Data

Given the breadth of approaches, differing formats and the numbers involved, analysis of the responses was complex and difficult to make quantitative. The data was primarily analysed in a qualitative way, presenting comments in discussion while assessing the predominance of views. Where a questionnaire had been used, it was possible to give a semi-quantitative 'score', but the methodology was not designed or expected to deliver this kind of measurement. The online material was subject to a sorting algorithm which enabled the flagging and grouping of particular comments, but, again, this was necessarily a 'soft' quantitation. The consultation was not aimed at finding absolute percentages; rather it was concerned with 'taking the temperature' and identifying key issues in relation to the ethics and practical implications of mitochondrial replacement.

Six headings were used to sort and group the data. The data from each methodology, or 'strand', was presented by the Oversight Group to the members of the HFEA's governing board in a series of substantial reports and conclusions [25–29].

These were appended to the main report and advice which went from the HFEA governing board to the Department of Health [24, 30]. Given the extensive nature of the reports, including the summarised data, what follows here is necessarily highly abbreviated.

1. *Permissibility of new techniques*

   The public representative survey showed a number of trends [26] with strong support for medical research, the care and treatment of those with genetic disease, good awareness of IVF treatment and clear support for genetic testing during IVF and for genetic testing to avoid children being born with serious disease. However, the proportion of people declaring they were unsure was significantly raised by questions relating to genetic testing as was the (smaller) number actively opposed. Answers to a specific question about treating mitochondrial disease by altering the genetic make-up of an egg or embryo reflected this latter type of response with 56 % positive, 33 % unsure and 10 % negative. It should also be noted that some three quarters of participants had not heard of mitochondrial disease and that 50 % believed that medical research had unforeseen negative side effects, with only 15 % dissenting.

   In the deliberative workshops [25], the first part was aimed at explaining the science behind mitochondrial replacement, while the second part explored ethical, social and regulatory dimensions. In general there was a positive response to the techniques though anxieties around 'playing God', 'aborting disabled people', 'designer babies' and the 'slippery slope' were expressed. When compared with currently available treatments such as egg donation (or adoption), overall participants favoured the new techniques as giving parents the opportunity to have a child that is genetically 'their own'. They also came down on the side of giving parents personal and individual choice.

   During the workshops concerns were raised around safety and efficacy and whether maternal spindle transfer was ethically preferable because it did not involve the creation and destruction of another embryo. These concerns also emerged during the open consultation meetings [28] and from the open consultation questionnaire [27]. Strong views were expressed by stakeholders at the London meeting representing a tension regarding the moral status of the embryo versus the right of affected parents to receive this treatment. In Manchester, where there were many science and law students, there was generally openness to the techniques, and in London, too, there was also a majority in support. The patient focus group advocated permitting both procedures, though questions about safety were raised and to what extent the first children born after this treatment would be 'an experiment' [29].

   The impact of stakeholder positions surfaced in the open consultation questionnaire [27]. There was a distinction between those who identified as 'students' or those with a 'family member or friend affected by mitochondrial disease' when compared to those who identified as 'other'; while the former were more often supportive, the latter were predominantly opposed. 275 responses were received, in different formats, which did not directly answer the questions posed and used similar wording in expressing opposition to both techniques.

With the exception of the open consultation questionnaire, where a small majority of responses were against the techniques, the general conclusion was of support [24]. However, their safety was frequently raised as a concern, and there was a preference expressed for MST over PNT by those concerned with the use of embryos. It was also evident that a significant proportion of those new to the subject wanted further information before making their mind up.

2. *Changing the germline*

If permitted, mitochondrial replacement transfer would modify the germline and pass donor mtDNA down the maternal line to succeeding generations—one of the major ethical dimensions raised by the proposed treatment. In the deliberative workshops, participants were introduced to this and provided with up-to-date information on the uncertainties and risks of altering the germline [25]. An 'ethics' questionnaire used in the workshops revealed that over 60 % of participants had no real concerns about changing the germline and that their views on this remained similar both before and after hearing about the ethical dimensions. These were largely informed by the significance participants gave to parents in making choices.

The place and responsibility parents have in making decisions for their children emerged in both of the open meetings [28]. In response to an argument from a member of the platform panel that it was morally unacceptable to alter the germline and that a child might have difficulties with the way they were brought into being, a member of the audience in London and one in Manchester spoke of how they would communicate their decision to any children born by mitochondrial replacement. Both emphasised their desire to do what they considered was best for their child, even if it might not be something with which the child agreed. This captured the view of almost everyone in Manchester and the majority in London. With respect to changing the germline itself, there were three general types of response: (1) that it would not be altered significantly because variation in mtDNA is limited and this could be minimised further by using a sequence (haplogroup) similar to the mother's; (2) that germline modification would pose serious risks, both to individuals and societies; and (3) that it would 'change the germline for the better' and reduce the incidence of mitochondrial disease. Those who took part in the patient focus group had limited concerns around altering the germline and again stressed parental decision-making. One participant expressed the view that an affected child might resent their parents for not taking an option which could have alleviated their pain and suffering.

A concern which emerged in the open questionnaire [27] was the way in which society might regard those who chose not to make use of these techniques: would there be pressure on parents to use them, or discrimination against them, or a generalised knock-on effect against disabled people? Alternatively, would those who chose to use them be treated differently, at least until the procedures moved from being new? Given that 'scientific understanding of genetics is far from comprehensive', concern was also expressed by some respondents about consequences from changing the germline which could be hard to predict and be severe and far-reaching.

The predominant ethical concern expressed by respondents was that these techniques would make altering the germline acceptable and, in the words of some, open the door to eugenics and cloning. All of the above emerged from a question on the possible social and ethical implications of changing the germline. Of the 1,115 respondents, those who were more positive about the techniques argued that the benefits outweighed the risks, that there were no implications or that the only implication was decreasing the incidence of an awful disease.

3. *Implications for identity*

The implications of having DNA from three people on identity raised more concern than germline alteration among participants in the representative survey [26]; 40 % were undecided and 15 % took a more negative view. Participants in the deliberative workshops, largely unfamiliar with the concepts beforehand, likewise responded with caution at first [25]. The use by presenters of analogies such as tissue donation, gamete donation or adoption helped participants to review their anxieties, and overall they moved to being less concerned about identity issues.

The impact of sensationalist reporting was raised in open meetings [28] and the patient focus group [29]. Variously described as 'emotive', 'misleading' and 'confusing', attention was drawn to the potential impact this might have on a child's sense of identity. The differences between the contribution of mitochondrial and nuclear DNA to a person's make-up were regularly emphasised, with the latter regarded as by far the most significant. At the open meetings, in contrast to claims that mitochondrial replacement created 'an artificially constructed identity', the conclusion of the 2012 Nuffield enquiry that these techniques did not raise any ethical issues for identity was referenced [13]. The point was also made that identity issues from mitochondrial replacement might be less than for adopted children or those conceived by donor eggs.

The concerns of contributors to the open questionnaire were linked to whether the donor of mtDNA was viewed as a 'third parent' [27]. If mitochondrial donation were equated to donor gamete-conceived children, the social and ethical implications would be comparable; if equated to blood or bone marrow donation, there would be no significant implications. However, the point was made by a few respondents that mitochondrial donation could not be fully equated with any other analogy, and the importance to a sense of identity of parents explaining how they came into being was expressed by many.

4. *The status of the mitochondria donor*

This was not included in the public survey, but in other strands responses to this issue reflected whether participants likened mitochondrial donation to that of blood, tissue or an organ or to the donation of gametes. In the open questionnaire, respondents were specifically asked how respondents viewed the status of the mitochondrial donor in comparison with other existing types of donor [27]. Equal numbers regarded this as comparable to tissue donation and distinct from gamete donation as those who described it as the very reverse. A second question asked respondents about the rules which should govern disclosure of information

about the mitochondrial donor to a child. Three alternatives were provided together with the options of describing another arrangement ('other') or registering opposition to mitochondrial donation. A majority chose this last option, while the remaining respondents divided evenly between the proposed alternatives.

Participants in the deliberative public workshops were divided regarding access of information on donors by the children [25]. Those in favour of donor anonymity stressed the importance of protecting a donor's rights and being given a choice as to whether their identity was made known to a child. Others emphasised the child's right to access information about the mitochondrial donor. At the conclusion of the workshops, 31 % supported access to information with 45 % disagreeing. The patient focus group were very clear that donation should remain anonymous and believed donors would desire this, too [29].

At the open consultation meeting in Manchester, most were emphatic that there was 'no relationship' between a donor and the child and a majority opted for non-traceability [28]. Participants in Manchester and London acknowledged that information relating to a person's origins was a fundamental human right and that mitochondrial donation represented a different category which could not properly be equated with either tissue or egg donation. The recognition of participants in Manchester that this was 'uncharted territory' resonated with participants' comments in London concerning the novelty of the science and a call by some for a donor register. However, a fear was expressed that permitting access to donors could strengthen the perception that they are a 'third parent'.

5. *Regulation of mitochondria replacement*

The requirement for a body to provide robust regulation was articulated in almost all strands, as was the pre-eminence of parents working with clinicians to make individual decisions. There were differing views about how to hold these in tension. In the public survey respondents were offered options on who should make the decision about treatment if the law were changed [26]. One quarter were unsure, while 36 % believed this should rest with parents. The remaining respondents (39 %) advocated the involvement of a regulator, split evenly between those opting for approval on a case-by-case basis and those in favour of specialists in approved clinics making the decision.

At the deliberative workshops [25] and open meetings [28], the need for strict control was linked with risks, uncertainties and the safety of mitochondrial replacement, together with concerns around inappropriate or illegal application of the technology. The potential burden of monitoring those treated, and whether it was realistic, was raised in workshops though regulation was deemed necessary and could ensure fairness and availability of treatment. At the end of the workshops, 40 % believed the decision should rest with parents and their clinician. The focus group did not specifically discuss the issue, but parental choice featured as a major concern for them [29]. Those attending the Manchester open meeting favoured a regulatory system akin to egg donation and affirmed the importance of parental choice; denying the possibility of treatment was regarded by some as unethical.

On regulation, the open consultation questionnaire offered three options plus one not permitting mitochondrial replacement [27]. Almost half chose the latter. Of the other options, a minority wanted case-by-case decisions made by the regulator. The remainder split evenly between a regulatory framework for specific diseases with clinics and patients making individual decisions (242 responses) and a system where patients and clinics made the decision they considered appropriate (232 responses). One of the motives given for the latter was the perceived lack of sensitivity by a central regulator to individual circumstances.

6. *Attitudes towards legislation change*

In the second part of workshops, the attitude of each participant towards mitochondrial replacement was measured three times during the process [25]. If the treatment were shown to be safe, participants were asked whether they would support or reject it being made available to families through HFEA-licensed clinics; a sliding scale of 1 to 10 was used with rejection at the bottom end. The mean score of all participants moved from 8.2 to 8.4 and then down to 7.8 at the end. The fall related to input at one workshop where a scientist in a video referenced work suggesting mtDNA in cytoplasm impacted the development of vertebrae in fish. This introduced concerns around the solidity of scientific understanding and demonstrated that for some confidence in the safety of the techniques was fragile and easily disturbed.

The majority of those who answered the open consultation questionnaire opposed legislative change, though there was a significant minority in favour [27]. A small number were supportive of one technique (MST) but not the other (PNT). The reasons for respondents' positions were largely those for earlier questions; however, the international context figured in relation to changes in the law. Those opposed were concerned that the UK would be the first or only nation to permit such techniques.

The safety and efficacy of the techniques featured prominently in responses to the questionnaire and in the workshops. As part of the workshops, participants prepared 'messages for the Secretaries of State' to consider before making their decision. Themes included affordability, availability, fairness, information and access to counselling. Although changing the law was not specifically addressed in other strands, there were reasonable grounds for confidence that a majority would allow mitochondrial replacement transfer.

A Long Journey to This Rubicon

The consultation process has been part of a long journey in the UK with many preceding elements [13, 30] and the Rubicon of authorising the alteration of DNA in a human's germline—a decision which rests with Parliament and not the HFEA. The language of 'genetic modification' has been highly controversial in the UK, notably

in relation to crops [31, 32], and easily misrepresented or misunderstood. Differentiating and distinguishing mitochondrial replacement from 'genetic engineering' has not been straightforward. The language of 'three parent embryos' (and sometimes 'four') [28] has added to this and been represented as a further Rubicon in the moral and ethical debate.

A unifying theme from all quarters has been the safety and efficacy of the techniques and the HFEA commissioned a third review of this in 2014 from their expert scientific panel [20, 33]. The phrase which has been used consistently in speaking of risk by the panel has been that there is *no evidence to show that mitochondrial donation is unsafe*. The expert panel have regularly set out where more experiments might be required for safety or efficacy, but at some point, with the appropriate legislative regulation, it will need to be tested in the clinic. The consultation provided evidence, if it were needed, of how fragile trust in scientific opinion can prove [25]. Caution may be difficult, especially for those whose window for conceiving a child is closing, but is vital for patients and researchers; gene therapy trials were put back years in the USA by an early experiment which proved injudicious [34].

On the basis of evidence from the consultation process, the HFEA has advised the UK Government that there is general support for both MST and PNT to be permitted [35]. In order to address concerns that this will 'open the door' to other germline modifications, the HFEA advised that changes in the law should specifically refer to mitochondrial replacement and the avoidance of serious disease while continuing prohibitions around nuclear DNA in treatment settings. In order to address concerns around safety and germline modification, the HFEA advised that mechanisms be put in place to allow for further research recommended by the expert panel and that centres licensed to offer the techniques be encouraged to follow up children and future generations born through their application [13, 35]. Such follow-up would encompass the welfare of the child and not be confined to issues of safety and efficacy; social research on how children might feel about their origins should be part of this.

With respect to regulation, the HFEA advised that the status of mitochondrial donors be regarded as similar to tissue donors [35, 36]. Children born from mitochondrial replacement techniques would not have a right of access to information identifying a donor at 18 years. However, access to non-identifying information should be provided to children and parents when the child reaches 16 years. In terms of record-keeping, systems for traceability of gametes and embryos used in fertility treatment would be applied to mitochondrial donation. To complement this regulation, the HFEA commended the setting up of local systems, built on mutuality of consent and perhaps involving professional bodies and charities to facilitate the voluntary sharing of information as happens around tissue donation.

Given the novel nature of mitochondrial replacement, the Government was advised that a case-by-case approval system be used and the law 'future-proofed' by giving the HFEA flexibility in designing a clinic-based approval process if appropriate.

Disagreement and Journeying Together

The consultation process, in all its variety and breadth, has endeavoured to bring together a broad range of perspectives, commitments, knowledge and experience. In order to assess how effective the process had been in achieving its aims, independent social scientists were commissioned to appraise the consultation from inception to completion, through observation and interviews, and submit a detailed evaluation. Though there were inevitably aspects which could have been improved, Watermeyer and Rowe's report [23] concluded that the process achieved its aims and represented a 'rarely seen' example of public engagement with an emotive, scientifically and ethically complex subject in a very 'human' way.

There was never any expectation of getting consensus, but the process of dialogue did not only deliver an outcome; dialogue has itself been an outcome and assisted learning and mutual understanding. This was something modelled through the co-working of the Oversight Group and, given stakeholder convictions, provided a significant human dynamic of respect and value at the centre of the process. Though some participants in the workshop held in Cardiff doubted that they would make much difference to policy decisions, many in the process expressed appreciation at being included and heard. The title of the consultation as a whole was 'Medical Frontiers: Debating Mitochondrial Replacement', and clearly the contributions of professionals and experts, whether in the disciplines of science or ethics, were vital for public discussion. However, finding ways of reaching further out was critical for increasing confidence in society and for legislators.

Remaining Human

The consultation was not designed to reopen debate on the desirability of IVF or its associated reproductive technologies—those Rubicons have been crossed and there is widespread support and acceptance for their use. In the Christian tradition to which the author belongs, there has been much careful theological and ethical reflection around what Bellamy has termed 'theological embryology' [37] with nuanced support for much practice in the UK, including mitochondrial replacement. Nevertheless, for many, whether of religious faith or none, ARTs present a threat to 'being human'. This was expressed most starkly by Torrance in 1984 when he stated, 'In experimentation with human foetuses, in the manipulation of human embryos, in test-tube fertilisation, in the cross fertilisation of human with non-human species, in surrogate motherhood, medical science has brought us to an ultimate boundary which a civilised and God-fearing society committed to the sanctity of marriage and the structure of the human family, may not go'; what was at stake was 'nothing less than the future of the human race' and 'the integrity of the scientific and moral conscience' [38].

It would be easy to reject this dire warning out of hand, but there are bioethicists who embrace ARTs as a means of completely reimagining social relationships, repositioning boundaries and redefining human possibilities [39, 40]. For them the production of human gametes in vitro provides the means of choosing an alternative biological and social future—such as two women conceiving their child—and are deemed consistent with the ethical decisions we already demonstrate around reproductive rights as a society. This may be dismissed as ultra-radical or even science fiction, but among some it reinforces fears of the slippery slope—if we permit this technique, what will come next? That may even be the view of some outside the UK, with 'we' becoming 'they'.

'Remaining human' at an ART-associated Rubicon such as mitochondrial replacement requires those who understand the techniques and ethical arguments to appreciate the overtones of 'Frankenstein' or 'Brave New World' which many intelligent people feel when hearing about the proposals and engaging with them rather than dismissing them. Assuming the efficacy of MST and PNT techniques proves comparable, some may see MST as a better ethical choice; not intrinsically or because PNT is 'less' ethical but in the light of the respect it shows to those who have serious objections to the 'destruction' of embryos.

To reassure more conservative concerns here or elsewhere [41], it is important to emphasise how significant the robustness of the regulatory environment in the UK has been in the journey across various Rubicons. A tension between holding boundaries (such as the 14-day limit on the use of embryos) and providing flexibility (such as permitting PTT on an embryo not at risk of a genetic condition) has been maintained. In doing so another very human tension has been held, that of judgement and risk. Maintaining that tension wisely has been at the heart of the UK journey. Eschewing an 'absolutist' stance towards the status of the human embryo and adopting a 'gradualist' position crossed a major Rubicon and made decision-making more complex [42]. Nevertheless, UK legislation is predicated on the special status of the human embryo; those involved in the regulation of ARTs and their practice must always ensure that human germ cells, gametes and embryos are afforded a dignity and protection which is not ascribed to other species.

It has been observed that IVF, which began as a treatment for infertility, has now become 'a wheelbarrow for genetic services' [18]. Inevitably advances in genetics will present further dilemmas and Rubicons to negotiate. The Christian tradition, to which the author belongs and is guided, reminds human beings of our complex character—'made in the image of God' and capable of wisdom and the very best virtues, yet also self-serving and self-justifying. It is worth reminding ourselves of this regularly and not least in approaching future Rubicons. Interests need to be acknowledged and owned in debating medical frontiers, as they were implicitly in the call for the UK consultation being made by the Secretary of State for Business, Innovation and Skills, as well as the Secretary of State for Health. Following this consultation on mitochondrial replacement, which has built on the remarkable work done by scientists and ethicists in the UK and across the world, the decision now rests with the UK Parliament as to whether this Rubicon is crossed. The author is trusting that the HFEA's advice will assist them, humbly but positively, to take that step.

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