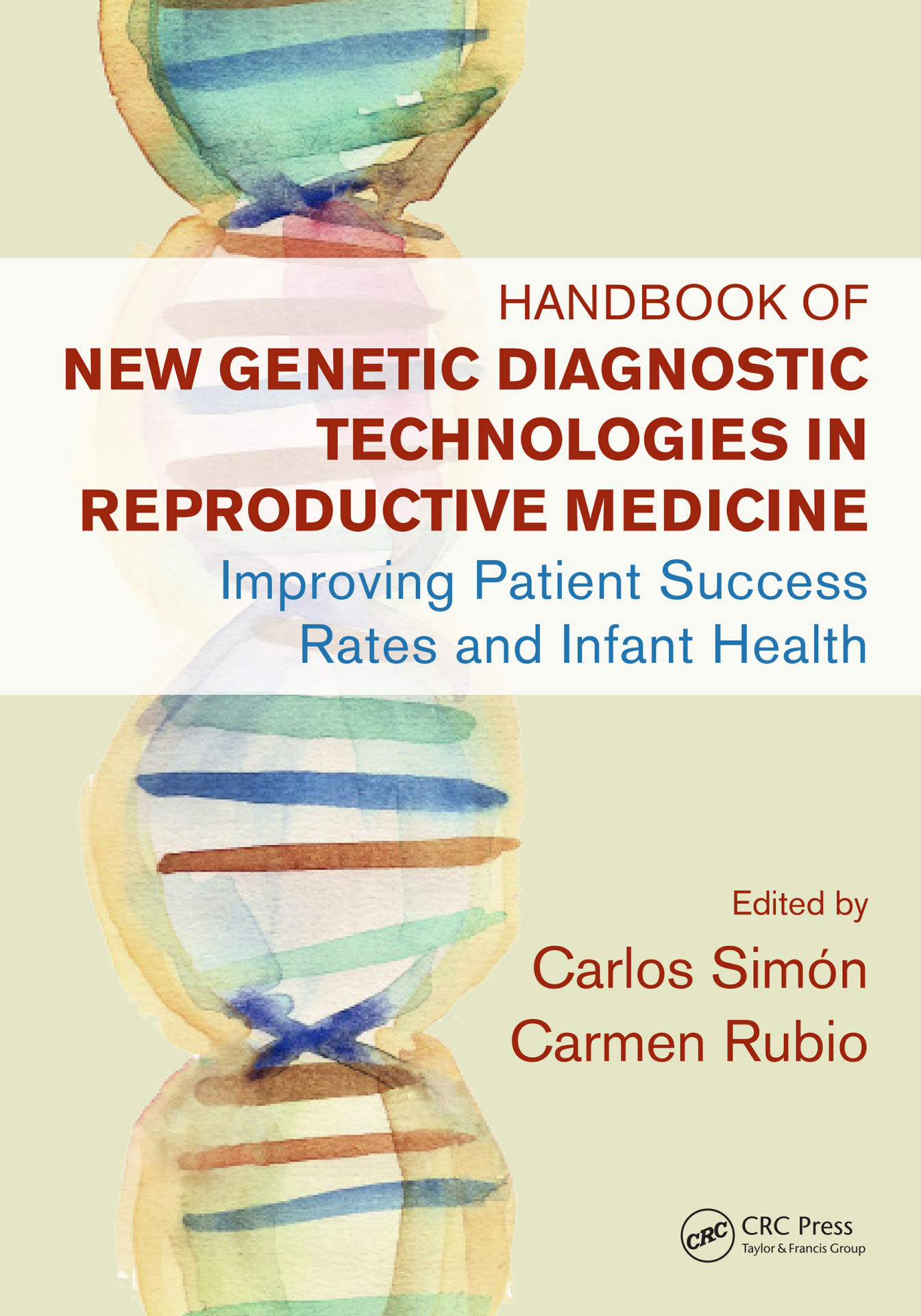

HANDBOOK OF NEW GENETIC DIAGNOSTIC TECHNOLOGIES IN REPRODUCTIVE MEDICINE

Improving Patient Success Rates and Infant Health

Edited by

Carlos Simón

Carmen Rubio

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Handbook of New Genetic Diagnostic Technologies in Reproductive Medicine

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Foreword: Views from a Pioneer

Does anyone doubt that discoveries in genetics are changing reproductive medicine? Recent decades have seen a plethora of technological and analytical advances. Combined with progress in sex steroid biology, embryo tissue culture, and cryopreservation, transformative changes have occurred in assisted reproductive technology and elsewhere in reproductive medicine.

Drs. Simon and Rubio are renowned for having contributed to this very transformation. At multiple levels, they have not only performed the basic research that has enabled this volume to be written, but also translated their work into improved clinical management. Their perspectives and experience have led them to identify those areas in which investigators and clinicians alike wonder just what genetic tests are available. And, when should genetic tests be ordered? What are their validities? How would results alter clinical management?

In male infertility, a host of these new tests exist, all with ostensible plausibility. To what extent does sperm aneuploidy matter? Is in vitro DNA fragmentation truly significant clinically? Is any given test preferable? Given that meiotic abnormalities virtually assure untoward outcomes, how can one best identify these? When would one proceed with intracytoplasmic sperm injection or other treatment modalities? In separate chapters, Garrido, Vidal and Rodrigo address these questions, providing salient details on technologies required.

Genetic screening before conception is expanding. Once relevant to only a few disorders in selected ethnic groups, genome-wide perturbations can now be sought. Martin places this potential wealth of information in perspective. In another chapter, Cervero discusses preimplantation genetic testing for single gene disorders and Rubio for aneuploidy. Selecting proper technology is crucial given so little DNA exists (1 or 5–10 cells), requiring technological adaptation that is lucidly described. Various approaches to 24 chromosome aneuploidy testing are discussed and compared. That preimplantation genetic testing-aneuploidy should accompany preimplantation genetic testing for single gene has become clear, and a protocol for accomplishing this is provided.

Cell free DNA in maternal plasma is increasingly interrogated to detect fetal aneuploidy and other conditions. Milan provides details on this rapidly changing technology. At present, noninvasive screening (i.e., venipuncture only) is applied once pregnant is achieved.

Finally, not all pregnancies succeed. Studying miscarriages remains a pivotal component of counselling and management in reproductive medicine. Al-Asmar traces the success using array comparative genomic hybridization or next generation sequencing to determine chromosomal status of miscarriages. These technologies are of great clinical usefulness because neither requires successful culture of products of conception.

Overall, this handbook covers the most important laboratory technologies encountered by reproductive specialists, lab directors, and staff. The editors are to be congratulated on the scope and details of this handbook, which belongs on the shelf (or computer screen) of all of us in the field.

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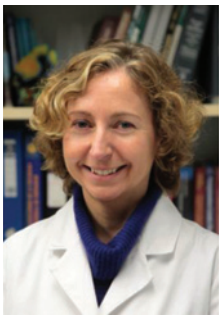
Editors

Carlos Simón is a Spanish reproductive endocrinologist and researcher. He is board certified and full professor of obstetrics and gynecology at the University of Valencia, Valencia, Spain; adjunct clinical professor, Department of Ob/Gyn, Stanford University School of Medicine, Stanford, CA; adjunct professor, Department of Ob/Gyn, Baylor College of Medicine, Houston, TX; and scientific director of Igenomix.

He has published 412 papers in peer-reviewed journals with an accumulated impact factor of 1,827.122, cited 14,355 times with an average of 35 citations/paper. His H-Index is 66.

He is the editor of 18 books in English, Spanish, and Portuguese, and the quality of his work has been recognized with awards by the American Society of Reproductive Medicine, the Society for Gynecological Investigation, the Spanish Society of Obstetrics & Gynecology, and the Spanish Fertility Society. He received the Prize Jaime I in Medical Investigation in 2011, and the ASRM Distinguished Research Award in 2016.



Carmen Rubio was trained in science and biochemistry at the University of Valencia, Valencia, Spain, and has a background in human embryology. She completed her PhD in 2004 in the field of reproductive genetics specializing in cytogenetic studies in gametes and embryos.

Becoming interested in chromosomal abnormalities in human embryos, she did her post-doctoral research in male and female meiosis and the mechanism underlying human aneuploidy at the laboratory of Drs. Patricia Hunt and Terry Hassold at the School of Molecular Biosciences (Washington State University, Pullman, WA).

At present, she is focused on embryo genetics and preimplantation embryo testing at Igenomix (Valencia, Spain). She has published more than 100 papers in the main peer-reviewed specialist journals in the field, book chapters, as well as numerous lectures at conferences worldwide. She is one of the most cited authors in the field, with more than 25 years professional experience.

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Carrier Screening for Single-Gene Disorders

Julio Martin, Beatriz Rodriguez, Arantxa Hervas, Ana Bover, and Roberto Alonso

CONTENTS

Introduction

The role of genetic variation in human diseases is well known, but the implementation of genetic studies in the practice of medicine has been challenging. Carrier screening allows us to identify individuals and couples at risk of conceiving children who will be affected by diseases traceable to single-gene mutations. Carrier screening is an important component of preconception and prenatal care.

Single-gene disorder screening was first proposed for condition-directed carrier testing for phenylketonuria in the 1960s, followed by sickle cell and Tay-Sachs diseases, both of which targeted high-risk populations. Other conditions such as cystic fibrosis were later included in the screening programs. This strategy resulted in remarkable declines in the incidence of severe diseases common in these populations. However, the continued increase in our genetic knowledge has allowed the decoding of close to 10,000 disorders with suspected Mendelian inheritance, approximately 1,150 of which are recessive-disease-causing genes (www.ncbi.nlm.nih.gov/omim), opening up the possibility of an extensive preconception testing approach.

Mendelian disease prevention by genetic screening is clinically pertinent. It accounts for 20% of infant mortalities and approximately 10% of pediatric hospitalizations [1,2]. Moreover, with the advent of efficient target capture methods, high-throughput next-generation sequencing (NGS), and bioinformatics advances, comprehensive preconception screening becomes more feasible, allowing simultaneous, efficient, and *affordable* testing for a large number of conditions that a family history would never detect. Using targeted NGS technologies each researcher or clinician can determine the appropriate approach to sequencing DNA (or RNA) to analyze sequence variation [3]. In this chapter, we will discuss the use of NGS for comprehensive DNA sequencing of genes causing Mendelian disorders as applied to test individuals and used to characterize their carrier burden and risk for descendants, as the best approach to ensuring a healthy baby.

What We Know Today

Carrier screening denotes genetic testing performed on an otherwise healthy, asymptomatic individual to determine whether that person has a mutation within a given gene associated with a disorder. It can be performed for a single condition, a number of specific conditions, or multiple disorders. Testing for a large number of conditions simultaneously is known as *expanded carrier screening*. Remarkably, expanded

carrier screening will identify most individuals who are at risk for the screened conditions, but a residual risk will still remain for both negative and positive results, here for the otherwise negative genes.

The ultimate goal of carrier screening is to provide individuals with meaningful information to consider their range of reproductive options based on their personal values [4]. Historically, preconception carrier screening has been recommended only for a handful of mutations targeting specific populations known to have prevalent conditions. The diseases most frequently screened for were cystic fibrosis, hemoglobinopathies, conditions associated with Ashkenazi ethnicity, spinal muscular atrophy, and, in women, fragile X syndrome [5]. The list of recommended disorders to be included in carrier screening tests is taken from the guidelines of the professional societies whose criteria are based on condition severity, race or ethnicity, prevalence, carrier frequency, detection rates, and residual risk. However, there is now a tendency toward the development of pan-ethnic genetic tests in consonance with current multiracial societies. Indeed, some authors believe that, with the tools and techniques available today, genetic analyses should test as many variants as possible [6]. Traditional and ethnicity-based tests were thought to have a higher mutation detection rate and be more cost-effective, but they have proved unsuitable for patients of mixed or unknown ethnic background [6,7].

The advantage of NGS-based genetic analysis is the possibility of designing a comprehensive assay to test all patients regardless their clinical history and ethnicity [8]. Concerning inheritance, most disorders included on current expanded panels are autosomal-recessive; however, some may be X-linked or even autosomal-dominant single-gene conditions [9]. In addition, the promotion of pan-ethnic carrier testing has extended to gamete donors, and they should undergo carrier screening before they become part of the screening programs [9,10].

What Genetic Conditions to Screen?

Traditional methods have focused generally on conditions that significantly affect life expectancy or quality because of cognitive or physical disabilities or a requirement for lifelong medical therapies, and that have a fetal, neonatal, or early childhood onset and a well-defined phenotype [9].

As part of a list of genes/mutations of variable size regarding genetic content, the expanded panels also follow those traditional clinical criteria and recommendations of professional societies for disease/gene inclusion, i.e., clinical utility, disease prevalence, disease severity, test accuracy and cost, reporting variants with a highly penetrant phenotype and mostly recessive and X-linked inheritance. Moreover, the inclusion criteria for the multigene panel must consider that multiple genes can cause a specific condition and therefore include more than one gene as causative or implicated in the pathogenesis of a condition. Digenic inheritance is another scenario to be considered. Expanded panels may also include other conditions with greater variation in their clinical presentation as well as rare conditions with still limited knowledge about carrier frequency and proportion of condition-causing variants that can be detected. Therefore, calculation of residual risk after a negative screening result is not possible for all conditions. It has been specified that it may be preferable to exclude conditions associated with adult-onset phenotype and variants with high allele frequencies and low penetrance, as well as variants of uncertain significance. However, it is not unusual to see high-prevalent monogenic diseases with moderate phenotypes included in the list. It is important that providers follow guidelines to ensure that actionable information is offered to individuals and expecting families [11].

Some authors have used NGS-based approaches to screen for more than 400 severe autosomal and X-linked recessive childhood disease genes, including disease genes, for low incidence and variable severity conditions [12]. Others have made calculations by modeling populations to demonstrate prevention and health cost reduction by using expanded carrier screening [13]. A significant decrease of 61% in the incidence of affected children has been reported when results for NGS-based testing were compared with couples without testing. Although more long-term implications must be investigated, combining this beneficial estimation of expanded screening NGS-based tests with reported evidence of preventing genetic conditions by carrier screening in high-risk populations [14] makes the use of current genetic knowledge and technologies very promising for offering greater beneficial healthcare opportunities to the general population.

Methods and Variant Interpretation

Array hybridization was initially explored for expanded carrier detection [15]. It is a cost-efficient test, but it is also a fixed-content method with significant analytical limitations, such as limited lists of mutations per gene. The type of mutations efficiently tested is limited mainly to nucleotide substitutions, which impacts the test's clinical utility since a significant portion of carriers will not be detected. Alternatively, target capture and NGS have shown efficacy and scalability for resequencing human exomes and genomes [12,16,17] with excellent analytical accuracy in terms of both sensitivity and specificity, including operational feasibility for carrier screening [7,10,12,18,19].

Regarding variant interpretation, although different providers may use their own criteria to determine the clinical impact of sequencing findings, here we describe generally accepted rules following standards and guidelines [20] for variant categorization. As a first step, pathogenic or likely pathogenic variants from annotated variants in databases (ClinVar [21] or HGMD [22]) must be selected. A curated list may allow for automatization of variant classification. These mutations typically correspond to variants reported in patients with solid medical evidence, and are classified as pathogenic. However, still more filtering steps are normally required. A second filter is related to allele frequency, implemented to classify detected variants as common or rare. Variants with an allele frequency >1% in dbSNP (www.ncbi.nlm.nih.gov/SNP) in the 1000 Genomes project (www.1000genomes.org) or in an in-house database are defined as common variants and are usually categorized as likely polymorphisms. Exceptions are made for well-described annotated pathogenic variants with allele frequency >1%. Variants with a frequency <1% are considered rare variants. Further filtering steps take into consideration the type of mutation and its functional impact, zygosity, disease prevalence, detection in patients vs. controls, etc. Regarding mutation types, rare missense SNVs and in-frame coding small insertion or deletions (indels) sequences with an allele frequency lower than the estimated prevalence of the corresponding conditions with no homozygous status ever detected in controls, but not reported in patients or reported but without clear evidence of causing disease, are normally classified as variants of unknown significance (VOUS). Finally, rare variants—typically below 1%—with severe functional impact (frameshift deletions, nonsense SNVs, and splice site variants) and with allele frequency below the corresponding disease prevalence with homozygous status ever detected in controls are classified as likely pathogenic.

Clinical Results

Several preclinical and clinical validation reports using NGS-based carrier screening have been published [7,10,18,19]. All studies are rather similar in terms of preclinical validation: a set of DNA samples previously characterized by a different method, usually Sanger sequencing, is reanalyzed. In our study [10], the selected DNA samples were positive for mutations affecting 27 genes of interest. Overall, the analytical sensitivity was >99%, with an estimated clinical sensitivity of 98%.

Regarding clinical results, a different carrier burden may be found due to various considerations such as gene/mutation content, method approach (array hybridization vs. NGS; amplicon vs. in solution enrichment), decision tree to classify sequence variation, etc. In our study [10] of individuals undergoing carrier testing as preconceptional screening in fertility clinics, a total of 2,570 tests were performed, on both patients and gamete donors. In total, 1,796 unique pathogenic or likely pathogenic variants were detected, and 13,785 variants of unknown clinical significance (VOUS) were defined. Of the 2,570 patients investigated, 2,161 (84%) were positive for at least one pathogenic variant. The average carrier burden of recessive or X-linked conditions was 2.3 mutations per sample. In 7 out of 138 couples using their own gametes, the carrier screening result identified a pathogenic or likely pathogenic variant in the same gene in both members. This accounts for 5% of the couples analyzed. In these cases, preimplantation genetic diagnosis (PGD) was recommended during post-test genetic counseling. In 6 out of 287 female patients, positive results for X-linked disorders were obtained, which accounts for approximately 2% of the total cohort. PGD was recommended here as well. Gamete donors who tested microbiologically negative were subjected simultaneously to karyotype analysis and fragile X (females only) investigation; abnormal karyotype individuals were not further tested and banned as donors. An additional 18 female donors

were excluded from the program because they carried a pathogenic or likely pathogenic variant in an X chromosome gene, including fragile X, representing 1.94% of the total tests requested. They received information on the adverse finding, including genetic counseling, and were discouraged from entering the donor program. The remaining donors were included in a blind-matching, informatically controlled database. By request, the match system always displayed a set of donors genetically compatible with the patient requesting gamete donation. The blinded system allowed us to include VOUS variants as criteria for assigning a matched donor. The system discouraged the assignment of donors having VOUS variants in the same gene where the patient was carrying a known pathogenic variant, or vice versa.

Limitations

Patients with genetic conditions constitute an important portion of the world population with special healthcare needs [2]. Based on this statement and current advances in genetic knowledge and technologies we could maintain that providing information about the carrier status is a major benefit for individuals, families, and society in general. However, there is little evidence that addresses reproductive outcomes when expanded carrier screening is used. Indeed, providing genetic information about risk for descendants to individuals and/or couples wishing to start a family may affect their ulterior decision in the event of a positive result. Moreover, residual risk is present, even for negative results. Some mutations are not covered by the technique. Mutations, the molecular basis of which remains unknown, must be explained to patients prior to the analysis and after results are available.

Other challenges are associated with the great amount of sequencing data obtained from these tests. Clinical interpretation for most genetic variation is still lacking (for example, regarding VOUS variants, especially in clinically variable phenotypes or incomplete penetrance conditions). This presents a challenge to the clinician as to how to guide the medical management of a patient in the context of an inconclusive test result. To overcome this issue, computational algorithms that help predict the pathogenicity of mutations have been developed. They are based on criteria such as evolutionary conservation of the nucleotide or amino acid changes, and consequences in the protein context like for example nonsense mutations that interrupt the addition of more aminoacids to the protein or frameshift mutations that change the open reading frame. These criteria may be weighted differently, thus resulting in a different classification of a variant among algorithms. The American College of Medical Genetics and Genomics guidelines [20] recommend taking as moderate/weak evidence of pathogenicity only those cases in which multiple algorithms coincide in their prediction. In addition, there is a need for data collection to create a curated data repository of variants and associated phenotypes. Together with technical differences in capturing and/or sequencing efficiency, variant interpretation across different studies is a further limitation to providing a definitive estimation of the carrier burden in humans.

Conclusions

Carrier screening is an important component of preconception care. It aims to identify couples at risk for passing on genetic conditions to their offspring. Today, high-throughput genotyping and sequencing approaches allow for efficient screening of many diseases simultaneously, namely expanded carrier screening. The comprehensive or expanded carrier genetic test described here is built upon the many advantages offered by next-generation DNA sequencing platforms.

Appropriate and accurate pre- and post-test genetic counseling is of utmost importance. Individuals and couples must understand the purpose of the genetic test, the disorders analyzed and their severity, and the fact that, even for a negative test result, a residual risk remains. In addition, they must be advised about disorders that arise due to a de novo mutation, mutations not included in the test, or a non-tested rare disorder. In the case of gamete donors, additional input may be needed. Gamete donors are typically young individuals attending the reproductive clinic for the purpose of donating, and they may not expect to be tested for genetic disorders. Even after pretest counseling and giving consent for testing, the possibility of being a carrier for a certain mutation may not be on potential donors' minds at all. Therefore, in the event of a positive result, the genetic counselor may have to deal with the potential donor's shock at being excluded from the donation program. In such instances, post-test genetic counseling must emphasize that,

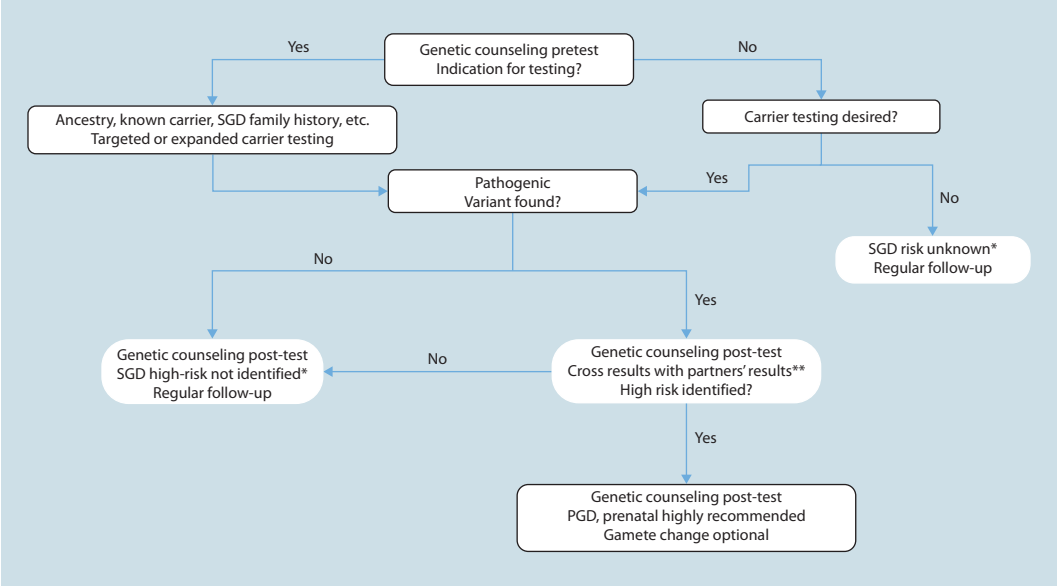

FIGURE 1.1 Algorithm for carrier screening. *Family wish is assumed during all processes of this algorithm, which refers to risk for the future offspring regarding AR and XL conditions. **Testing partner is assumed. AR, autosomal recessive condition; SGD, single-gene disorders; XL, X-linked condition.

generally, there is no clinical risk for the tested individual but at the same time must indicate the clinical relevance of the information for future family planning (see Figure 1.1).

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2

Meiotic Abnormalities in Infertile Males

Zaida Sarrate, Joan Blanco, and Francesca Vidal

CONTENTS

Overview of Meiosis

Meiosis is a key process in spermatogenesis, with the final goal of production of gametes with haploid genetic content. A significant number of exhaustive revisions of this process have been published in the specialized literature [1–14]. In meiosis, DNA replication is followed by two consecutive cell divisions that reduce the number of chromosomes by half. The first meiotic division is reductional and involves the segregation of homologous chromosomes to opposite poles, while in the second meiotic division sister chromatid segregation occurs (Figure 2.1). Prophase of the first meiotic division includes highly complex events and consists of different substages (leptotene, zygotene, pachytene, diplotene, and diakinesis). During prophase I, homologous chromosomes pair; synaptonemal complexes are assembled and disassembled; and reciprocal exchanges between homologous chromatids take place, a phenomenon called meiotic recombination. In male meiosis, specifically at the zygotene and pachytene stages, chromosomes X and Y form a peculiar structure called a sex vesicle. At the end of prophase I, chromosome bivalents show chiasmata, which should be interpreted as the visible manifestation of recombination. Next, at metaphase I, bivalents reach maximum condensation, chiasmata are still visible, and bivalents appear arranged at the equatorial plate to segregate the homologous chromosomes to the opposite poles during anaphase I. As a result of the first meiotic division, two haploid cells are produced (each chromosome with two sister chromatids). During the second meiotic division, which starts without previous DNA replication, the cohesion between sister chromatids is lost, and the segregation results in four haploid cells (each chromosome with one chromatid).

The meiotic process is highly regulated, involving different cell control mechanisms (checkpoints) designed to detect anomalies in chromosome pairing and segregation. The pachytene checkpoint prevents cells from overcoming this stage if chromosome pairing anomalies are present [2,15], and spermatogenic arrest at this stage has been associated with defects in recombination and/or in homologous chromosome synapsis [8,16]. The spindle assembly checkpoint regulates the transition from metaphase

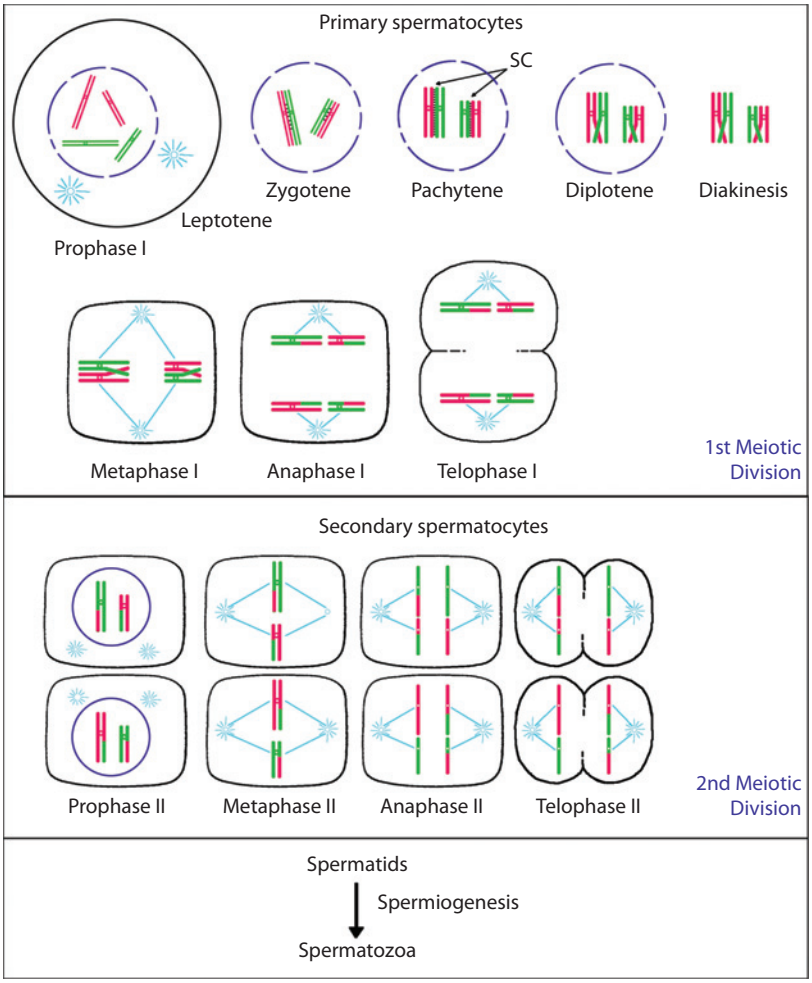

FIGURE 2.1 Schematic overview of meiotic process in spermatogenesis. SC, synaptonemal complex.

I to anaphase I and acts to retain cells at metaphase I until all bivalents are properly orientated to the spindle [17,18], which is a prerequisite for correct segregation.

Methods of Study

Meiotic cytogenetic studies address the detection of abnormalities that exclusively affect the germline. Thus, studies are performed in testicular tissue cells. Biopsies are usually obtained under local anaesthesia and are disaggregated using the appropriate protocol according to the type of subsequent analysis performed. Since the 1980s, several cytogenetic techniques have been implemented. Essentially, these techniques address the study of synaptonemal complexes and the analysis of the meiotic chromosomes [19].

Immunostaining of the synaptonemal complex elements and of MLH1 recombination foci (Figure 2.2) has been used to evaluate synapsis and chromosome recombination at the pachytene stage [20]. Moreover, individual identification of each synaptonemal complex using centromere-specific multicolor fluorescence in situ hybridization (cenM-FISH) or subtelomere labeling [21–23] has contributed to a better understanding of the synaptic process in normal and abnormal scenarios. However, these studies have limited application in clinical contexts and will not be discussed in this chapter.

In contrast, studies of meiotic chromosomes using uniform staining have been implemented in some laboratories. Briefly, this protocol consists of mechanical disaggregation of the testicular tissue in a hypotonic solution (0.075 M KCl), followed by cell fixation using methanol:acetic acid (3:1). The cell suspension obtained is dropped onto slides, and samples are stained with Leishman stain (20%) for evaluation with an optical microscope (Figure 2.3). This methodology allows the analysis of chromosome features at prophase I, metaphase I, and metaphase II, identifying meiotic abnormalities in these stages (Figure 2.4).

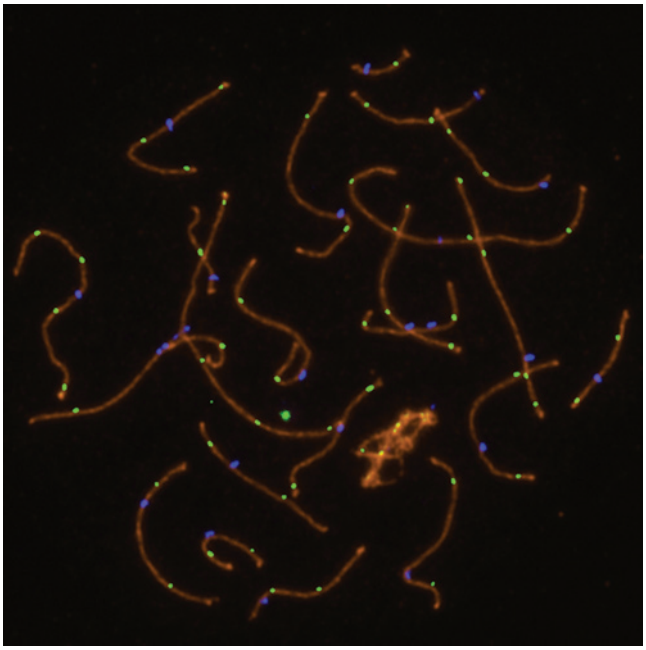

FIGURE 2.2 Human pachytene spermatocyte immunostained. Synaptonemal complexes are immunolabeled in red (SYCP3), centromeres in blue (CREST), and MLH1 recombination foci in green. (Image courtesy of V. Peinado.)

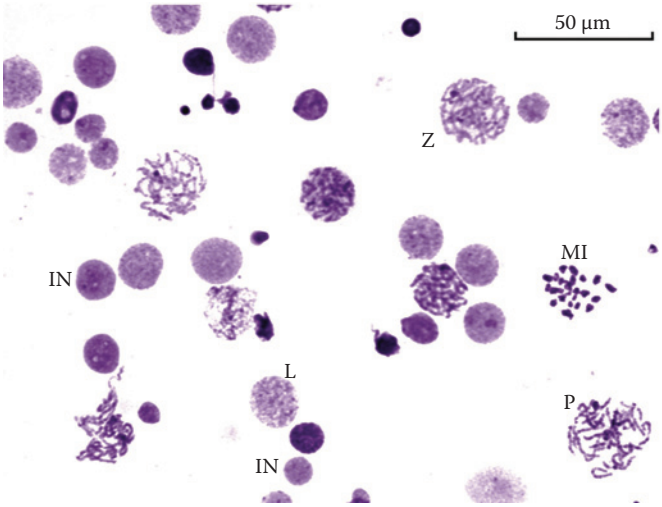

FIGURE 2.3 Leishman-stained spermatogenic cells obtained from human testicular tissue using cytogenetic protocols. IN, interphase nucleus; L, leptotene; MI, metaphase I; P, pachytene; Z, zygotene.

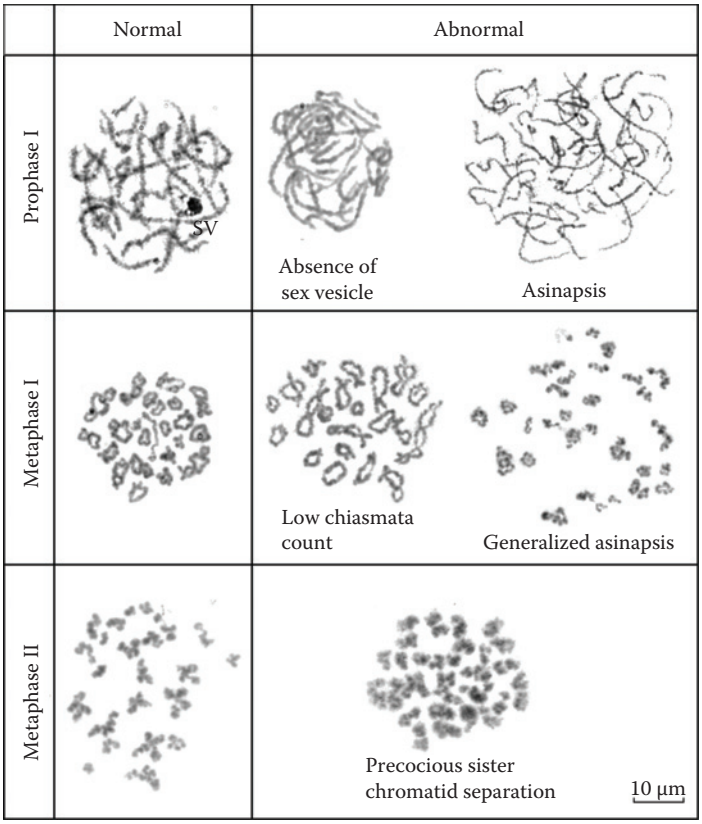

FIGURE 2.4 Leishman-stained meiotic figures from human testicular biopsies. SV, sex vesicle

The implementation of multiplex fluorescence in situ hybridization protocols (M-FISH) has allowed the unequivocal identification of chromosomes at metaphase I and metaphase II [24] (Figure 2.5). This methodology, limited to the research field due to the disproportionate relationship between cost and information obtained, has provided new data on the characterization of chromosome meiotic abnormalities described using uniform staining [25,26].

Classification of Meiotic Anomalies

Meiotic anomalies are usually classified in two categories: asynapsis and desynapsis. Asynapsis is the abnormal pairing of chromosomes from early prophase I with no formation of the sex vesicle, anomalies in synaptonemal complexes, and large reductions in the number of chiasmata in metaphase I [27,28]. Desynapsis is the apparently normal pairing of chromosomes up to the pachytene stage displaying sex vesicle formation, but with visible anomalies in some synaptonemal complexes and low chiasmata count in metaphase I [28,29]. Focusing on bivalents in metaphase I, abnormalities may affect one, several, or most of them. Furthermore, these anomalies may involve all cells or coexist with normal cell lines [19].

In a clinical scenario, the analysis of uniform staining preparations enables us to reach a diagnosis of "normal meiosis" or "altered meiosis." In normal meiosis, all spermatogenic stages are present in normal proportions, showing normal cell features. In abnormal meiosis, a proportion of the spermatogenic cells is abnormal (meiotic arrest), usually due to an accumulation of prophase I stages or metaphase I cells. Abnormal meiosis also arises if anomalies such as low chiasmata count or presence of univalents are observed.

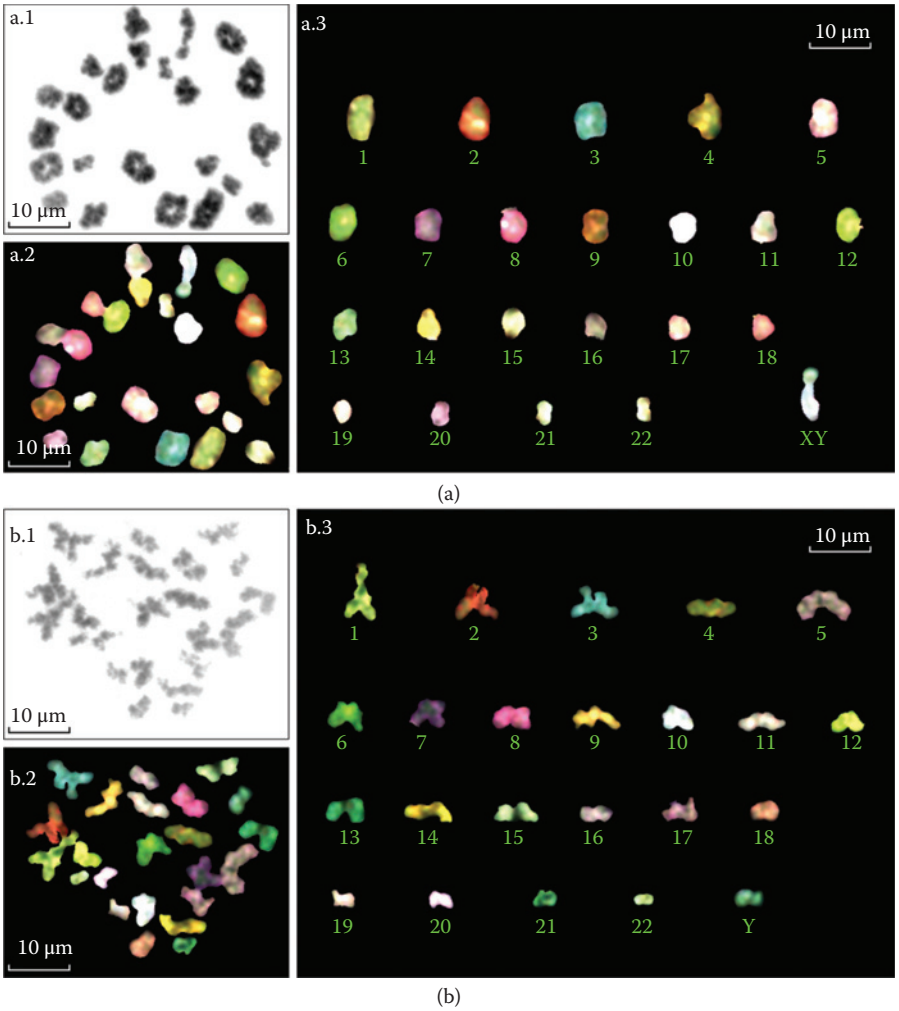

FIGURE 2.5 Chromosome identification at metaphase I and metaphase II using sequential Leishman stain and M-FISH procedures. (a) Metaphase I. (b) Metaphase II. a.1, b.1: Leishman staining. a.2, b.2: M-FISH images. a.3, b.3: M-FISH karyotypes.

Low Chiasmata Count

The reduction in the number of chiasmata per bivalent can influence their orientation along the metaphase plate and therefore affect the segregation of homologous chromosomes during anaphase I [1]. This phenomenon, combined with failures in the control mechanisms, could increase the proportion of chromosomal abnormalities in spermatozoa [30].

The relationship between male infertility and low chiasmata count has been described by different authors [19,31–37], affecting the reproductive fitness of these individuals [38–41]. Use of M-FISH techniques has established that bivalents formed by medium and large chromosomes are the most susceptible to this phenomenon [25]. Despite this, since these chromosomes have a basal number of chiasmata higher than two [32], the reduction rarely leads to the presence of univalents. Accordingly, sperm FISH studies do not show preferred increased values of chromosomal abnormalities in medium and large chromosomes [37]. Therefore, the presence of at least one chiasma seems to guarantee, in most cases, a correct segregation of chromosomes during meiosis.

Presence of Univalents

The lack of chiasmata between homologous chromosomes compromises their orientation at metaphase I, resulting in segregation errors and leading to chromosomally abnormal gametes [42].

Several studies have reported the presence of univalents in infertile individuals [19,32,37]. This abnormality, which results from an extreme reduction in the number of chiasmata, is the most frequently observed anomaly in spermatocytes and usually involves sex chromosomes and small autosomes [25]. It is expected that defects in the processes of pairing, synapsis, and/or recombination of homologous chromosomes during prophase I could lead to the production of achiasmatic chromosomes [5,7]. Due to small autosomes (F and G group) and to sex chromosomes often showing a single chiasma [32], it is not surprising that these chromosomes were the ones observed more frequently as univalents [35]. This meiotic behavior is also consistent with the sperm FISH studies that described the highest aneuploidy rates in sex chromosomes and in G group chromosomes (especially chromosome 21) [37].

Meiotic Arrest

Meiotic arrest, characterized by the presence of unexpected proportions of some spermatogenic stages, has been described by different authors in infertile patients [19,43]. The activation of checkpoints in pachytene and metaphase I/anaphase I stages would arrest and remove cells with meiotic abnormalities [44]. Depending on the severity of the alterations and the effectiveness of control mechanisms, a total or partial arrest of spermatogenesis could occur, resulting in azoospermia or more or less severe oligozoospermia. It is expected that a low number of spermatozoa is related to an increased rate of chromosome abnormalities, probably due to the inefficiencies in control mechanisms.

Relationship between Meiotic Anomalies and Male Infertility

Published data underscore the fact that meiotic abnormalities occur frequently in infertile males [19]. Studies of diakinesis/metaphase I in a series of 1,100 patients, including azoospermic to normozoospermic individuals with a normal somatic karyotype who were seeking advice about infertility, described synaptic abnormalities in 6%–8% of males [45]. The incidence of these anomalies was higher (17.5%) in a study performed on 103 infertile males with severe oligoasthenozoospermia [46]. Another study also described synaptic anomalies in 27% of the 60 normozoospermic males with a long history of sterility or with previous IVF failures evaluated [19]. Moreover, a study of 31 infertile males that incorporated M-FISH techniques to better characterize meiotic abnormalities suggested that these figures could reach an incidence of 48.4% and be a significant association with oligozoospermia [25].

According to all of this evidence, the best candidates for a meiotic study would be infertile males with a normal karyotype and unexplained infertility, among them males with normozoospermia and long-term sterility or with IVF failures (embryonic factor, no fertilization, repeated IVF failures) or infertile males with a severe oligozoospermia factor [45].

Meiotic Studies in the Clinical Diagnosis of Male Infertility

Limitations of the Technique

The technique is cheap, fast, easy to perform, and reliable. The analysis of meiotic images is tricky but anomalies are easily identified by experienced personnel.

Nevertheless, sample features often result in limitations to the study due to the small amount of material obtained, the few cells under division, or, in the case of partial arrest during prophase I, the small numbers of metaphase I and II spermatocytes available for analysis. Moreover, uniform chromosome staining procedures, together with the characteristic appearance of metaphase I and metaphase II chromosomes, make it difficult to identify the chromosomes affected by a given anomaly. Another limitation is that, occasionally, the characteristics of the preparations obtained are not compatible with a cytogenetic analysis.

Finally, it is important to note that, to ensure accurate analysis results, it is strongly recommended to establish internal laboratory limits of normality for the different meiotic alterations.

Interpretation of the Results

Meiotic studies allow for determining whether the meiotic phase of spermatogenesis progresses correctly, but anomalies observed in this process do not always correlate with an increased rate of abnormalities in the resulting spermatozoa. In this sense, when results from meiotic studies and FISH on sperm analysis performed on the same individuals were compared, a clear reduction in abnormal cells across the process of spermatogenesis was observed in 74% of patients [37], probably due to the activation of checkpoints that would selectively eliminate aneuploid cells [47,48]. Even so, an altered meiosis diagnosis would have to be interpreted as evidence of abnormalities in the pairing, recombination, and/or segregation of meiotic chromosomes, indicating that spermatogenesis is compromised.

Taking all of this into consideration, individuals diagnosed as having an altered meiosis should be advised about their reproductive risk, and it is highly recommended they have a sperm FISH study performed (see Chapter 3, this volume) to establish the final outcome of chromosome abnormality at the end of the spermatogenic process.

Acknowledgments

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3

Chromosomal Analysis of Sperm

Lorena Rodrigo Vivó, Vanessa Peinado, Lucía Marín López, Rupali Chopra, and Rajni Khajuria

CONTENTS

Introduction

Infertility problems affect 10%–15% of couples of reproductive age. In 1992, the World Health Organization determined that 10%–30% of the infertility cases were due to male causes [1]. Most of the factors associated with male infertility have a genetic component, such as chromosomal abnormalities and genetic defects, that affect the reproductive system or spermatogenesis. Errors in synapsis, recombination, and DNA repair processes during male meiosis can generate abnormal segregation of homologous chromosomes at meiosis I or sister chromatids at meiosis II, leading to spermatozoa with numerical chromosome abnormalities such as aneuploidies or diploidies.

Several checkpoint mechanisms that regulate the different stages of male meiosis can eliminate defective germ cells and ensure spermatozoa with a normal chromosome content. For this reason, spermatogenesis can be partially arrested in any of the maturation stages, resulting in *oligozoospermia* (reduced sperm number), or it can be completely arrested, resulting in *azoospermia* (absence of sperm). However, if these control mechanisms are deficient, any of the abnormal cell lines can evade the checkpoints and give rise to chromosomally abnormal spermatozoa.

In couples with severe male factor infertility, intracytoplasmic sperm injection (ICSI) increases the chance of pregnancy. It is important to note that prenatal testing from ICSI pregnancies has indicated an increased incidence of de novo sex chromosome aneuploidies and structural rearrangements [2,3]. Most chromosomal aberrations in these pregnancies are of paternal origin [4,5] and are attributed to the sperm quality of the infertile males [6,7]. Between 2% and 26% of infertile men with a normal karyotype exhibit cytogenetic anomalies confined to the germ cell line [8,9], which makes sperm chromosome studies particularly relevant.

The first chromosome studies in sperm appeared in 1970 with the use of differential staining of specific chromosome regions. The total aneuploidy rate was estimated at 38%, with 1.4% for sex chromosomes and an average of 2% for autosomes [10]. These rates were considered excessively high and were attributed to the low chromosomal specificity of the technique. In 1978, the development of a technique to fuse sperm with hamster oocytes without zona pellucida [11], which was standardized in 1982 [12], provided information on the full chromosome content of sperm. However, this technique was complex and laborious and was limited to the analysis of spermatozoa capable of fertilization. The mid-1980s gave rise to the development of in situ hybridization techniques using specific DNA probes labeled

with radioisotopes [13]. Later, in the 1990s, the use of DNA probes labeled with non-radioactive isotopes allowed the visualization of sperm chromosomes with the fluorescence in situ hybridization (FISH) technique [14].

FISH Technique for Chromosomal Analysis of Sperm

FISH uses fluorescently labeled DNA probes directed to specific DNA sequences in the interphase sperm nuclei. By visualizing the hybridization signals using fluorescence microscopy, it is possible to identify numerical chromosome abnormalities in the nucleus of ejaculated, epididymal, and testicular sperm. The simultaneous use of multiple probes specific for different chromosomes allows a rapid and relatively simple evaluation of a large number of sperm, enabling the detection of structural and numerical abnormalities [15,16]. Before applying the FISH protocol, spermatozoa must be fixed and spread on glass slides, avoiding overlapping. The disulfide bridges between protamines that condense the nuclear chromatin must be broken by reducing agents to decondense the nucleus and to allow access of the DNA probes. Then the sperm double-stranded DNA and the FISH probes are denatured by incubation at high temperature. Finally, the co-incubation and hybridization of sperm nuclei and DNA probes form a duplex of complementary strands (see Figure 3.1).

FISH in sperm is commonly performed using centromeric, locus-specific, and sub-telomeric fluorescent DNA probes. For segregation studies in structural rearrangements, specific combinations of these three types of probes are designed for each specific rearrangement. However, in carriers of numerical sex chromosome abnormalities and also in normal-karyotype infertile men, the most widely analyzed are chromosomes 13, 18, 21, X, and Y, the aneuploidies of which are compatible with life (see Figure 3.2).

In normozoospermic men with a normal karyotype, the total sperm aneuploidy rate has been estimated at 6%, with 0.31% disomy for the sex chromosomes and an average frequency of disomy for the autosomes of 0.12% [16]. Due to this low aneuploidy rate, it is recommended to score a minimal number of 1,000 sperm per sample for clinical applications; however, this number may be smaller in cases of low sperm count such as cryptozoospermia and azoospermia. After evaluation, an abnormal FISH result is considered in cases where the sample shows a significant increase in sperm with numerical chromosome abnormalities (aneuploidies and/or diploidies) when compared to a control population of normozoospermic fertile males.

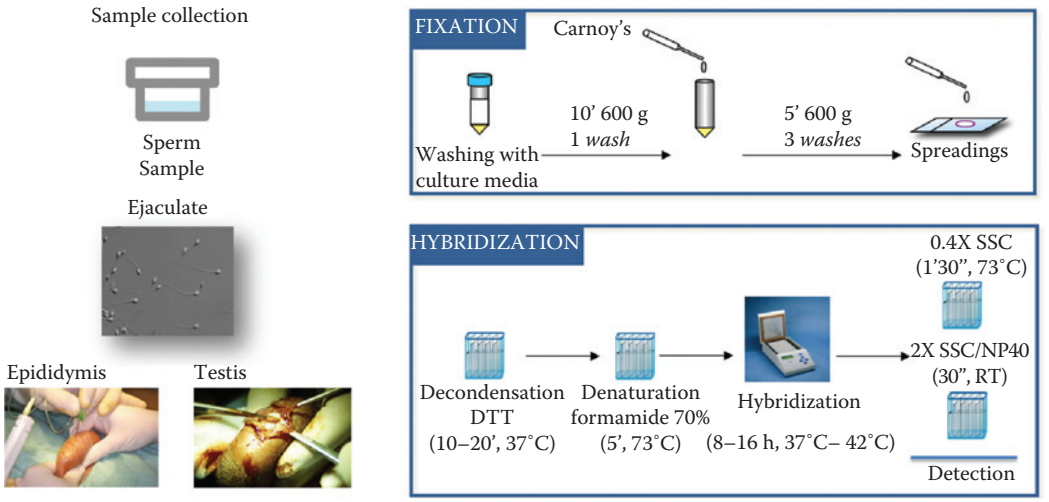

FIGURE 3.1 Sperm sample extraction and preparation for FISH analysis. After sample collection from the ejaculate, epididymis, or testis, spermatozoa are fixed and spread on glass slides. Hybridization is performed using specific fluorescently labeled probes for the analysis.

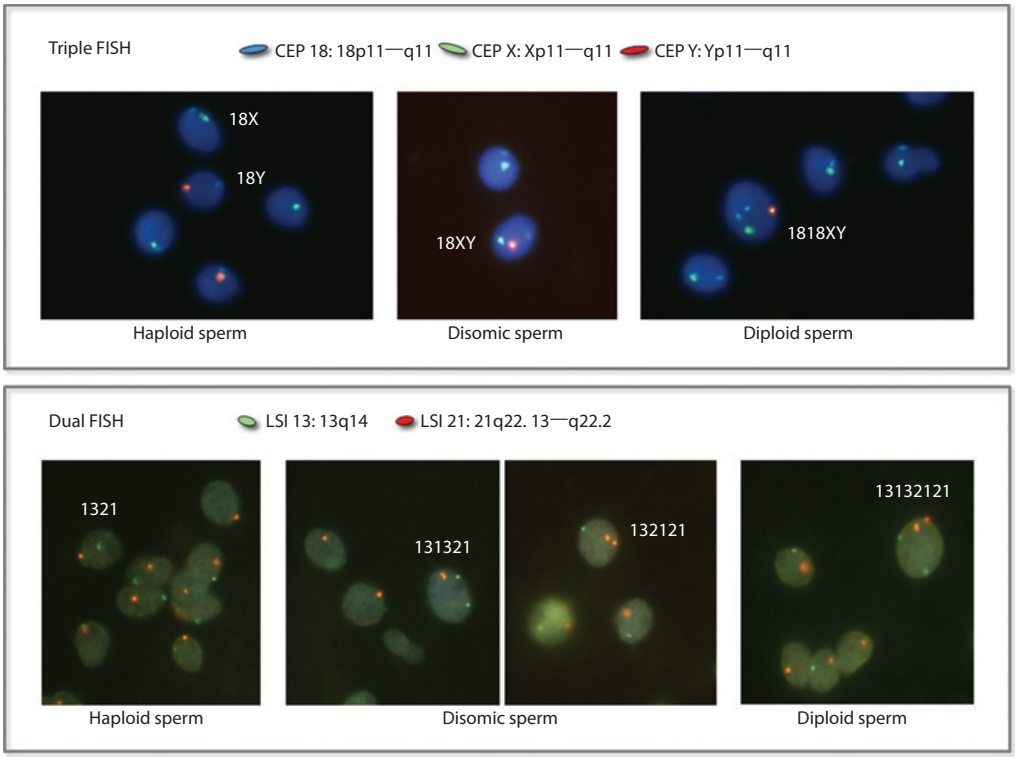

FIGURE 3.2 Evaluation of FISH signals using epifluorescence microscopy. Spermatozoa are hybridized using a triple FISH with centromeric enumeration probes (CEPs) for chromosomes 18, X, and Y, and a dual FISH with locus-specific identifier (LSI) probes for chromosomes 13 and 21. Spermatozoa with one signal for each of the autosomes evaluated and one signal for the sex chromosomes (X or Y) are considered *normal haploid*; spermatozoa with two signals for one chromosome and one signal for the remaining ones are considered *abnormal disomic*; and spermatozoa with two signals for all the chromosomes analyzed are considered *abnormal diploid*.

Indications

Currently, FISH in sperm is used in diagnosis protocols of male infertility, allowing the evaluation of the transmission risk of chromosomal defects to the offspring. Discriminating between infertile males with normal and abnormal karyotype, candidates for receiving sperm FISH analysis would be the following.

1. *Infertile men with abnormal karyotype*
   - *Carriers of numerical abnormalities for sex chromosomes. Klinefelter (47,XXY)* and *47,XYY syndromes* are at risk of low sperm production with poor sperm quality and abnormal chromosome constitution [17–20]. Blanco and coauthors described incidences of 1%–20% of spermatozoa with aneuploidies for the sex chromosomes and 1% diploid sperm in these males [21].
   - *Carriers of structural chromosome abnormalities*. Carriers of *balanced chromosomal rearrangements* such as Robertsonian or reciprocal translocations and inversions, even with a variable range of alterations during gametogenesis, *may result* in normozoospermia, oligozoospermia, or even azoospermia. After spermatogenesis, the spermatozoa can be chromosomally unbalanced in a variable range [22]. The incidence of unbalanced sperm

for the chromosomes of the rearrangement is 10%–40% in Robertsonian translocations, 50%–65% in reciprocal translocations, and 1%–55% in inversion carriers [23].

2. *Infertile men with normal karyotype*
   - *Impaired meiosis in testicular analysis.* Low recombination frequency in meiotic pachytene cells has been related to high aneuploidy frequency in sperm [24], mainly for the sex chromosomes [25]. In fact, a significant correlation has been described between cells with sexual vesicles without recombination sites and sex chromosome disomy in sperm [26]. Peinado and coauthors also recently observed that 81.2% of non-obstructive azoospermic males with lower recombination levels compared to post-vasectomized obstructive azoospermic controls had an abnormal sperm FISH result, with a fourfold increase in disomy for all the chromosomes analyzed compared to controls [27].
   - *Impaired sperm parameters.* Most of the studies performed in *oligoasthenozoospermic* males describe increased incidences of aneuploid and diploid sperm compared to normozoospermic men [28–35]. The sex chromosomes are the most affected, and the incidence of aneuploidy seems to be directly correlated with the severity of the oligozoospermia, being higher in patients with a sperm concentration lower than 5 million [36–46] (see Figure 3.3 [47]). The same correlation has been observed in testicular sperm from azoospermic patients, mainly in those with *non-obstructive azoospermia* [37,44,48–52] where up to 42% of the men have an abnormal FISH result [53].

     However, this correlation seems less clear regarding motility or morphology in sperm. Different studies have centered their attention on *isolated asthenozoospermia*, finding no correlation with meiotic errors [42] even when FISH analysis is performed on motile and non-motile sperm of the same sample [54,55]. Nevertheless, a higher correlation with sperm meiotic defects has been observed in those cases of isolated severe asthenozoospermia [35,56] with specific deformities involving sperm flagella [57]. Regarding *isolated teratozoospermia*, an increased incidence of aneuploidy and polyploidy seems to be associated with isolated teratozoospermia and specific morphological defects such as large-headed and multiple-tailed spermatozoa [58–63].
   - *Chemotherapy and radiotherapy treatments.* Most *chemotherapy* or *radiotherapy* treatments have gonadal toxicity and affect spermatogenesis to a variable degree, depending on the type and duration of treatment. Reports indicate fivefold increases of diploid sperm and sperm with aneuploidies for autosomes and gonosomes after 6 months of treatment

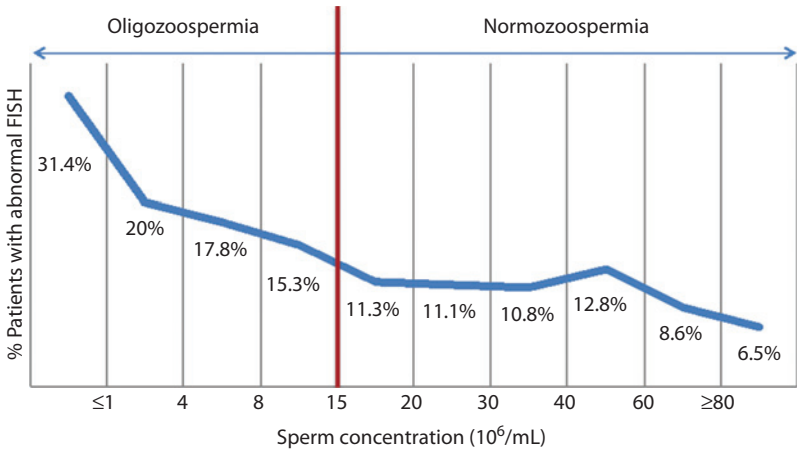

FIGURE 3.3 Effect of sperm concentration on the outcome of FISH results. The figure represents an inverse correlation between sperm concentration and the percentage of patients with increased sperm aneuploidy. (Data from Rodrigo L. et al., Why Preimplantation Genetic Screening [PGS] improves clinical outcome in couples with low sperm counts? Impact of spermaneuploidy. 14th International Conference on PGDIs, Chicago, IL, 2015.)

compared to their basal level [64–66], and, in general, these rates decline to basal levels 18–24 months post-treatment [67]. Several studies have also described an association between Hodgkin's lymphoma and impaired spermatogenesis, some of them finding a significant increase in aneuploid sperm before any treatment [68,69]. These data suggest that the emergence of cancer itself induces problems in meiosis.

- *Clinical history of unknown recurrent pregnancy losses.* Approximately 66% of the abnormal karyotypes from miscarriages have a male origin [70]. Meiotic abnormalities [8,50] and sperm aneuploidy have been reported in recurrent pregnancy loss (RPL) patients [42,71–76]. Most of the reports describe increases in the incidence of sperm with sex chromosome disomy, and an increase in the diploidy rate was noted in a subset of patients with recurrent miscarriage after ovum donation [72]. Moreover, the proportion of men with increases in aneuploid sperm is higher in couples with RPL [77].
- *Clinical history of repetitive implantation failure.* It is thought that oocyte fertilization by a chromosomally abnormal sperm may cause implantation failure [30]. A study carried out in patients with three or more failed ICSI cycles reported that 31.6% of the patients had an increase in spermatozoa with sex chromosome disomy [42]. Later studies have related an abnormal FISH result in spermatozoa with a decrease in pregnancy and implantation rates in ICSI cycles [78–80].
- *Previous pregnancy with chromosomopathy.* Men with *chromosomally abnormal offspring of paternal origin,* such as Down syndrome (trisomy 21), Klinefelter syndrome (trisomy XXY), and Turner syndrome (monosomy X), have shown incidences of 1%–20% aneuploid sperm, with the altered chromosome affected [81–84].

Clinical Impact of Sperm Chromosomal Abnormalities

As a clinical diagnostic tool, sperm FISH analysis should offer a clinical prognostic value to evaluate reproductive possibilities in the infertile couple. Therefore, an abnormal FISH result should be evaluated in terms of how much it affects the clinical outcomes.

At the *clinical outcome level*, an increase in spermatozoa with chromosomal abnormalities has been associated with a decrease in pregnancy rates and higher miscarriage risk in infertile couples undergoing ICSI cycles. Rubio et al. (2001) [42] compared the outcome of 108 ICSI cycles from patients with a normal sperm FISH result with 23 ICSI cycles from patients with an abnormal result. The results indicated similar fertilization rates (71.5% vs. 74.5%), a higher pregnancy rate (36.5% vs. 23.6%), and a lower miscarriage rate (54.8% vs. 80.0%) in the group of patients with normal FISH. Similarly, Burrello et al. (2003) [79] analyzed the clinical results in a series of 48 patients undergoing ICSI. They observed lower pregnancy (34% vs. 75%) and implantation (13% vs. 34%) rates and a higher miscarriage rate (38.9% vs. 11.1%) in patients with higher incidence of aneuploid sperm compared to patients with normal incidence (considering normal below 1.55%). However, fertilization rate and embryo quality were similar in both cases. More recently, Rodrigo et al. [46] retrospectively analyzed the reproductive outcome of male factor infertility couples without any history of recurrent miscarriage or implantation failure. Conventional IVF/ICSI cycles in couples with an abnormal sperm FISH result showed significantly lower embryo transfer rates (64.0% vs. 84.8%), higher mean number of transferred embryos (2.3 ± 0.9 vs. 2.0 ± 0.6), and lower pregnancy (22.9% vs. 30.8%) and implantation rates (12.4% vs. 21.4%) than patients with normal sperm FISH result. Nicopoullos et al. [80] also found a significantly higher sperm aneuploidy rate in patients who did not achieve pregnancy compared to patients who achieved pregnancy after ICSI (2.37% vs. 1.18%). Moreover, the probability of achieving a clinical pregnancy decreased by 2.6 times for every 1% increase in the sperm aneuploidy rate. In another study, Petit et al. [78] found higher rates of aneuploidy and diploidy in sperm of men who did not conceive after ≥4 ICSI cycles compared with those who got pregnant after 1–3 ICSI cycles.

At the *embryo level*, preimplantation genetic testing for aneuploidies (PGT-A) has allowed the evaluation of the impact of higher rates of sperm numerical chromosome abnormalities on the chromosomal constitution of embryos. Several reports described higher rates of abnormal embryos, noting a high incidence of aneuploidy for sex chromosomes and mosaic embryos [17,85–90]. The percentage of abnormal embryos ranged between 43% and 78% in patients with oligozoospermia and azoospermia in which an abnormal FISH in sperm or an impaired meiosis were reported [17,86–94]. Patients with severe oligozoospermia or cryptozoospermia who were carriers of Y-chromosome microdeletions showed a higher increase in aneuploid embryos, highlighting monosomy X [95]. In particular, different effects on embryo chromosome constitution have been described according to the type of sperm chromosomal abnormality detected. Therefore, an increase in the percentage of spermatozoa with sex chromosome disomies has been associated with an increase in embryo aneuploidies compatible with life (Patau, Edwards, Down, Klinefelter, and Turner syndromes, and trisomies XXX and XYY). In contrast, an increase in diploid spermatozoa has been related to an increase in triploid embryos, which mostly miscarry before delivery [17].

In translocation carriers, a correlation has been observed between the percentage of abnormal gametes and the percentage of abnormal embryos [96]. In normal karyotype patients, a retrospective analysis performed in 249 PGT-A cycles in couples with isolated severe male factor infertility showed a linear increase in the percentage of abnormal embryos with the increase in the percentage of total aneuploid sperm [97].

At the *offspring level*, several studies performed in parents of children with Down syndrome, Klinefelter syndrome, and Turner syndrome have shown increases in sperm chromosomal abnormalities associated with the chromosomopathy observed in the children. In a study conducted on two fathers of children with Down syndrome of paternal origin, the sperm disomy 21 rates were reported as 0.75% and 0.78% [81]. Similar studies in couples with miscarriages or children of carriers of sex chromosome abnormalities (Turner or Klinefelter syndrome) have reported high incidences of sperm aneuploidy for sex chromosomes, ranging from 0.20% to 24.7% [82–84,98–100]. (See also Figure 3.4.)

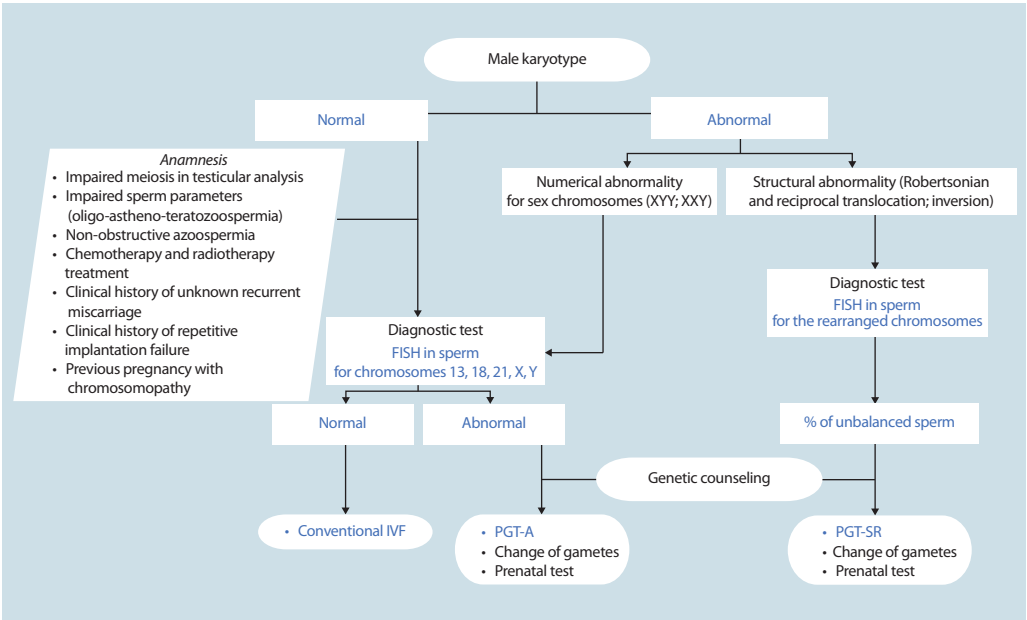

FIGURE 3.4 Algorithm of testing options. FISH, fluorescence in situ hybridization; IVF, in vitro fertilization; PGT-SR, preimplantation genetic testing for structural rearrangements; PGT-A, preimplantation genetic testing for aneuploidies.

Clinical Reproductive Counseling

As described in this chapter, the presence of numerical chromosome abnormalities in sperm can be translated into clinical consequences, such as infertility problems or genetic risk for the offspring. Therefore, when an abnormal FISH result is found in sperm, genetic counseling should be offered to the couple. In addition, depending on the severity of the abnormalities observed in the sperm, several clinical options can be proposed.

1. For significant increases of sperm aneuploidies compared to the control population, PGT-A has been proposed as an alternative to improve the possibility of healthy pregnancies [86–88]. A retrospective evaluation of PGT-A cycles analyzing a battery of 9 chromosomes in patients with abnormal FISH in sperm showed higher pregnancy (39.7% vs. 28.3%) and implantation rates (33.8% vs. 21.4%) than patients with a normal sperm FISH result, despite a lower mean number of embryos transferred (1.6 ± 0.6 vs. 1.7 ± 0.6). Interestingly, patients with normal FISH in sperm had similar clinical results regardless of conventional IVF/ICSI or PGT-A; however, patients with abnormal FISH in sperm showed better pregnancy and implantation rates after PGT-A [46]. Moreover, aneuploidy screening of the 24 chromosomes in couples with male factor infertility offers even better clinical results, with 83.6% of cycles having at least one euploid embryo to transfer, resulting in a pregnancy rate per transfer of 62.9%, an implantation rate of 54.2%, and a take-home baby rate of 50.9% [94].
2. In cases of severe meiosis impairment resulting in extremely high increases in abnormal sperm, sperm donation would offer better clinical results and a higher chance of healthy babies (see also Figure 3.4).

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4

DNA Fragmentation in Sperm: Does It Matter?

Nicolás Garrido, María Gil, and Rocío Rivera

CONTENTS

Background

The Need for Sperm Quality Tests

Sperm DNA fragmentation is a broad concept that involves various situations where DNA sequence integrity in sperm is affected, linked to specific techniques developed to detect and quantify it.

In recent decades, the rapidly evolving field of assisted reproduction techniques together with technical developments concerning therapeutic interventions has increased the need to improve diagnostic tools to estimate pregnancy chances or forecast reproductive outcomes. Moreover, the availability of appropriate diagnostic tools may positively impact reproductive success by avoiding repeated failures or offspring with health problems.

These diagnostic tests seem especially relevant for infertile males, who are thought to be responsible for at least 50% of reproductive failures. Evidence indicates that sperm function is multifactorial. The fact that you may need to select spermatozoa prior to IVF or ICSI, or even have the possibility of selecting among different ejaculates from the same male, makes the development of these biomarkers to assess sperm function especially relevant [1].

Sperm DNA integrity appears critical to releasing paternal genetic content properly to the oocyte and permitting embryo development, implantation, and the growth of a healthy child.

Nevertheless, the increasing number of papers that report the relationship of reproductive outcomes with DNA integrity have resulted in a confusing body of literature, often extremely difficult to interpret and of limited clinical applicability [2,3]. This was the result of the relative low cost of sperm DNA fragmentation tests and the ease of obtaining biological samples to study. The popularization of such studies resulted in myriad research works that mixed different technologies, thresholds employed, main outcomes measured, sample sizes and statistical significance, extent of effect, experimental designs, and potential biases, which may confound the clinician as a reader, user, or prescriber of these tests.

In this chapter we describe the rationale and current knowledge regarding the status of DNA fragmentation analysis in sperm, its utility, and its link with reproductive success and children's health. We provide evidence-based recommendations for its use as a complementary tool to basic sperm analysis in appropriate situations in the clinical environment.

What Is Sperm DNA Fragmentation, What Causes It, and Why Is It Important?

The pioneering studies in the United States in the early 1980s on DNA fragmentation first suggested that assessment of DNA integrity in sperm could function as an independent biomarker for animal and human reproductive ability [4]. Several later findings tended to support this hypothesis, given the higher DNA damage in men with severe sperm defects [5] and the cases of decreased natural fertility and poor results following assisted reproduction techniques when sperm DNA quality was impaired [6,7].

These findings made sense at that time, given the theoretical relevance of sperm DNA integrity in the proper transmission of the paternal genome to the early developing embryo.

We can distinguish three types of sperm DNA damage: (1) the formation of 8-hydroxy-2'-deoxyguanosine (8OHdG) adducts, mainly as an effect of oxidative species on DNA bases; (2) single DNA strand breaks; and (3) double DNA strand breaks (see Figure 4.1) [8].

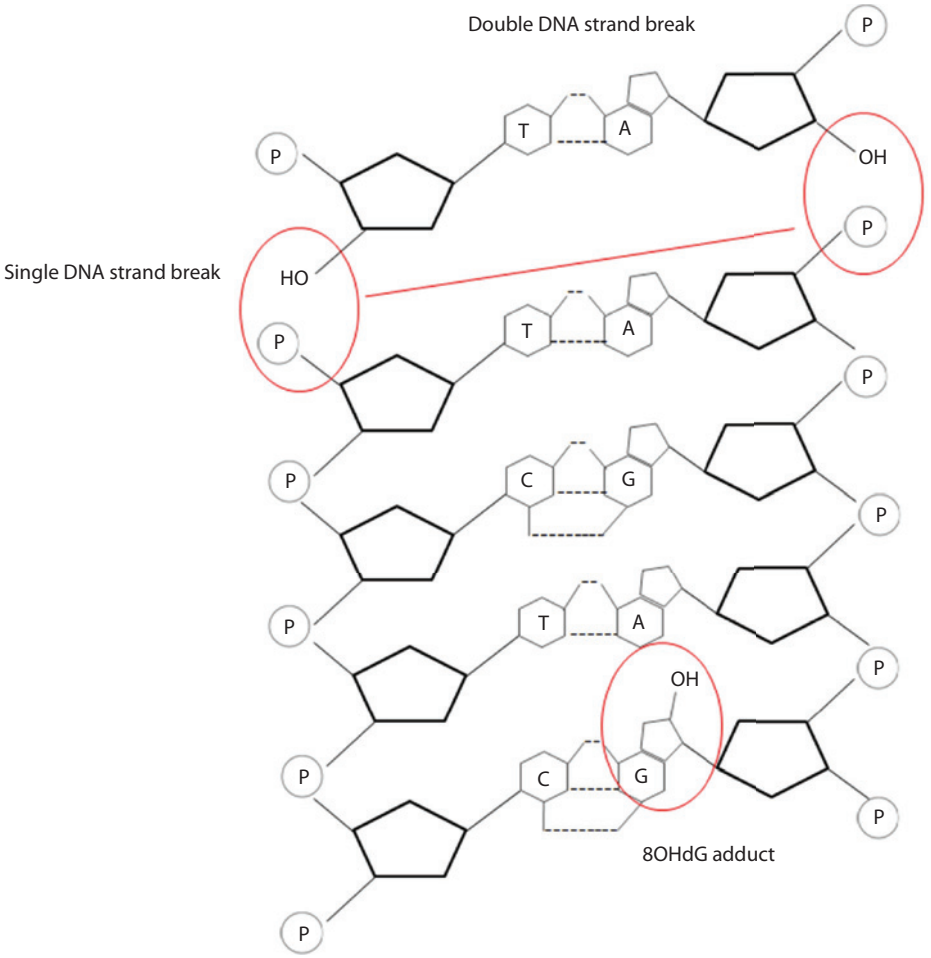

FIGURE 4.1 Types of damage that may occur in sperm DNA molecules.

As many studies demonstrate, this DNA damage can be caused by several internal and external factors or combinations of both.

Specific defects in spermatogenesis may lead to the production of sperm with damaged DNA, although frequently even a small percentage of spermatozoa from fertile men may possess some degree of levels of DNA damage [6,9–11].

Double-strand breaks are thought to be endogenously induced during normal spermatogenesis in the meiosis phase to facilitate meiotic crossovers, and during spermiogenesis, when the chromatin of the haploid round spermatids is compacted after substituting histones by protamines during the later steps of spermatogenesis [12]. Afterward, sperm may suffer additional DNA damage during maturation and storage in the epididymis [13].

Protamine structure deficiency has also been found to be related to sperm DNA damage [14,15], a finding correlated with the fact that several infertile men present enhanced or complete protamine deficiency [16], leading to abnormal chromatin compaction [17] and greater susceptibility to DNA damage.

Similarly, defective reparation of transient DNA nicks resulting from spermatogenesis has been described as a cause of DNA strand breaks in spermatozoa [18].

Apart from DNA breaks caused by alterations in spermatogenesis, the presence of abnormally high reactive oxygen species (ROS) levels is also a cause of DNA fragmentation, as previously demonstrated in different studies. These ROS may be naturally generated by ordinary sperm metabolism, but excesses caused by defective spermatozoa and by semen leukocytes both provoke sperm dysfunction. The association between sperm DNA damage and sperm-derived ROS suggests that DNA damage may be due to a defect during spermiogenesis [19], while ROS derived from leukocytes may exert a post-testicular defect, possibly related to infection or inflammation [20]. Both excessive ROS production and decreased seminal antioxidants may cause this misbalance [21,22].

Aborted apoptosis is a term, initially suggested by Sakkas, that exemplifies the circumstances where apoptosis has been initiated, leading ultimately to DNA impairment, without having compromised cell viability. Apoptosis, which maintains homeostasis and avoids production of abnormal sperm forms, may be retarded in some spermatozoa, leading to the presence of living spermatozoa with DNA damage [23].

Advancing age and gonadotoxins have been associated with reduced levels of germ cell apoptosis in the testicle and an increase in the percentage of ejaculated spermatozoa with DNA damage, suggesting that, in these men, both spermatogenesis and apoptosis have been disrupted [24].

Advancing age has been associated with increased sperm DNA damage [25–27] as well as behavioral styles that result in obesity and diseases such as diabetes [28,29].

Men with cancers such as Hodgkin's lymphoma or testicular cancer may present significant sperm DNA damage [30,31] that may be enhanced due to cumulative doses of chemotherapy [32,33], persisting long after chemotherapy has ceased and sperm production has been recovered.

Male genital tract infection and/or inflammation, as in cases of orchitis or prostatitis, with an enhancement in leukocyte counts, also increase ROS and subsequent DNA damage in sperm [34]. In addition, varicoceles have been described as related to increased DNA fragmentation through oxidative stress [35–37], while DNA integrity has been shown to improve after varicocele repair [38].

Hormonal defects have also been demonstrated to be linked with DNA fragmentation, such as serum testosterone levels, inversely related to sperm DNA damage in infertile men [14,15].

Extrinsic factors that harm sperm DNA include the effect of recreational or medical drugs (e.g., cigarette smoking or chemotherapy, respectively), environmental exposures, lifestyle choices, and habits. Cigarette smoking is linked to an increase in abnormal sperm forms and sperm DNA damage [39,40], probably via increased leukocyte ROS production. Pesticides and air pollution may produce the same situations [41,42].

An increase in scrotal temperatures may also cause significant sperm DNA damage [43,44]. Iatrogenic but preventable DNA fragmentation induction can be caused by the intervention of assisted reproduction laboratories, for example, by unnecessarily delaying sperm preparation and following established sperm preparation protocols [45,46].

In summary, there are several causes of DNA fragmentation that may be initially identified in infertile patients just by means of an exhaustive anamnesis. Counseling for this can be conducted even before a sperm DNA fragmentation test is recommended. The importance of maintaining DNA integrity is vital to passing our healthy genomes to future generations.

How Can We Measure Sperm DNA Fragmentation?

The initial and promising associations between DNA damage and low reproductive outcomes have led to the introduction of sperm DNA integrity testing in tandem with the World Health Organization's basic sperm analysis for the clinical assessment of male fertility.

To this end, various techniques measuring different aspects or characteristics of sperm DNA damage can be utilized. These assays are categorized in several ways, including the molecular basis of the test, the kind of damage, the parameter or indicator measured, or the molecular biology techniques employed [47].

Available assays include direct DNA damage assessment by means of terminal deoxyuridine nick end labeling (TUNEL) assay, comet, and in situ nick translation (ISNT) or indirect assessment with FISH, sperm chromatin dispersion (SCD), acridine orange, and either visual or flow cytometry sperm chromatin structure assay (SCSA) and sperm DNA fragmentation assay (SDFA) [10,48–50].

The comet, TUNEL, and ISNT assays detect actual DNA strand breaks, while the SCSA and SCD mainly measure chromatin integrity and the susceptibility of DNA to denaturation [11,51–53]. The formation of single-stranded DNA from native double-stranded DNA is based on the premise that nicked DNA denatures more easily compared with double-stranded DNA.

The basis of the TUNEL assay lies in the quantification of the breaks by means of incorporating the 3'OH of broken DNA breaks of fluorescence-labeled modified nucleotides (deoxyuridine triphosphate, dUTP), enabling detection of either a single- or double-strand break by a terminal deoxynucleotidyl transferase enzyme independent of a template [54]. Measuring fluorescence by microscopy or flow cytometry provides information about the number of broken sites. This fluorescence is generated by fluorophores as FITC combined with propidium iodide (PI) or 4',6-diamidino-2-phenylindole (DAPI) or amplified by marked anti-dUTP antibodies (see Figure 4.2a).

The comet assay may also be used to study single- or double-strand DNA actual breaks to determine the electrophoretically migrated DNA remains of single sperm in the electric field. The extension of the tail reared apart from the core nucleus, which resembles a comet (and hence the name), represents the amount of fragmented DNA [55] (see Figure 4.2b).

This can be followed by fluorescence microscopy using, for example, dedicated imaging software stained with ethidium bromide or SYBR® Green. Sperm cells are embedded in agarose on a glass slide and treated with lysis buffer, and afterward protamines and histones are removed. The distance the fragments of damaged DNA migrate into the comet tail depends on their size, and information regarding tail length (from the leading edge of the head), from the tail DNA (percentage of DNA found in the tail compared to the head), and the olive tail moment (OTM, tail DNA multiplied by the distance between the means of tail and head fluorescence) can be obtained [8].

The ISNT technique incorporates biotinylated dUTP at only single-stranded breaks using DNA polymerase I, following a template.

SCSA determines the extent of DNA damage by measuring the metachromatic shift from green fluorescence to red fluorescence after acidic denaturing conditions (see Figure 4.2c).

Chromatin with strand breaks in an acidic environment will denature due to reduced stability, thus indicating DNA strand breaks [56]. Native DNA and denatured DNA will differ in the fluorescence color (fluorescent green and fluorescent yellow/red, respectively) and are measured using flow cytometry or fluorescence microscopy.

The DNA fragmentation index (DFI) represents the percentage of the sperm population with medium to high DNA damage. It is calculated by establishing the ratio of red to total (both red and green). Spermatozoa exhibiting high levels of green fluorescence have high DNA stainability (%HDS) [57,58].

The SCD or halo assay tests for chromatin dispersion. It is the simplest and most convenient technique because it may be conducted in every laboratory. It uses fluorescence or bright-field microscopy to estimate the amount of DNA damage by looking at the formation of a halo under determined denaturing conditions and nucleoprotein removal by means of Dithiothreitol [59,60] (see Figure 4.2d).

The SCD assay is based on the principle that sperm with fragmented DNA fail to produce the characteristic halo following acid denaturation and removal of nuclear proteins [50].

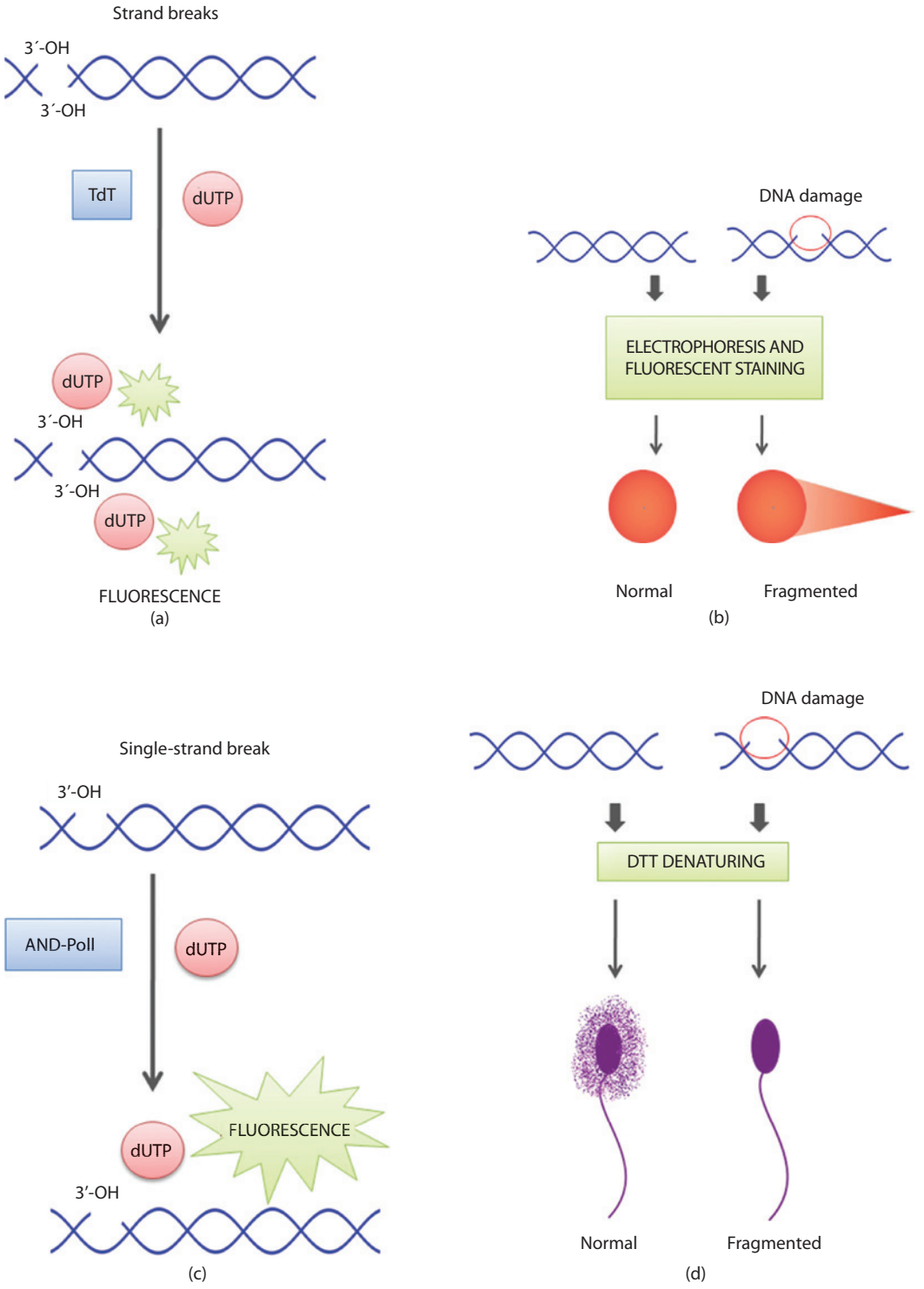

FIGURE 4.2 (a) TUNEL assay. (b) Comet assay. (c) ISNT. (d) SCD. Samples (a minimum of 500 sperm) are evaluated under the ×100 objective of the microscope, based on the fact that relaxed loops of DNA, attached to the central core of the nucleoid, will be dispersed in an agarose, ultimately producing a kind of halo with absence of DNA fragmentation. Fragmented DNA does not produce such a halo [61].

The lack of agreement in the literature is partially due to the diversity of sperm DNA tests, lack of standardized protocols, inter-laboratory variations, consideration of wide ranges of threshold values, and to some extent, the limited understanding of what each sperm DNA assay actually measures [3,51,62].

Despite several differences in the assay methodology, all results obtained using the different approaches largely correlate with one another, except for the manual acridine orange test [63].

That said, we must also keep in mind that correlation of the different techniques of the "sperm DNA fragmentation tests" is not necessarily desired. Reproducibility is the objective. Statistical confirmation should not rest on Spearman's correlation alone. To determine whether two or more different tests are measuring the same, the kappa coefficient or intraclass correlation coefficient (ICC) must be addressed. This information is typically lacking in the literature.

Molecular biology techniques that measure DNA fragmentation may include expensive equipment such as flow cytometers. This has the advantage of testing thousands of cells per experiment, reducing time and increasing robustness, but cost and availability mean that fertility clinics may not have access to the expensive facilities needed. Other tests are based on electrophoresis and/or bright field or fluorescence microscopy, which are readily available.

How Is Sperm DNA Fragmentation Related to Reproductive Outcome?

Forecasting Reproductive Failure/Success

Sperm DNA fragmentation tests are aimed at forecasting reproductive success or failure. The first use to be tested is as a predictive test. For a test to be considered "predictive," several practical characteristics are mandatory: (1) it should be compared with a universally accepted gold standard outcome, in this case a clinical pregnancy, live birth, or miscarriage; (2) the study population should be one in which the test would be applied in clinical practice, in this case male infertility; (3) the test should be replicated accurately in the laboratory; and (4) optimal threshold values must be determined by looking at test characteristics and optimizing sensitivity and specificity using receiver operator characteristic (ROC) curves.

Apart from robustness and reproducibility, the test should have the capability of classifying the expected outcome beforehand, on the basis of the obtained results. This would permit us to know the likelihood of reproductive success before a sperm sample is employed in ART. If a negative result is found, several measures could be taken, such as using other ejaculate or changing gametes for the donor's spermatozoa.

This can be measured statistically by means of ROC curve analysis, a graphical plot that illustrates the performance of a binary classifier system as its discrimination threshold varies, creating the curve by plotting the true positive rate (TPR, sensitivity) against the false-positive rate (FPR, 1−specificity) at all threshold settings. ROC analysis is an important consideration in cost/benefit analysis of diagnostic decision making.

In sperm DNA fragmentation analysis, a diagnostic test is administered to determine whether a couple will achieve parenthood after using the studied sample. A false positive occurs when the sperm sample tests positive, but actually does not reach parenthood. A false negative occurs when the person tests negative, suggesting the sperm are healthy when they actually reach it.

The best possible prediction method is one with an area under the curve of 1, representing 100% sensitivity (no false negatives) and 100% specificity (no false positives).

Depending on the nature of the test, one can be more permissive regarding sensitivity and specificity, as well as AUC. Typically, only tests with AUC >0.85 are considered good, while sensitivities or specificities above this number are also good, and perhaps sufficient to merit implementing the test as routine for infertile patients.

We can compare this information to the previously published results. There are approximately 650 citations in EMBASE and Pubmed about DNA fragmentation and reproductive results. A careful study of the most comprehensive reviews and metaanalysis is recommended. An essential topic is ROC curve analysis to predict intrauterine insemination, in vitro fertilization or ICSI, that shows varying AUC values but rarely above 0.85, and sensitivities or specificities ranging from 0.06 to 0.96. Detailed lists of papers published since 2000 and their corresponding data are available elsewhere [64–68]. Various circumstances may make it impossible to compare results and difficult to extract universal conclusions about the question that is the title of this chapter (see Table 4.1).

TABLE 4.1

Factors Acting as Effect Modifiers or Confounders in Studies Relating Sperm DNA Fragmentation with Reproductive Outcomes

Potential Bias Sources for Studies Concerning DNA Fragmentation in Sperm
• Population studied
• Sample analyzed (either raw or prepared sperm)
• Time passed between ejaculation and analysis controlled or not
• Main outcome measured (biochemical pregnancy, clinical pregnancy, ongoing pregnancy, live birth, biochemical miscarriage, clinical miscarriage)
• Techniques employed to detect DNA fragmentation
• Assisted reproduction techniques employed (simplifying IUI, IVF, or ICSI, with or without PGS)
• Considering or not the contribution of surplus embryos frozen/thawed in subsequent cycles
• Threshold/cutoff value employed to catalog the sample as pathological or not (may range from 4% to almost 60%–80%)
• Blind or unblind studies (those collecting the samples are not the same as those conducting the tests or analyzing the results)
• Effect size (with odds ratio between positive and negative samples ranging from 0.2 to 10.0, 55, and 76 and confidence intervals from 0.11 to almost 100), varying sample sizes (from 12–20, to almost 400)
• Controlling female factor (with inclusion criteria, or controlling if using own or donated oocytes)
• You measure many sperm, but use only one sperm

Generally speaking, there are approximately the same number of studies assessing embryo quality analysis with some poor embryo quality indicator related to DNA fragmentation as there are studies unable to find any correlation.

The meta-analysis by Li et al. [64] concluded that sperm DNA damage is associated with IVF clinical pregnancy rates but not with ICSI outcomes. Another meta-analysis by Collins et al. [66] concluded that assessment of sperm DNA damage is not strong enough to provide any clinical advantage for these assays to evaluate infertile men. The Practice Committee of the American Society for Reproductive Medicine [2] concluded that the existing data do not support a consistent relationship between abnormal DNA integrity and ART outcomes.

Forecasting Miscarriage

It is becoming increasingly apparent that DNA testing is essential because high DNA damage causes increased risk of pregnancy loss, regardless of which test (comet, TUNEL, or SCSA) is used.

There is a statistically significant OR of 2.5, 95% CI 1.5-4.0, corresponding to a pregnancy loss rate of 10% with a normal (negative) test and 37% with an abnormal (positive) test. Despite these encouraging numbers, it should be made clear that the assay failed to identify 60% of the pregnancy loss cases due to a sensitivity of only about 40% [56,69].

To this end, only papers reporting miscarriage rates have been included, and a wide heterogeneity again is found across the papers available. The same authors acknowledge that they cannot advise a couple not to proceed with IVF/ICSI because of a high DNA fragmentation finding, given the fact that there is an approximaely two-thirds chance that any pregnancy will end well. In addition, because there are no proven therapies to improve DNA integrity in most patients, the test result will not affect case management. This is one of the most relevant points to consider when deciding whether or not to request these tests.

Forecasting Offspring Harm

Another concern is the possibility of causing harm in the offspring's health that may appear later as a consequence of involuntarily using spermatozoa with damaged DNA, and considering the magnification effect after applying these techniques in an increasing number of couples worldwide [8,29]. Direct evidence is lacking; only animal experimentation or indirect evidence may produce some preliminary clues.

A higher incidence of hematological cancers such as leukaemia has been found in offspring conceived by men who smoked and smoking has been found to be related to increased DNA fragmentation in

sperm. There is a general awareness of the effects of chemotherapy treatments on the offspring, which may cause DNA fragmentation, but cohorts of children conceived naturally from parents in remission after cancer treatment do not support this hypothesis.

Damaged DNA, together with higher miscarriage rates after the use of ICSI, has been linked to increased incidence of severe disorders [29]. Nevertheless, the information available is still weak, and strong conclusions cannot be drawn.

So, Whom Should We Test?

Initially, the ideal situation could be to test those patients identified to be at relevant risk of having elevated DNA fragmentation.

Infertile males, due to their condition, are potential candidates, but this would mean a massive screening, which seems unnecessary from the cost/benefit perspective, as determined by previously published guidelines.

Several causes or inducers of DNA fragmentation described above may be identified by exhaustive anamnesis (habits, disease, etc.), while others will not (spermatogenesis defects, abortive apoptosis, etc.). Unfortunately, in some cases, no further tests are available, but most important, no therapeutic techniques appear to exist.

On the other hand, patients with repeated reproductive failure, mainly in those cases where other causes are discarded, may be candidates for further DNA testing exploration. A recent meta-analysis by Zhao et al. [65] suggests recommending assays detecting sperm DNA damage to those suffering from recurrent failure to achieve pregnancy.

What Can We Do When We Find Elevated DNA Fragmentation Levels?

Once high sperm DNA fragmentation is detected, you may infer that the worst reproductive results can be expected, probably including an increased risk of miscarriage and/or offspring at a significantly (but perhaps not clinically) higher risk of some (rare) diseases. There is no clear plan for counseling patients.

Two different approaches may be used: treating the sample or treating the male.

With regard to treating the sample, very few therapeutic techniques are available to select sperm cells with lower sperm DNA fragmentation. Magnetic activated cell sorting has been demonstrated as useful when apoptosis caused fragmentation, intracytoplasmic morpologically selected sperm injection (IMSI) in cases of high vacuolation, and pretesting of sperm for ICSI (PICSI) when immature sperm are present with low hyaluronic acid receptors. This means that additional testing should be offered to attempt to identify the cause. Detailed lists of methods to improve overall sperm quality may be found elsewhere [1], although many of them may be thus far considered experimental and without sufficient evidence to support their use.

With regard to treating the male, there is some evidence of significant decrease in sperm DNA fragmentation after medical treatment. Cochrane Reviews on the use of antioxidants seem clear.

The situation is even more difficult because direct causes in individuals will probably not be detected. If it is possible to eliminate the cause, we may at least expect a decrease in sperm DNA fragmentation. This is the case for DNA fragmentation caused by environmental pollutants, toxic habits, obesity, recreational or therapeutic drugs, smoking, or oxidative stress. Behavioral change may be beneficial. Such change is frequently a low cost and common sense remedy.

Regarding specific therapies, in some situations, ICSI rather than IVF has emerged as an option, with limited evidence supporting it. This may be unusual for some centers where ICSI treatments are the main reproductive therapies employed.

Recent papers support the hypothesis that obtaining sperm directly from the testis in cases where the harm is caused in the epidydimis enhances DNA quality and reproductive results [70–72].

Extreme cases may lead to change of the oocyte. Our results indicate that oocyte donation will buffer the negative effects of sperm, due to the repairing capabilities of young and healthy oocytes.

In summary, given that causes are multiple and not always identifiable, and treatments are not always available, there is no single solution, and in-depth investigation of DNA fragmentation causes in individuals may not be cost-effective.

Conclusions: Does It Matter?

Sperm DNA fragmentation is to some extent increased in infertile patients and linked to harmful habits, exposures, and disease, leading to increased miscarriage indexes, and perhaps (only perhaps) an increased risk of harm to offspring. Existing data do not support a sufficiently strong relationship between abnormal DNA integrity and reproductive outcomes to significantly modify reproductive counseling and strategies.

Moreover, the results of sperm DNA integrity testing only are unable to sufficiently predict pregnancy rates achieved to date through either natural conception or assisted reproduction technologies with IUI, IVF, or ICSI.

The use of sperm DNA integrity analysis as part of a routine sperm analysis is not recommended because the confusing evidence regarding its relationship to an increased risk of failure, miscarriage, or disease is not strong enough. From the information retrieved, there is no clear post hoc management or decision due to the absence of evidence-based techniques to either improve DNA quality or select those sperm with better DNA integrity within an ejaculate.

Conversely, sperm integrity analysis may be used in DNA fragmentation for high-risk cases defined after initial infertility work-up and be useful in cases after repeated IVF failure with no evident cause and probably with bad embryo quality when additional information to take clinical decisions is needed [2,3].

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5

Aneuploidy in Human Oocytes and Preimplantation Embryos

Eva R. Hoffmann, Alan H. Handyside, and Antonio Capalbo

CONTENTS

Introduction

A Historical Context for Human Aneuploidy

After 30 years of widely held belief that humans had 48 chromosomes, the refinement of karyotyping techniques in the 1950s facilitated the discovery that humans have 46 chromosomes, including the XY sex chromosomes [1]. Using improved cytogenetic techniques, Jacobs and Strong [2] reported that Klinefelter syndrome in males was caused by an extra X chromosome [2]. In the same year, Jejeune, Gauthier, and Turpin [3,4] and the Jacobs group [5] independently discovered that Down syndrome was caused by an extra chromosome 21. Ford and colleagues [6] found that Turner syndrome was caused by the loss of an X chromosomes (45,X) in females and also reported the first mosaic individual (XXY/XX) [7]. These studies, reported in 1959, led to an explosion in the investigations into aneuploidy [8–12] and initiated epidemiological and extensive cohort studies of both spontaneous miscarriages as well as live births (Figure 5.1). Of spontaneous miscarriages, nearly 50% are chromosomally abnormal, mainly due to aneuploidy (one in

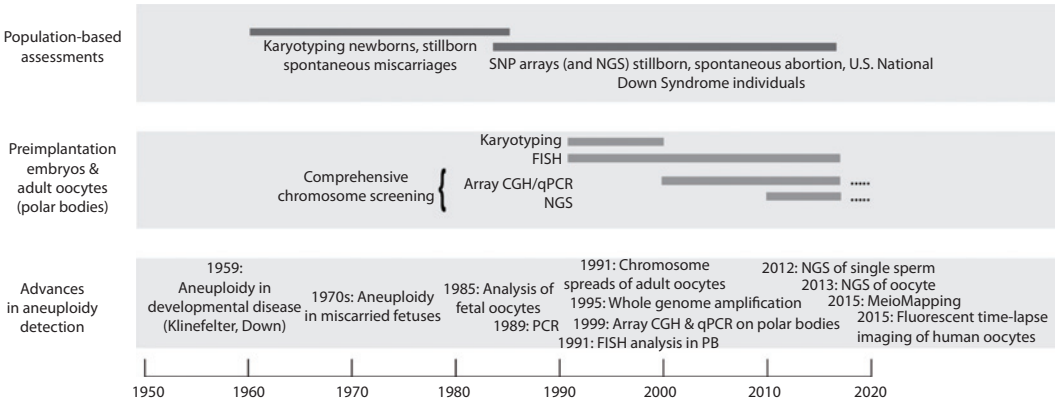

FIGURE 5.1 Historical overview of developments in the importance and detection of aneuploidy in human health. Array CGH, comparative genomic hybridization; FISH, fluorescence in situ hybridization; NGS, next-generation sequencing; qPCR, quantitative polymerase chain reaction; SNP, single-nucleotide polymorphisms. MeioMapping allows recovery of both chromosome content and genetic variants (SNPs) from the same cell in oocytes and matched polar bodies.

three), but triploid conceptions are also common [13]. To date, the most comprehensive cohort study is the U.S. National Down Syndrome Project, which was initiated by Terry Hassold and Stephanie Sherman [14].

The population-based studies have revealed three important facets of human aneuploidies. First, the trisomic conceptions that reach clinical recognition predominantly derive from the mother and their incidence increases with maternal age. The chromosomes also display different aging profiles, suggesting complex interactions between general aging features of oocytes and chromosome-specific susceptibilities [15]. Second, in addition to maternal age, recombination has emerged as a critical factor that influences the chromosomal content of the egg [16]. Chromosome-specific recombination patterns could thus contribute toward the different age-related aneuploidy curves displayed by specific chromosomes. Consistent with the importance of recombination, higher maternal (but not paternal) recombination rates are associated with increased reproductive success (more children) [17]. The implication is that recombination, which occurs during fetal development, affects chromosome segregation decades later in adult life, when oocytes ovulate. Third, the larger cohort studies have allowed other epidemiological factors to be uncovered, including the role of socioeconomic status [18]. This raises important questions about how lifestyle and health issues might influence the quality of human eggs and embryonic development.

The J-Shaped Curve of Human Aneuploidies

The incidence of human aneuploidies is characterized by a J-shaped curve according to maternal age (Figure 5.2). First reported for Down syndrome [19], the J curve displays a triphasic rate of trisomic conceptions in clinically recognized pregnancies as a function of the mother's age (Figure 5.2) [20]. Aneuploidy rates are slightly higher for teenagers, then drop slightly before the onset of the linear increase (20–30 years), followed by the shift to an exponential rate around 30–35 years.

The J curve is also seen in studies of oocytes or polar bodies as well as in preimplantation embryos (Figure 5.2, red curve). The magnitude in preimplantation embryos is much greater than in clinically recognized pregnancies, also known as "products of conception" [22–27]. The elevation in magnitude is in part due to the selection against embryos with chromosome constellations that are incompatible with embryonic and early fetal development, such as monosomies of the autosomal chromosomes and complex aneuploidies, where multiple chromosomes are affected [20]. Thus, population-based assessments are a vast underestimate of the genomic diversity generated in gametes and embryos. Indeed, in oocytes from "younger" women (under the age of 30), maternal aneuploidy rates are estimated to be 10%–30%. For women of advanced maternal age (35 or above), aneuploidy rates range between 30% and 85% (Figure 5.3) [25,27–37]. This wide range reflects the exponential curvature of the aneuploidy rate according to the age distribution of women included in different studies. However, the use of different

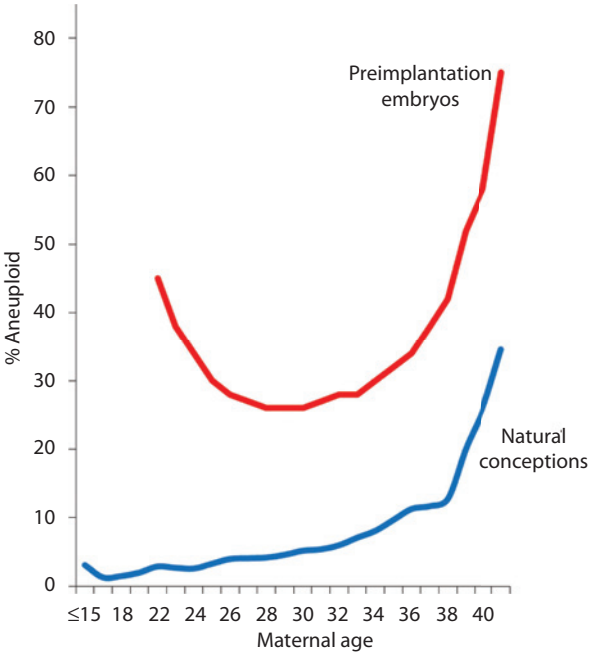

FIGURE 5.2 J curve of aneuploid conceptions. Blue indicates % trisomic conceptions in clinically recognized pregnancies. Red indicates % blastocysts with aneuploidy in day 5 blastocysts trophectoderm samples. (Data from Hassold T and Hunt P, *Nat Rev Genet*, 2, 280–291, 2001; from Franasiak JM et al., *J Assist Reprod Genet*, 31, 1501–1509, 2014.)

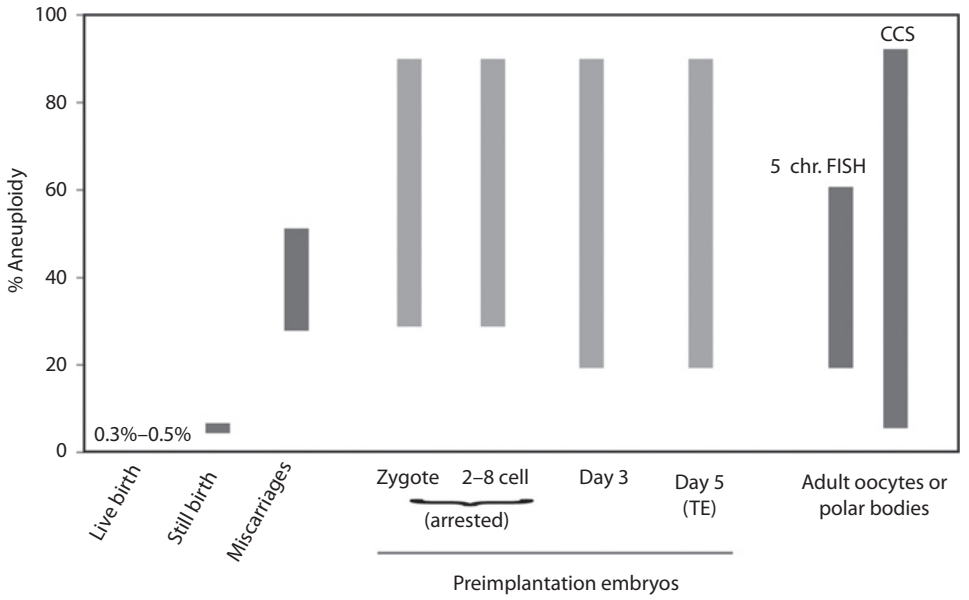

FIGURE 5.3 Variability in reported aneuploidy rates. chr., chromosome; CCS, comprehensive chromosome screening; TE, trophectoderm biopsy; FISH, fluorescence in situ hybridization. (Data obtained from Franasiak JM et al., *Fertil Steril*, 101, 656–663. e1, 2014; McCoy RC et al., *PLoS Genet*, 11, e1005601, 2015; Pellestor F et al., *Hum Reprod*, 17, 2134–2145, 2002; Lim AS and Tsakok MF, *Fertil Steril*, 68, 265–271, 1997; Kuliev A et al., *Cytogenet Genome Res*, 111, 193–198, 2005; Anahory T et al., *Mol Hum Reprod*, 9, 577–585, 2003; Kuliev A et al., *Reprod Biomed Online*, 22, 2–8, 2011; Fragouli E et al., *Cytogenet Genome Res*, 114, 30–38, 2006; Fragouli E et al., *Reprod Biomed Online*, 19, 228–237, 2009; Munne S et al., *Reprod Biomed Online*, 14, 628–634, 2007; Gabriel AS et al., *J Med Genet*., 48, 433–437, 2011; Handyside AH et al., *Eur J Hum Genet*, 20, 742–747, 2012; Magli MC et al., *Reprod Biomed Online*, 24, 331–338, 2012.)

methodologies may also influence the variability in estimates of aneuploidy rates. For example, in some cases, fluorescence in situ hybridization (FISH)–based assessments have been plagued by suboptimal processing [38]. Regardless of this, egg donation programs, where oocytes from younger women result in live births at rates equivalent to the donor's age, as opposed to the recipient's age, have firmly implicated chromosome errors in the egg as causative of infertility and pregnancy loss [39,40].

Chromosome-Specific Effects and Equal Contributions from Monosomies and Trisomies in Human Oocytes and Preimplantation Embryos

The genomic studies on oocytes and preimplantation embryos are concordant with population-based studies that the exponential phase starts from around the mid-30s. Moreover, not all chromosomes are created equal with regard to their risk of aneuploidy and their maternal age-related curves [21, 25–27]. This includes the preponderance of aneuploidies for specific chromosomes, especially the two smallest chromosomes, 21 and 22, but also 15 and 16. In general, size correlates negatively with age-related aneuploidy [27]. In population-based studies, chromosomes display substantial differences in the meiotic division (first or second) in which the segregation errors occur [16]. Collection of these data requires chromosome fingerprinting and such data have yet to be obtained for a large set of oocytes and preimplantation embryos.

The direct assessment of aneuploidies from oocytes and embryos have allowed new insight into their origins. Several important findings from ART settings include that aneuploidy rates for monosomies are at least as common as trisomies, if not more so [24,27,28,30,41]. In contrast, among natural conceptions, the only viable monosomy is 45, X0 (Turner syndrome), suggesting selection against or poor developmental potential of chromosomally abnormal conceptions at peri-implantation stages and during gestation [20,42]. Aneuploid embryos may fail to implant due to their aberrant triggering of calcium signaling in decidual cells at implantation [43].

Features Influencing the J Curve in Oocytes and Preimplantation Embryos

In addition to the shift in magnitude, three additional features shape the J curve in oocytes and preimplantation embryos compared to clinically recognized pregnancies. First, the J curve appears much more exaggerated in comparison to natural conceptions (Figure 5.2). The differences in the J shape likely reflect multiple factors, including that all chromosomes are assessed in embryos (Figure 5.4), whereas the J curve is highly dependent upon chromosomes 13, 16, 18, and 21 in trisomic conceptions. Since both mitotic and meiotic errors are present in preimplantation embryos, age-dependent changes to mitotic rates could also contribute to the differences in the J shape. Since the first two to three cell divisions in the cleavage embryo are driven by maternal proteins, features in the egg could influence the fidelity of mitosis (Figure 5.5).

Second, the origin of aneuploidies in human eggs due to meiotic errors can be accurately inferred when all three cells from a single meiosis are assessed together (MeioMapping) [44,45]. Such "trio" analyses have revealed that errors during meiosis I can be "corrected" by a compensating error at meiosis II, resulting in a euploid egg. Approximately half of the segregation errors result in an aneuploid egg, whereas the polar bodies are affected in the remaining cases [44,45]. As a result, when analyzing embryos or fetuses, we capture only half of the segregation errors that occurred during meiosis.

Third, for preimplantation embryos, the shape of the J curve is heavily dependent upon which stage during development the embryo is sampled. High rates of aneuploidy due to mitotic errors (postzygotic) were originally identified using fluorescence in situ hybridization (e.g., [46]). Using genomic assessments, day 3 cleavage-stage embryos have been reported to have a high rate of aneuploidy, frequently characterized by chaotic karyotypes [27,47,48]. In contrast, day 5 embryos have a lower rate of chaotic karyotypes, which has been proposed to be due to selection against embryos with high levels of chromosome instability at the day 3 cleavage stage [27]. Consistent with this, a relatively high rate of preimplantation embryos arrest at day 3 (20%–30%), although some of these are also euploid [48]. The causes of chromosome instability and embryonic arrest are currently not clear. A genome-wide association study suggested that common variants of PLK4 may contribute to mitotic instability in early preimplantation embryos [49],

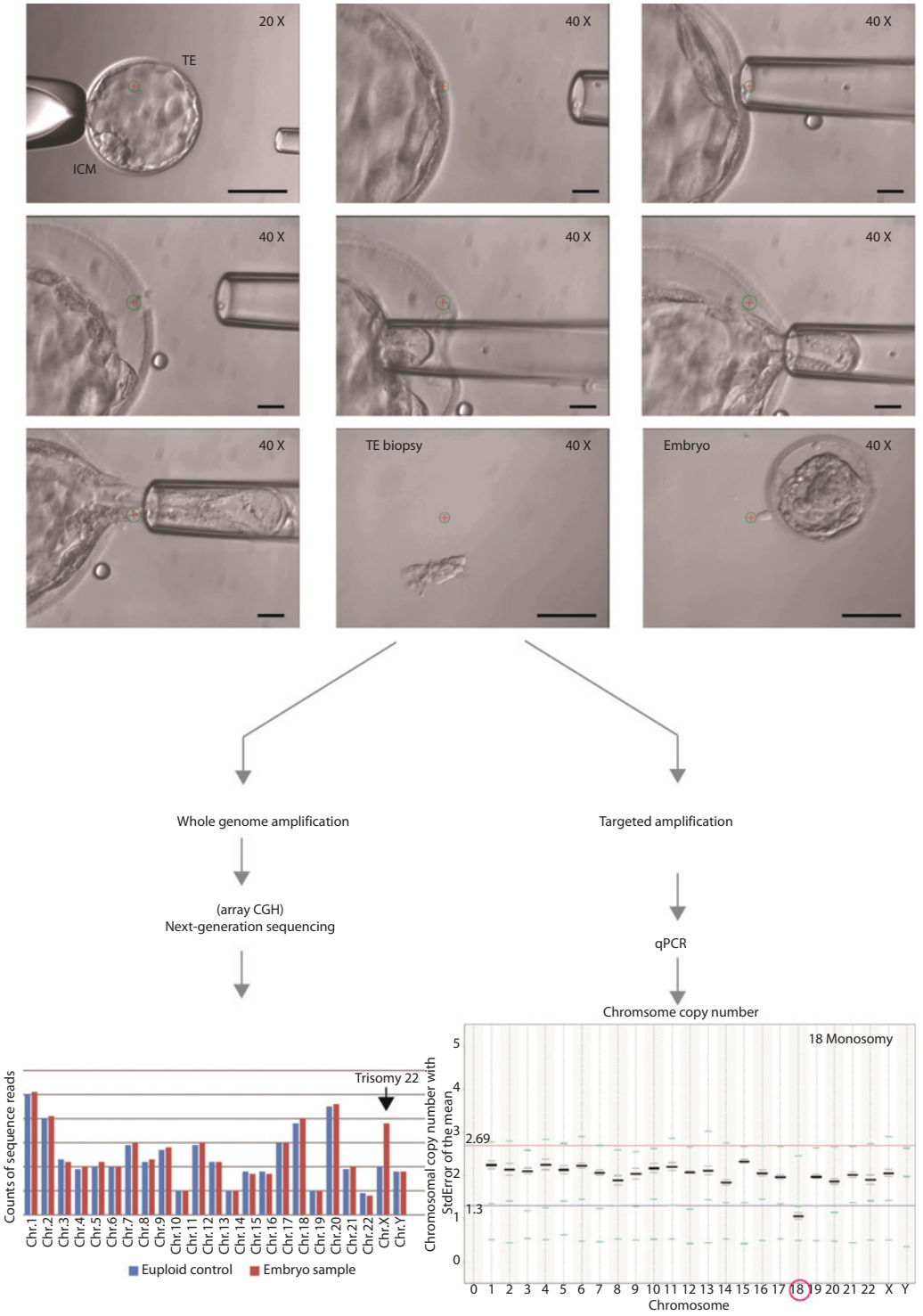

FIGURE 5.4 Detection of chromosome content in trophectoderm samples from blastocyst embryos. A sample of 5–10 cells is biopsied from the trophectoderm. One method uses whole-genome amplification coupled with next-generation sequencing (left), whereas the other uses a qPCR method (right). The whole-genome amplification method has been used to obtain sequence and chromosomal content on single cells, including oocytes and polar bodies. (From Hou Y et al., *Cell*, 155, 1492–1506, 2013; Ottolini CS et al., *Nat Genet*, 47, 727–735, 2015.)

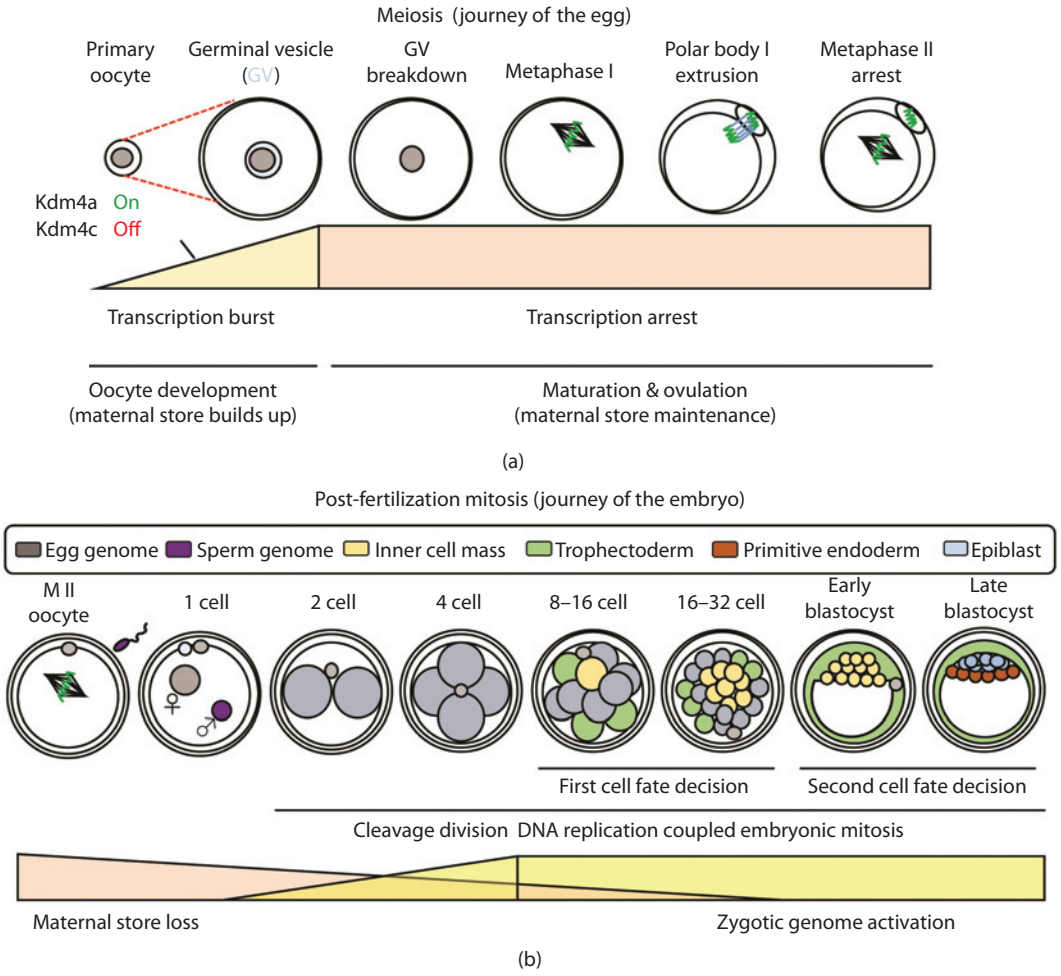

FIGURE 5.5 Human embryo development. A summary of the seminal events occurring during preimplantation development in humans. (Image courtesy of Dr. Aditya Sankar.)

but functional assessment and mechanistic studies are yet to be conducted on how specific PLK4 alleles would alter the cell divisions. DNA damage during the embryonic divisions may also contribute to the high arrest rate of cleavage-stage embryos [50]. This is consistent with the transcriptional upregulation of cell cycle and DNA damage response genes in mature oocytes [51], which drive the first two to three mitotic divisions until the embryonic genome activation (EGA) (see Figure 5.5) [52].

Other factors may also contribute to the highly variable rates of aneuploidies in preimplantation embryos reported in the literature [53]. These include methodological differences such as whether the hormone stimulation used to treat women affects aneuploidy rates [54–57], the type of culture medium, and the technology used to assess chromosomal content (reviewed in [58]). A substantial degree of variability in aneuploidy rates across different centers is also seen in oocyte donors [59], suggesting that the variability in aneuploidy rates may not be specific to female patients undergoing fertility treatment. Variability in aneuploidy rates across different geographical settings is also seen for natural conceptions [60]. Understanding whether such differences are biologically meaningful is important since they may reveal new insights into the origins of aneuploidies. As our methods to detect whole chromosome as well as "segmental" aneuploidies improve, our focus will shift to understanding the biological phenomena that drive chromosome instability in preimplantation embryos and their functional impact on the embryo (Table 5.1).

TABLE 5.1

Estimated Incidence and Types of Aneuploidies at Different Stages of Human Reproduction

	Incidence	Most Common	References
Oocytes			
Whole chromosome aneuploidies	20%–90%		*Fragouli et al., 2011*
– *Trisomies*	*55.0% of aneuploidies*	±15, ±16, ±18, ±19, ±21, ±22	*Gabriel et al., 2011*
– *Monosomies*	*45.0% of aneuploidies*		*Fragouli et al., 2013*
Complex aneuploidies	4%–30% of aneuploid oocytes	ND	
Ploidy alteration	ND	NA	
UPDs	ND	NA	
Pathogenic CNVs	ND	NA	
VOUS	ND	NA	
Mosaic	NA	NA	
Preimplantation embryos			
Whole chromosome aneuploidies	25%–90%		*Franasiak et al., 2014*
– *Trisomies*	*50% of aneuploidies*	±15, ±16, ±18, ±19, ±21, ±22	*Alfarawati et al., 2011*
– *Monosomies*	*50% of aneuploidies*		*Capalbo et al., 2014, 2016*
Complex aneuploidies	5%–30% of aneuploid embryos	ND	*Gueye et al., 2014*
Ploidy alteration	<2%	Haploidy, Triploidy	
UPDs	<2%	ND	
Pathogenic CNVs	ND	NA	
VOUS	ND	NA	
Mosaic	4%–5%	ND	
POCs (miscarriages ≤12 gestational weeks)			
Whole chromosome aneuploidies	40%–70%		*Rodriguez-Purata et al., 2015*
– *Trisomies*	*85% of aneuploidies*	+13, +14, +15, +16, +17, +18, +21, +22, 45X	*Wang et al., 2016*
– *Monosomies*	*15% of aneuploidies*		*Levy et al., 2014*
Complex aneuploidies	Absent	NA	*Huang et al., 2009* *Fritz et al., 2001*
Ploidy alteration	<2%	Triploidy	*Wapner et al., 2012* *Baird, et al., 1988*
UPDs	<2%	ND	*Hook, et al., 1983*
Pathogenic CNVs	2%–5%	ND	
VOUS	2%–3%	ND	
Mosaic	<2%	2, 8, 16, sex, structural (7, 13, 18, 20, 21)	
Ongoing pregnancies (>12 gestational weeks)			
Whole chromosome aneuploidies	<5%		*Huang* et al., 2009
– *Trisomies*	*95% of aneuploidies*	+13, +18, + 21, 45X, 47XXX, 47XXY	*Forabosco et al., 2009*
– *Monosomies*	*5% of aneuploidies*		*Wapner et al., 2012*
Complex aneuploidies	Absent	NA	*Martin et al., 2015*
Ploidy alteration	Absent–0.01%	Triploidy	*Sudmant et al., 2015*
UPDs	0.01%	ND	
Pathogenic CNVs	≈0.5%	del 7q11.23, del/dup 8p23.1, del 15q11.2q13, del/dup 17p11.2, del 17q21.31, del 22q11.2	
VOUS	2%–3%	dup 4q35.2, dup 8p22, del 15q11.2, dup 15q13.3, del/dup 16p13.11, del 16p11.2, dup Xp22.11	
Mosaic	<0.5%	16, sex, structural (7, 13, 18, 20, 21)	

(*continued*)

TABLE 5.1 (*CONTINUED*)

Estimated Incidence and Types of Aneuploidies at Different Stages of Human Reproduction

	Incidence	Most Common	References
New-borns			
Whole chromosome aneuploidies	0.1%–4%		*Hassold et al., 1996*
– *Trisomies*	*95% of aneuploidies*	+13, +18, +21, 45X,	*Wellesley et al., 2012*
– *Monosomies*	*5% of aneuploidies*	47XXX, 47XXY	*Martin et al., 2015*
Complex aneuploidies	Absent	NA	*Sudmant et al., 2015*
Ploidy alteration	Absent	NA	*Dolk, et al., 2010, Hamerton*, et al., *1975*
UPDs	<0.01%	ND	
Pathogenic CNVs	≈0.5%	del 7q11.23, del/dup 8p23.1, del 15q11.2q13, del/dup 17p11.2, del 17q21.31, del 22q11.2	
VOUS	2%–3%	dup 4q35.2, dup 8p22, del 15q11.2, dup 15q13.3, del/dup 16p13.11, del 16p11.2, dup Xp22.11	
Mosaic	<0.5%	Sex	

Note: UPD, UniParental Disomies; CNVs, Copy Number Variations; VOUS, Variants Of Unknown Signficance; POCs, Product Of Conceptions; ND, Not Dermined; NA, Not Applicable.

The Origins of Maternal Chromosome Errors

Oocytes and spermatocytes halve their chromosome numbers by a specialized division known as meiosis. Although the chromosome content in sperm and activated oocytes are the same, their development to mature gametes is highly dimorphic. Primordial germ cells migrate to the genital ridges during fetal development and the gonadal environment determines whether the cells induce meiosis (oocyte) or remain arrested until puberty (males). Mouse studies have shown that fetal oocytes initiate meiosis and differentiation due to the presence of retinoic acid in the fetal ovary [61,62]. The fetal oocytes replicate their DNA, forming sister chromatids that are cohesed together. During meiotic prophase I, the homologous chromosomes align and recombine [63], which forms the bivalent chromosome structure (Figure 5.6). The oocytes then enter a prolonged arrest (dictyate) until menarche, when a single follicle matures and the egg completes the first meiotic division. Oocyte numbers within the ovary are depleted from fetal development until the onset of menopause. Only 450 of the 5–7 million fetal oocytes complete the first meiotic division, and even fewer go on to segregate sister chromatids after fertilization induces the completion of the second meiotic division [64].

Aneuploidies originating from female meiosis have traditionally been classified as meiosis I or meiosis II errors, pending upon whether the extra chromosome contained genetic information from both or only one of the maternal chromosomes, respectively (chromosome fingerprint) (Figure 5.7). Meiosis I errors are more frequent compared to meiosis II [44,45]. Direct assessment of human oocytes and polar bodies has revealed segregation patterns that predispose to aneuploidy in the eggs: meiosis I non-disjunction, precocious sister chromosome segregation or pre-division, meiosis II non-disjunction, and more recently, reverse segregation [45].

Errors in Meiosis I Are Predominantly Due to Pre-Division in ART Oocytes

One of the most surprising findings from studies of human adult oocytes and polar bodies is that the precocious separation of sister chromatids (PSSC), also dubbed pre-divisions by Darlington (1937), appears to be 2- to 10-fold more frequent than classical meiosis I non-disjunction. When human oocytes became available, cytogenetic techniques of spreading the metaphase chromosomes were used to assess chromosome structure and their numbers [65–67]. "Fresh," unfertilized metaphase II–arrested oocytes were found to contain extra whole chromosomes, consistent with meiosis I non-disjunction [67]. In 1991, Angell and colleagues reported a preponderance of extra chromatids, not chromosomes, in meiosis II oocytes from laparoscopic surgeries or meiosis II oocytes that failed to fertilize in IVF settings [68,69]. Subsequently, approaches that do not rely on spreading

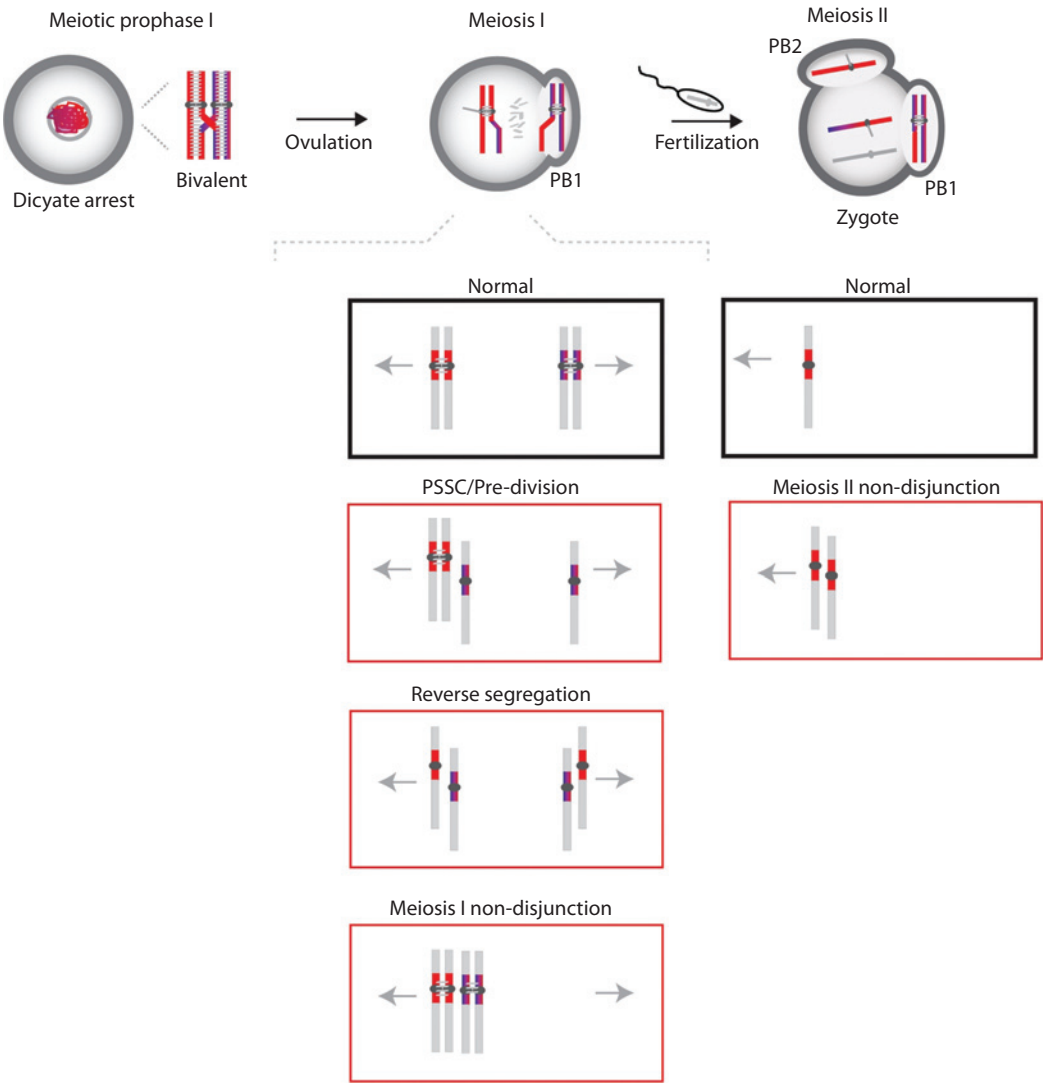

FIGURE 5.6 Chromosome segregation errors in human female meiosis. The two homologous chromosomes are shown in red and blue, respectively. Recombination occurs in fetal oocytes, which arrest at the dictyate stage, once bivalent chromosomes have formed. Their formation depends upon recombination as well as cohesion between the two sister chromatids. Different chromosome segregation patterns can be discerned when the genetic variants (SNPs) are available. (From Hou Y et al., *Cell*, 155, 1492–1506, 2013; Ottolini CS et al., *Nat Genet*, 47, 727–735, 2015.)

the chromosomes but measure DNA content (Figure 5.1) also confirmed the conclusion that pre-division is common in human oocytes, at least those obtained from gonadotropin stimulation in women [25,27–37,44,45].

Two recent studies that mapped the genetic information as well as chromosomal content in all three cells for female meiosis (oocyte and the two corresponding polar bodies) (Figure 5.7) revealed that pre-division or PSSC of single chromatids outnumber classical meiosis I non-disjunction about 5 to 10 [44,45]. This implies that pre-division is common, at least in the ART population.

Reverse Segregation: A Novel Segregation Pattern

Chromosome fingerprinting of all three meiotic cells has firmly established that homologous chromosomes segregate at meiosis I and sister chromatids at meiosis II (classical meiosis). Fingerprinting relies on the assumption that crossing over (or recombination) is suppressed near centromeres, such that two sister

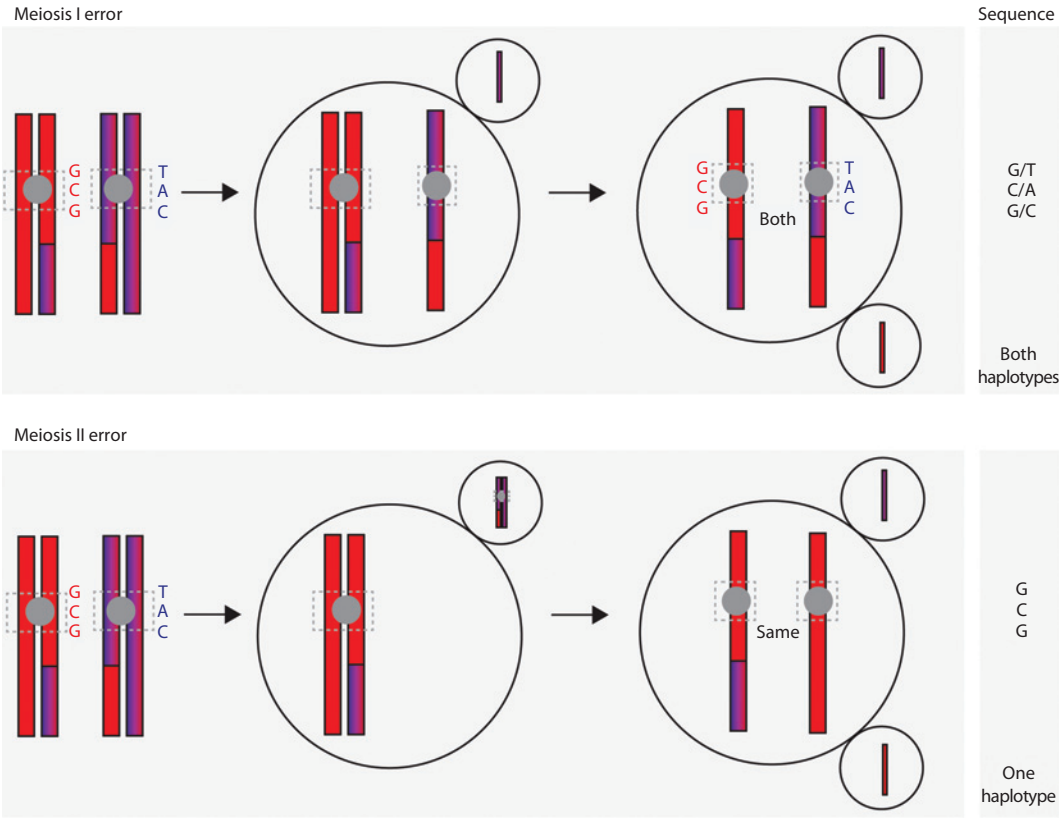

FIGURE 5.7 Chromosomal fingerprinting allows inference about the origin of extra chromosomes. Recombination between homologous chromosomes causes shuffling of the genetic material. However, because recombination is suppressed near centromeres, the origin of the chromosome is preserved (gray box, red and blue). The genetic markers, such as single-nucleotide polymorphisms around the centromere, can be used as a chromosomal fingerprint. Meiosis I errors lead to two maternal chromosomes with information from both blue and red chromosomes (upper panel). In contrast, meiosis II errors lead to a same chromosomal fingerprint of both maternal chromosomes.

chromatids share the same genetic markers such as single-nucleotide polymorphism (SNP). After meiosis I, the oocyte should contain two sister chromatids (one chromosome) and the first polar body should contain two sister chromatids of the second homolog. However, Ottolini et al. [45] found that the first polar body frequently contained two non-sister chromatids, i.e., chromosome fingerprints from both maternal chromosomes. The frequency of this event increased by more than 100× compared to the incidence expected from two independent pre-division events, which suggests a common origin. Moreover, it was also reported that there was a weak preference for the two non-sister chromatids to segregate in a balanced fashion at meiosis II, such that the oocyte and the second polar body contained one chromatid each. Thus, overall, the entire meiosis was chromosomally balanced. However, the pattern of segregation was reversed: sisters segregated at meiosis I, followed by homologs in meiosis II (Figure 5.8). The report also showed that only a single or few of the chromosomes followed a reverse segregation pattern within oocytes [45]. This suggests chromosome-specific vulnerability to aneuploidies amid general aging effects of the oocytes as a whole.

Time-Lapse Imaging Reveals Chromosomal "Aging" Defects May Precede Errors in Segregation

How does reverse segregation occur? Analyses of fixed chromosomes have revealed that the bivalent chromosome configurations either not established properly during fetal development [63,70–72] or frequently deteriorated in adult oocytes. In particular, univalents have been reported as "vulnerable"

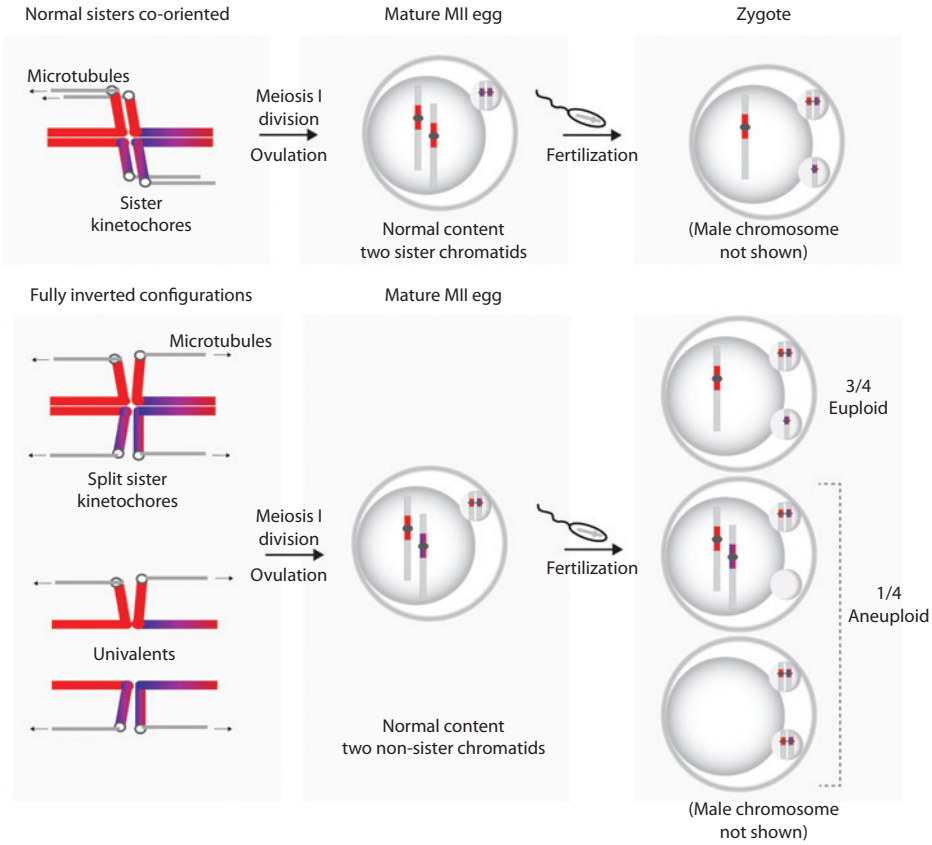

FIGURE 5.8 Reverse segregation, a new segregation pattern in human meiosis. Genomic analyses of oocytes and their matching polar bodies revealed that reverse segregation is the most common non-canonical segregation pattern in human oocytes. Rather than having a single chromosome and genotype in the first polar body, two non-sister chromatids are often present (purple and red). The "fully inverted" configuration may precede segregation of sister chromatids to opposite poles for both homologs, mimicking a mitotic-like segregation pattern. In particular univalents are at-risk of a mitotic-like division in meiosis I. After the second meiotic division, the segregation of the two non-sister chromatids appear to be more efficient that predicted by chance alone (3 in 4 are euploid rather than half).

chromosome configurations (Figure 5.9). Time-lapse imaging in mouse oocytes revealed that univalents are at risk of meiosis I non-disjunction as well as PSSC/pre-division [73]. In humans, the recent development of time-lapse imaging where oocytes are injected with mRNA of fluorescently labeled kinetochores and tubulin showed that sister kinetochores often "invert" at meiosis I. Thus, rather than being co-oriented, sister kinetochores are attached to microtubules from opposite spindle poles. Fully inverted configurations occur when both chromosomes display the behavior. Half-inverted configurations occur when only one of the homologs is affected. In several instances, univalent formation is associated with bi-orientation of sister kinetochores (Figure 5.8). It is possible that univalent formation precedes bi-orientation, indicating that the inverted kinetochores may be a response to bivalent deterioration.

The putative loss of cohesion and structural integrity of bivalent chromosomes is age dependent and is one manifestation of "chromosomal aging" in human oocytes [74–76]. However, the molecular mechanism underlying loss of cohesion is currently not known. Studies in rodents suggest that loading of cohesin complexes may be restricted to fetal development [77–79] and subsequently affected during the extended dictyate arrest [80,81]. Intriguingly, SMC1β, a meiosis-specific component of meiotic cohesin complexes, is required for maintaining the bivalent configuration in mouse oocytes [82,83]. Whether depletion of meiotic cohesin complexes underlies cohesion loss in human oocytes is not clear since loss of cohesin staining does not appear to preferentially affect the chromosomes that have lost their bivalent

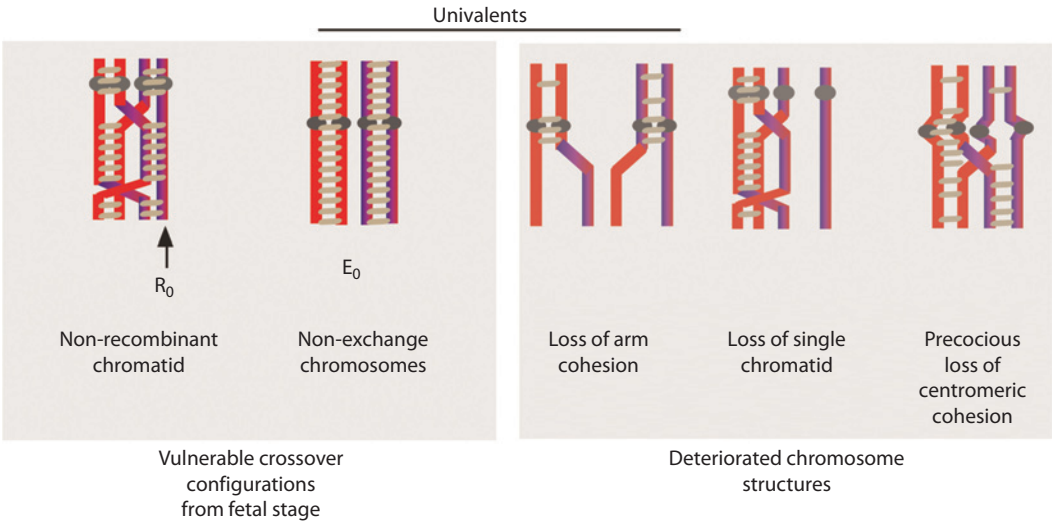

FIGURE 5.9 Vulnerable chromosome configurations in meiosis.

structure [84]. Since only a proportion of cohesin complexes are thought to mediate sister chromatid cohesion in mitotic cells [85,86], it is possible that the cohesive function of the cohesin complexes, an acetylated form of SMC3, may be affected during the extended dictyate arrest.

Recombination Affects Chromosome Segregation in Human Females

The findings that univalent formation precedes errors in segregation in both mouse and human oocytes can explain the importance of recombination in aneuploidy [76,87]. In population-based studies, altered recombination patterns and maternal age remain the two major factors that influence aneuploidy in human conceptions [16]. Although defects in female meiosis are more pronounced, altered recombination also influences chromosome segregation in sperm [88]. As chromosome fingerprinting such as SNP arrays and next-generation sequencing (NGS) have become available, recombination patterns can also be studied. Recombination, which occurs during fetal development, has been inferred to influence chromosome segregation in complex and chromosome-specific ways. Studies on miscarriages and in trisomic individuals revealed three patterns associated with increased risk of aneuploidy: (1) failure to recombine (non-exchange or achiasmate); (2) recombination points near chromosomal ends (telomeric); and (3) recombination in the vicinity of centromeres.

The recombination patterns associated with meiotic errors and aneuploidy are highly chromosome-specific. All three types of recombination patterns were associated with errors in segregation of chromosome 21, whereas chromosome 18 was predominantly due to the non-exchange pattern [16]. Since recombination together with sister chromatid cohesion physically link homologous chromosomes prior to their segregation at anaphase I, the non-exchange or achiasmate pairs were suggested to predispose to classical meiosis I non-disjunction (Figure 5.9). Achiasmate pairs would generate univalents, and analyses of fetal oocytes have suggested that up to 20% of fetal oocytes lack an MLH1 focus on chromosome 21 [63,70,72,90]. The cytological analyses have provided tremendous insight into the recombination program. However, the approach is inherently limited by a lack of genomic resolution of crossover resolution and its association with chromosome segregation.

Recombination Rates Are Established during Fetal Life and Affect Chromosome Segregation in Adult Oocytes

Recent studies on single oocytes have allowed inferences about recombination and chromosome segregation at the DNA sequence level [44,45]. Oocytes with higher recombination rates are more likely

to be euploid. Thus, selection against aneuploid conceptions during the peri-implantation stages can explain why children born to older mothers tend to have higher recombination rates compared to their siblings born while the mother was young [17]—aneuploid eggs tend to have lower recombination rates and therefore do not develop to healthy babies. One model is that as age-related loss of cohesion occurs, chromosome pairs with multiple crossovers may be more likely to retain their bivalent configuration as oocytes age (Figure 5.10).

Meiomapping also revealed some unexpected features of recombination and its effect on chromosome segregation. Normally, crossing over has been considered on a per bivalent basis such that if a crossover occurred between the two homologous chromosomes, chromosome segregation would proceed with high fidelity. This was referred to as the "obligate" crossover (e.g., [89]). However, Ottolini et al. [45] found that what happens to individual chromatids also influences their segregation. Non-recombinant chromatids that did not engage in recombination reactions (although its sister chromatid did) are vulnerable and at increased risk of undergoing PSSC/pre-division (Figure 5.9). These findings open new areas of investigation as access to oocytes and new single-cell technologies improve.

Paradoxically, fetal oocytes have more vulnerable configurations compared to spermatocytes despite higher overall global recombination rates [63]. However, oocytes display extreme heterogeneity in crossover rates [70], and the two smallest acrocentric chromosomes 21 and 22 are frequently (5%) without an MLH1 focus [72,90]. This pattern of non-exchange is virtually never seen in spermatocytes [72,90]. Thus, despite the higher rate of recombination in female germ cells, their distribution among chromosome pairs appears to be less regulated. The defect in patterning of crossovers has been proposed to be due to inefficiency in the maturation of crossovers [71], which would generate vulnerable crossover configurations at increased risk of chromosome errors, decades later in adult life.

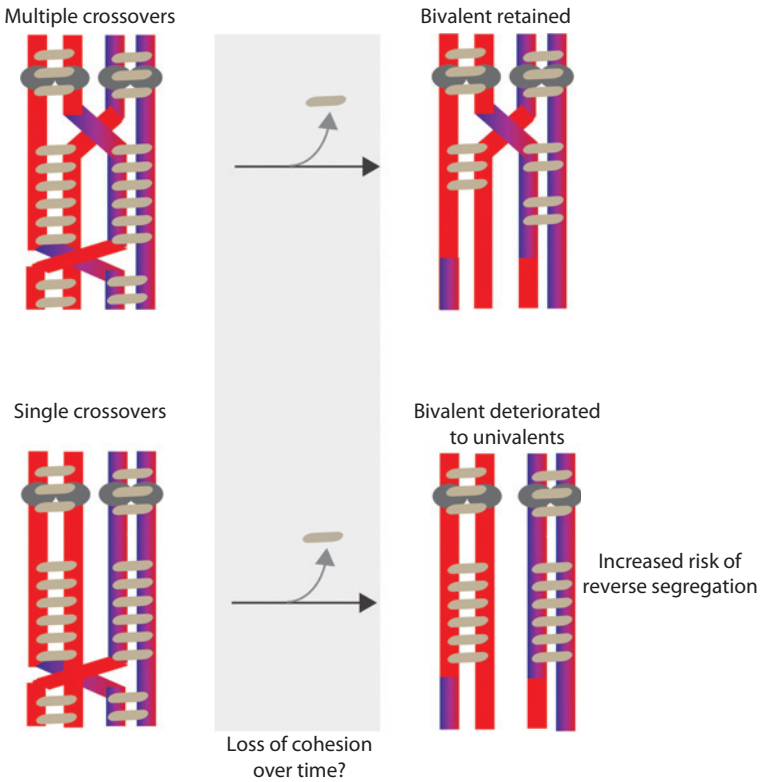

FIGURE 5.10 A hypothetical model for how recombination may be protective of bivalent structures as oocytes age.

Meiotic Spindles Are Inherently Error-Prone in Human Female Meiosis

In mitotic cells, spindle formation is driven largely by centrosomes, microtubule organizing centers (MTOC) from which microtubules emanate and capture chromosomes by their kinetochores. However, in many species, oogenesis is acentrosomal, although MTOCs are formed [91]. In human oocytes MTOCs are not apparent and the spindle formation is driven by the chromosomes. The first metaphase I takes an astonishing 12–15 hours [74,92] and the spindles are highly unstable [92]. Although spindle instability correlates with missegregation, this facet of human oogenesis does not appear to be age related [92].

Microtubules are composed of alpha and beta tubulin subunits. Recently, mutations in *TUBB8,* the major β-tubulin expressed only in oocytes and preimplantation embryos, were shown to be associated with maturation arrest [93] as well as defective divisions in both oocytes and embryos [94,95]. *TUBB8* is particularly interesting because it evolved in the primate clade and could thus contribute to the substantial differences in spindle dynamics in mouse and human oocytes.

General Aging Features of Human Oocytes

Approaches to studying aging features of human oocytes that may predispose to aneuploidy have largely derived from transcriptional comparisons or immunocytological staining of factors identified in mouse oocytes or from human cell lines [51]. The spindle assembly checkpoint (SAC) is essential in mouse oocytes to facilitate accurate chromosome segregation [96], and it has been suggested that it is affected by aging in human oocytes [97]. Similarly, removal of acetylation marks on histone H4K12 or H3K9 is important for chromosome compaction in human oocytes and is associated with aberrant meiosis [98,99]. This higher level of histone H4 acetylation in oocytes from women of advanced maternal age [98] indicates a general decrease in the capacity of aged oocytes to remove histone acetylation marks. Collectively, several cellular and chromosomal factors conspire to cause the high levels of aneuploidy in human eggs. Understanding aneuploidy will require sophisticated approaches. It is sobering, however, that we still do not have biomarkers that allow precision medicine for women at risk of aneuploid conceptions.

Mitotic Chromosome Errors in Preimplantation Embryos

Mitotic errors in chromosome segregation lead to mosaic embryos. The incidence of mosaicism in human embryos is currently debated [58,100], but in contrast to our growing knowledge of basic biological mechanisms that lead to aneuploidy in human oocytes, analysis of cell divisions and chromosome segregation errors in preimplantation embryos is less developed. Many models are inferred from knowledge obtained in mitosis in cell lines (e.g., reviewed by [101]). However, ethical regulation and technical challenges of lineage tracing and single-cell genomics limit our ability for direct observations in embryos. In particular, classical loss-of-function and gain-of-function analyses that are used to infer gene function are challenging in embryos. This area of research will likely evolve rapidly in the coming years since ethical permission has been granted in several countries to use CRISPR-Cas9 for gene editing. Despite the limitations, major insights into chromosomal aneuploidy in embryos have been obtained from the use of preimplantation genetic screening and diagnosis (PGD/PGS) to prevent aneuploid conceptions and inheritance of monogenic diseases.

The current emerging picture is that DNA damage, cellular depletion [50], and aneuploidy [102] cause developmental arrest in 20%–30% of preimplantation embryos. Individual blastomeres can display highly chaotic karyotypes (mosaicism, Figure 5.11), especially during the cleavage stage [27,47,103,104]. Confocal microscopy has revealed that normally fertilized zygotes can undergo tripolar divisions, which would give rise to highly chaotic karyotypes [105].

Expanded blastocyst embryos are less mosaic than cleavage-stage embryos and contain fewer chaotic karyotypes [27]. Nevertheless, meiotic aneuploidies, including “complex” events that affect several chromosomes, can persist to the blastocyst stage [24,27,45,106]. The relatively low rate of whole aneuploidy mosaicism in day 5 blastocyst embryos is concordant with the low level (less than 1%) in chorionic villi biopsies [107]. Thus, aneuploidies per se do not appear to influence preimplantation development. The emerging picture, however, is complex, since clinics report highly variable aneuploidy rates not only for patients but also for oocyte donors [53,59]. It is currently unclear what factors cause the variability, but understanding its causes is critical, especially for preimplantation genetic testing [108].

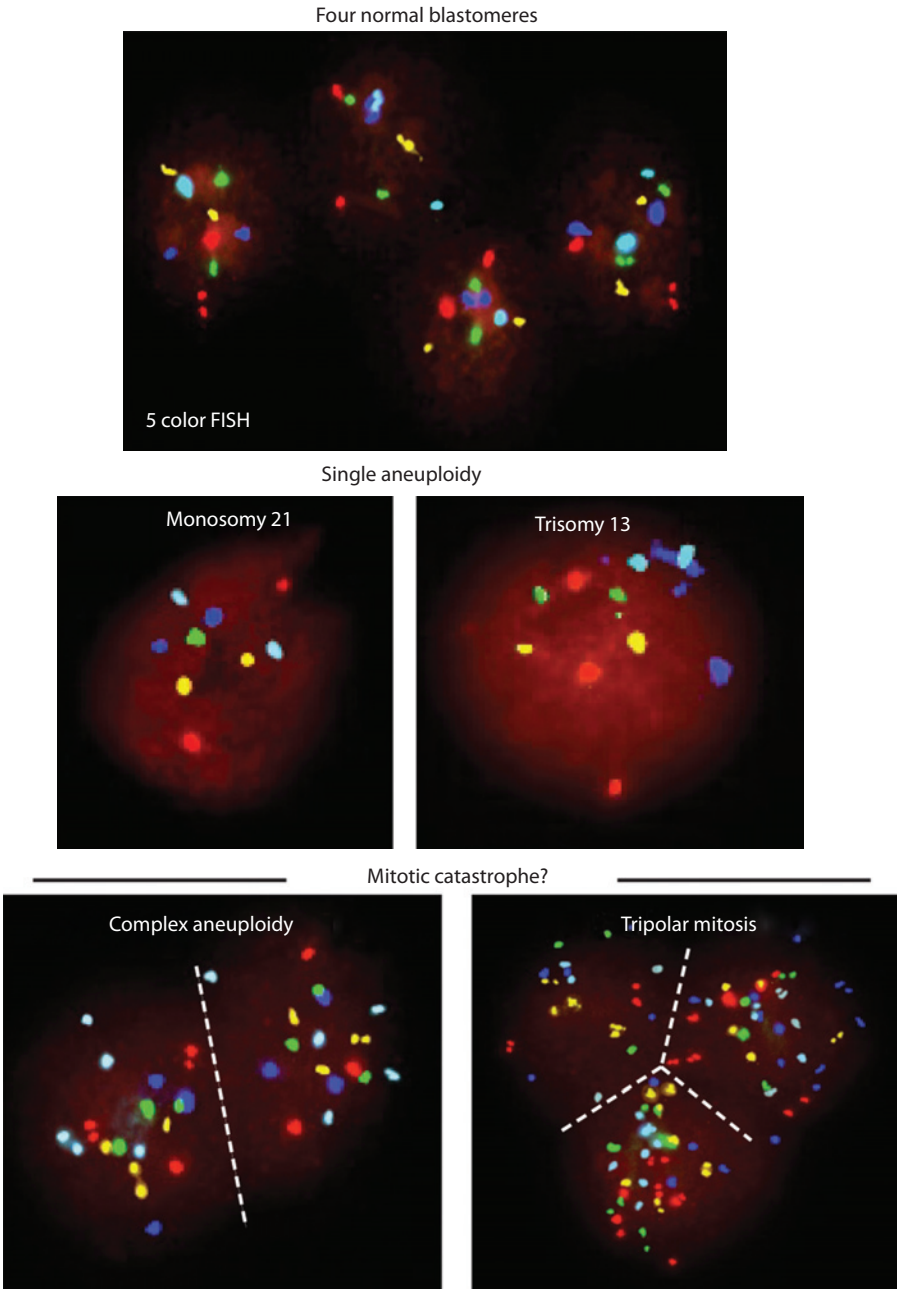

FIGURE 5.11 Tripolar mitosis and "chaotic" karyotypes in a human preimplantation embryo. FISH used against five chromosomes. The chromosomes have segregated in highly chaotic fashion into the three daughter cells.

Genome-Wide Association Study to Map Variants That Affect Chaotic Mitotic Aneuploidies

The first genome-wide association study (GWAS) to identify common variants that affect aneuploidy in preimplantation embryos has been conducted. Using aneuploidy data from around 46,000 embryos (a mix of day 3 and day 5) from 4,700 individuals, McCoy and colleagues found no association with putative maternal meiotic origin and maternal genotypes at the statistical significance threshold used.

However, they found a 600 Kb region (quantitative trait loci, QTL) of low recombination on chromosome 4 that was associated with multiple complex aneuploidies of mitotic origin (they followed paternal chromosomes, since meiotic error rate is very low in sperm). The QTL contained common variants of *PLK4*, a polo-like kinase that regulates centrosome numbers and whose dysregulation can lead to large-scale chromosome missegregation [109,110]. Importantly, it was the maternal *PLK4* variants that influenced mitotic chromosome segregation, consistent with the model that maternal factors drive the initial mitotic divisions in human preimplantation embryos [52,111,112]. Although the QTL contains seven other gene variants, *PLK4* is an attractive candidate since it regulates centriole duplication, a critical part of the centrosome cycle, and also mediates spindle formation during the initial cell divisions in mouse and bovine embryos [109,110]. Thus, *PLK4* variants may cause tripolar spindle formation that results in chaotic karyotypes (Figure 5.11). Importantly, tripolar spindles originating from normally fertilized human embryos have been observed [105].

The first follow-up clinical study of the rs2305957 minor variant of *PLK4* in the Chinese population revealed significant association with blastocyst formation in women with the AA genotype undergoing IVF as well as in a cohort of women suffering from early recurrent pregnancy loss [113]. It is intriguing, however, the although the efficiency of blastocyst formation was decreased in the women with the AA genotype undergoing IVF, implantation, early miscarriage rates, and live birth rates of the blastocysts that were transferred were not affected by the minor variant. One possibility is that the rs230597 is a risk factor that results predominantly in aberrant cell divisions resulting in embryonic arrest; thus, embryos that developed to day 5 were genomically stable. Although this cannot be ruled out, such a model cannot explain why women carrying the rs2305957 allele would be at elevated risk of early pregnancy loss. Further studies on the chromosomal constitution will be required to determine possible links between *PLK4* variants and aneuploidies that sustain fetal development. Another possibility is that the other variants within the QTL contribute to genome instability or embryonic loss as several of the genes have been implicated in cell cycle regulation or embryogenesis. As functional studies become feasible, assessing the function of the minor variant allele and determining whether alterations in *PLK4* actually occur in human oocytes or during early divisions are critical next steps in assessing cause and effect.

Aneuploidy and Embryonic Arrest

The association of complex and often chaotic aneuploidies with embryonic arrest in early-stage preimplantation embryos suggests that grossly aneuploid blastomeres may have poor viability. Indeed, aberrant cell divisions leading to gross cellular and genomic defects may cause arrest [50]. In such cases, aneuploidy is a result of other cellular defects. In other cases, aneuploidy may drive embryonic arrest or affect embryo development. The effect of aneuploidy on embryo development and the assignation of aneuploidy cells to specific tissues are studied by the generation of chimeras [114–121]. For cells carrying a single chromosomal aneuploidy, there is no or little evidence for the preferential assignment of aneuploidy cells to the placental precursor line (trophectoderm) or the fetus. Even when chaotic karyotypes are produced by inhibiting components of the spindle assembly checkpoint, there is no evidence for the active allocation of aneuploid cells to specific lineages [122]. However, in mouse embryos with chaotic chromosome constitutions, blastocysts display a depletion in cell numbers and the mechanism may depend upon lineage. Aneuploidy in the fetal lineage leads to apoptosis whereas senescence limits aneuploidy within the placental lineage [122]. It will be interesting to elucidate whether human embryos display similar mechanisms and, in particular, what causes apoptosis versus senescence in the two different lineages.

Future Directions

In this chapter, we have reviewed current findings and emerging themes in the human aneuploidy field in the context of our knowledge of population genetics. We envisage that technology-driven investigations into aneuploidy and its intersection with other cellular features, such as epigenetics, will shape our increasing appreciation of our genetic inheritance. Early embryonic divisions are largely governed by maternal factors, until the activation of the embryonic genome [111,123]. It follows that the robustness in

the developmental program of mature oocytes as well as early embryos is important for genome stability in the germline. With current advances in embryology, stem cells, and gene editing, it may soon be possible to use some of these tools to gain fundamental insight into mechanisms and cellular causes of aneuploidy, which afflicts such a large proportion of human conceptions and limits reproductive lifespan in women.

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6

Preimplantation Genetic Testing for Aneuploidy: All You Need to Know

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CONTENTS

Introduction

Aneuploidy is the most common genetic abnormality in human embryos. Preimplantation genetic testing for aneuploidy (PGT-A) can increase pregnancy rates in infertile couples requiring assisted reproductive technology (ART). This technique has also been called preimplantation genetic screening (PGS) and comprehensive chromosome screening (CCS), among other names. Large datasets from comprehensive aneuploidy testing of preimplantation embryos demonstrate that over half of embryos produced by in vitro fertilization (IVF) are aneuploid [1–3]. Several techniques have been applied for aneuploidy screening and embryo selection in IVF, including screening procedures for numerical or structural chromosome abnormalities.

However, in the early 2000s, publication of several randomized clinical trials (RCTs) using fluorescence in situ hybridization (FISH) raised controversies regarding the usefulness of PGT-A. The concerns were mainly due to technological limitations that only allowed analysis of a small number of chromosomes and inherent issues analyzing FISH signals on a single cell biopsied from an embryo containing 6–8 cells. Subsequently, new diagnostic technologies, such as array comparative genomic hybridization (aCGH) and next-generation sequencing (NGS), which interrogate all 23 chromosome pairs, became available. Three pilot RCTs that tested trophectoderm (TE) biopsy and aCGH on patients with a good prognosis for live birth show significant improvements in ongoing pregnancy rates and have changed the opinion of the PGT-A field [4–6]. In addition, new technologies allow better discrimination of copy number for each chromosome and have opened the possibility to identify the presence of embryonic mosaicism and sub-chromosomal abnormalities.

In this chapter, we will discuss approaches for embryo biopsy, applied technologies, clinical indications, and the impact of mosaicism in current practice.

Biopsy Strategies: Tips and Tricks

PGT-A can be applied to different preimplantation developmental stages, including polar bodies, day 3 blastomeres, and TE biopsies. Figure 6.1 presents a summary of advantages and limitations of each strategy.

- *First and second polar bodies* are usually retrieved the day of fertilization. This approach restricts detection of chromosome abnormalities to the female. Several groups have applied this methodology mostly to study monogenic diseases and translocations [7,8], and for aneuploidy screening using FISH [9] and aCGH, mainly for females of advanced age [10–12].
- *Cleavage-stage biopsy* involves the removal of a single blastomere from a day 3 embryo, usually with 6–8 cells and a low fragmentation degree (<20%). This approach allows identification of both maternal and paternal contributions. Embryo transfer can be performed two days later, on day 5 of development, and this strategy of fresh transfer has been the common policy for day 3 biopsies. However, several groups have strongly criticized this approach due to the potential detrimental effect on embryo viability [13,14]. Nonetheless, several aspects related to good practice could explain optimal results achieved by some groups [15–17]. In addition, two RCTs with FISH [18] and one with aCGH [19] have shown improved live-birth rates with day 3 biopsies compared to transfer of untested blastocysts.
- *Trophectoderm biopsy* is performed at the blastocyst stage, on day 5, day 6, or sometimes day 7. With this strategy, a diagnostic result must be obtained within 24 hours after biopsy or blastocysts must be cryopreserved. At this stage, embryos have undergone their first cellular differentiation, resulting in two cell lineages: the inner cell mass (ICM) (cells will form the embryo from this stage) and TE cells. Thus, biopsy of TE cells does not adversely affect embryo development [14]. Moreover, previous studies have demonstrated identical

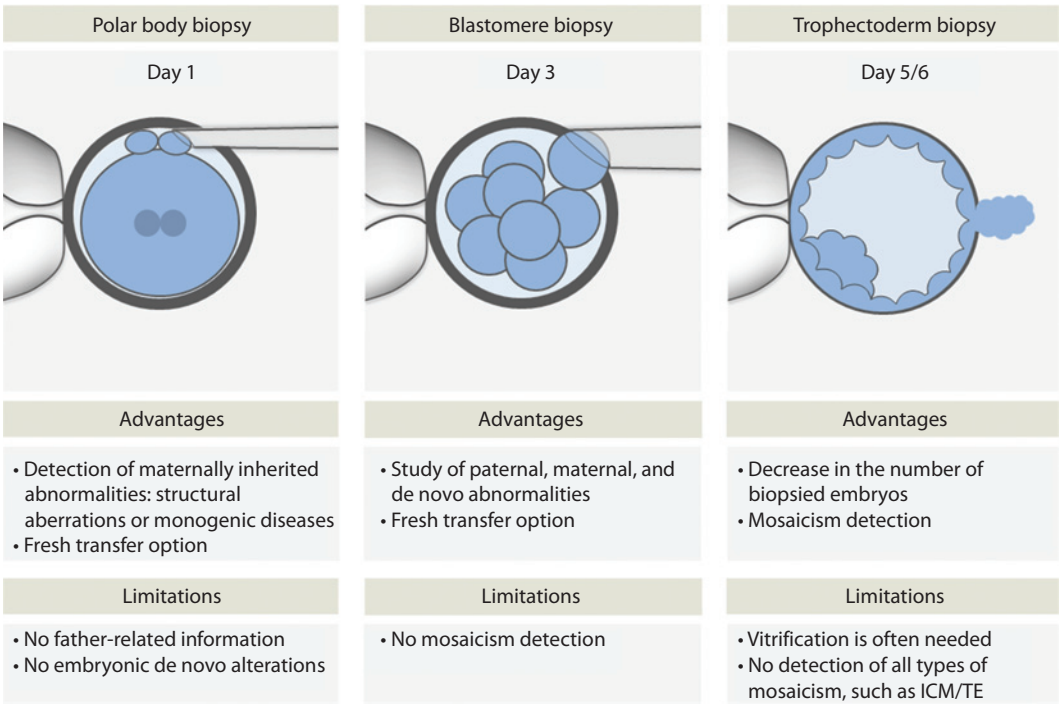

FIGURE 6.1 Polar body, day 3 blastomere, and trophoectoderm biopsies: advantages and limitations of each strategy.

genetic constitution of both ICM and TE from the same embryo [20]. Improvements in embryo culture conditions as well as in vitrification systems [21] have paved the way for the current trend of TE embryo biopsies. With TE biopsy, multiple cells can be sampled from each embryo, which can improve accuracy in the genetics laboratory. This approach has spread with the development of molecular analysis of 24 chromosomes, and several RCTs using this combination have shown improvements in delivery rates [4,22,23]. More recently, a new type of embryo biopsy that is thought to be less invasive, *blastocentesis*, has also been proposed. However, further results are needed to ensure reliability of the results [24] before widespread clinical application.

For each approach, the zona pellucida must be perforated. This can be accomplished by several methods: (1) mechanically, by cutting through the membrane with a micropipette; (2) chemically, by dissolving part of the membrane with an acid solution (i.e., Tyrodes); or (3) by laser, via the optical system of a microscope. The laser approach is most commonly used because it is faster, safer, and more reproducible among technicians. However, the number of cells removed and number of laser shots impact quality of the samples, and, if damaged cells are analyzed, results of the molecular genetic analysis can be inconsistent. Proper training and optimal IVF lab conditions, including embryo culture and vitrification, can yield reproducible results among groups and practitioners [25]. For these reasons, previous validation of the embryologist is also compulsory for successful results. Embryologists should be validated with several mock biopsies in discarded embryos, including control tubes with media from washing droplets.

Evolution of Technology Applied to PGT-A: The NGS Era

Several methods allow study of aneuploidies in human embryos, ranging from FISH, which provides limited information for a few chromosomes, to single-nucleotide polymorphism (SNP) arrays, quantitative polymerase chain reaction (qPCR), aCGH, and NGS, which analyze all 23 chromosome pairs. The evolution of aneuploidy screening techniques has provided more information about genetic status of the embryo in addition to more reliable and faster results, which enable transfer of euploid embryos during the same cycle (Figure 6.2).

- *PGT-A version 1.0:* From the 1990s to 2010, FISH was performed on polar bodies or cleavage-stage embryos. Numerous retrospective studies claim that the technique works, and thousands of cycles have been conducted worldwide [26–30]. FISH assays use fluorescent nucleic acid probes complementary to DNA to visualize regions of interest. However, FISH cannot be performed on all chromosomes simultaneously; therefore, it is targeted to those chromosomes related to spontaneous miscarriage or compatible with live birth, such as chromosomes 15, 16, 17, 18, 21, 22, X, and Y [31]. However, these chromosomes must be assessed over multiple rounds of hybridization, and informativity rates and accuracy of results depend on morphology and integrity of a single nucleus fixed onto a glass slide.
- *PGT-A version 2.0:* New technologies have facilitated the transition from FISH analysis of a limited number of chromosomes to analysis of all 23 chromosome pairs simultaneously in a single cell. Among these technologies, qPCR, SNP arrays, and aCGH have been most widely published to date [32]. This approach has been mainly incorporated in day 3 embryos [16,19] and blastocyst biopsies [4,20]. aCGH technology allows analysis of chromosome DNA copy number variations from an embryo compared to a reference sample. First, DNA from a single blastomere or 4–6 TE cells is amplified via whole genome amplification (WGA). Amplified DNA is then labeled with different fluorescent probes, combined, and hybridized onto a slide containing specific bacterial artificial chromosome (BAC) probes that span the length of chromosomes with ~1 Mb coverage. Chromosome loss or gain is revealed by the color of each spot after hybridization. Fluorescence intensity is detected using a laser scanner and data processing software, which can anvalyze whole chromosome aneuploidy and sub-chromosomal structural imbalances [16]. SNP arrays also utilize an array setup, although they interrogate specific SNPs in the genome

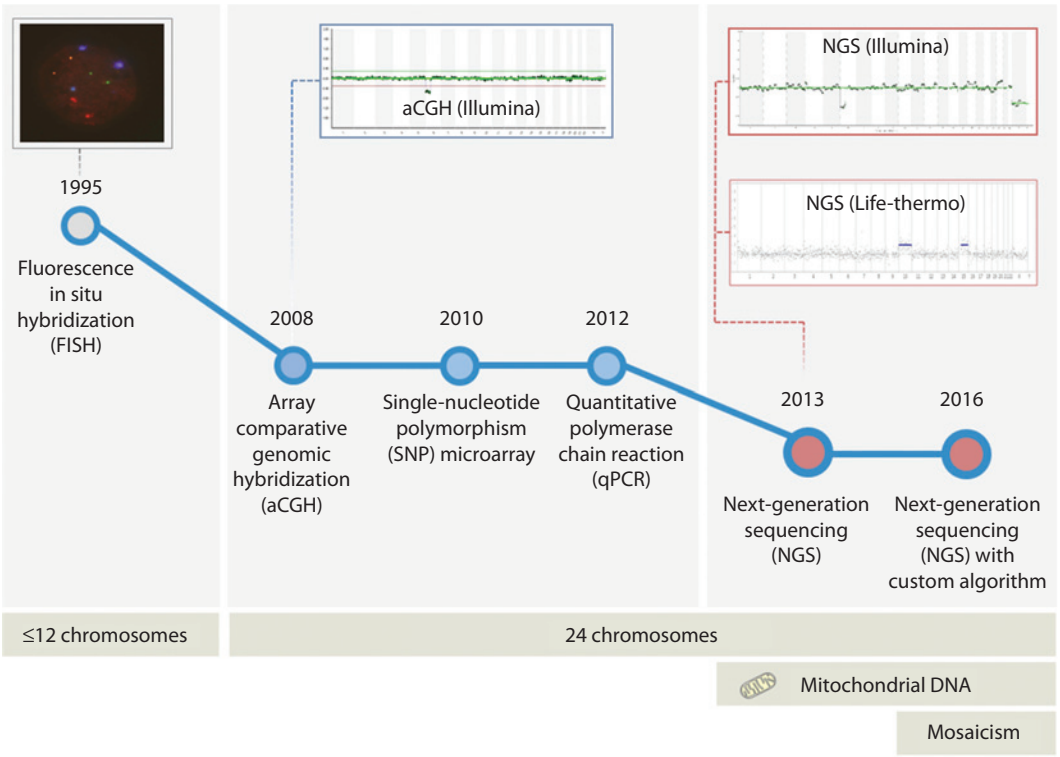

FIGURE 6.2 The evolution of aneuploidy screening techniques.

and compare these data to SNP patterns of maternal and paternal partners to arrive at a ploidy call [33]. For qPCR, specific PCR primers amplify a limited section of each chromosome on all 23 chromosome pairs in replicates. By analyzing the relative amount of DNA from each PCR product, a ploidy status can be inferred and assigned to each chromosome [34].

- *PGT-A 3.0:* This could be considered the latest approach with the possibility to use NGS in a small number of cells from TE biopsies. Decreased cost of genome sequencing has positioned NGS as one of the most promising platforms to study not only aneuploidies but also mitochondrial DNA or gene disorders in simultaneous analyses [35]. For NGS, most extended protocols share the first steps with aCGH protocols, starting with WGA. A barcoding procedure follows this, in which different samples are labeled with unique sequences, so that they later can be mixed, sequenced, and matched to their original patient and embryo. This barcoding process allows 24–96 biopsies to be pooled in a sequencing run, optimizing cost per sequenced embryo. After sequencing, each sequence is aligned with a reference human genome, and copy number variations for whole chromosomes and small deletions/duplications are established using specific software [36–39]. Depth of sequencing is also an important aspect to consider, especially for simultaneous study of aneuploidies and single gene disorders, which requires high coverage on those regions of interest.

In addition, the sequence of mitochondrial DNA has also been related to embryo quality [40,41], and NGS can detect lower levels of mosaicism in TE biopsies than previous technologies [42]. However, current bioinformatic analysis of sequencing data has difficulty clearly discriminating low-degree mosaicism from experimental noise related to the quality and quantity of biological samples and amplification artifacts. There is consensus among most groups to report mosaicism in >30% of estimated aneuploid cells. In addition, customized software and algorithms are being developed to improve robustness and objectivity among observers to identify mosaicism and develop mitochondrial DNA scores [43].

Mosaicism: Impact on Diagnostic Accuracy and Clinical Outcome

Mosaicism refers to the presence of cells with different chromosomal genotypes within a single embryo. It can originate from the first embryo cleavage and can be identified in PGT-A of a blastocyst biopsy with analysis of several cells from the TE (day 5, day 6, or day 7 embryo biopsy).

The first evidence of mosaicism in preimplantation embryos comes from re-analysis studies of aneuploid embryos. Several groups used aCGH to compare results of day 3 biopsies to FISH results on the remaining cells of day 5 embryos, showing low false-positive rates of 1.9%–2.7% [44–46]. Another study that isolated cells from the ICM and re-analyzed them using FISH shows a false-positive rate of 2.9% compared to TE biopsy results [47]. These results are also consistent with another blinded study in which PGT-A on day 3 embryos and TE biopsies shows high confirmation rates (98% for day 3 embryos, and 97.6% for TE biopsies) [48].

All samples showing discrepancies in those studies were diagnosed as chromosomally abnormal in the embryo biopsy but chromosomally normal in re-analysis of the full blastocyst. Therefore, the most feasible explanation is that they are "euploid-aneuploid" mosaic. However, it is challenging to calculate the real incidence of mosaicism in preimplantation embryos because of technical limitations [45,49,50] and because biopsies represent a small percentage of total number of cells of the embryo and will only partially represent the whole blastocyst, depending on percentage and distribution of euploid and aneuploid cells in the ICM and TE (Figure 6.3). Nevertheless, patients undergoing PGT-A have high pregnancy and implantation rates as well as reduced miscarriage rates [4,14,16,18], in addition to a very low clinically recognizable error rate [51]. Using a mouse model that generated "euploid-aneuploid" mosaic embryos, a previous study proposed that there is a threshold number of "tolerable" abnormal cells in a mosaic embryo and that pregnancy will be interrupted only when the number of chromosomally abnormal cells exceeds that threshold [52].

More recently, studies have proposed the possibility of transferring some types of mosaic embryos. Greco et al. [53] showed that some mosaic embryos can develop into healthy euploid newborns, suggesting that mosaic embryos could be considered for transfer in women who undergo PGT-A that results in no euploid embryos. However, the authors are cautious and state that additional clinical data must be obtained before this approach can be extended. Fragouli et al. [54] reported that transfer of mosaic embryos has significantly poorer clinical outcomes compared to a contemporary control group with the transfer of euploid embryos (ongoing pregnancy of 46.2% versus 15.4%; $p = 0.003$). The authors concluded that embryo viability is compromised by the presence of aneuploid cells. Finally, Scott and

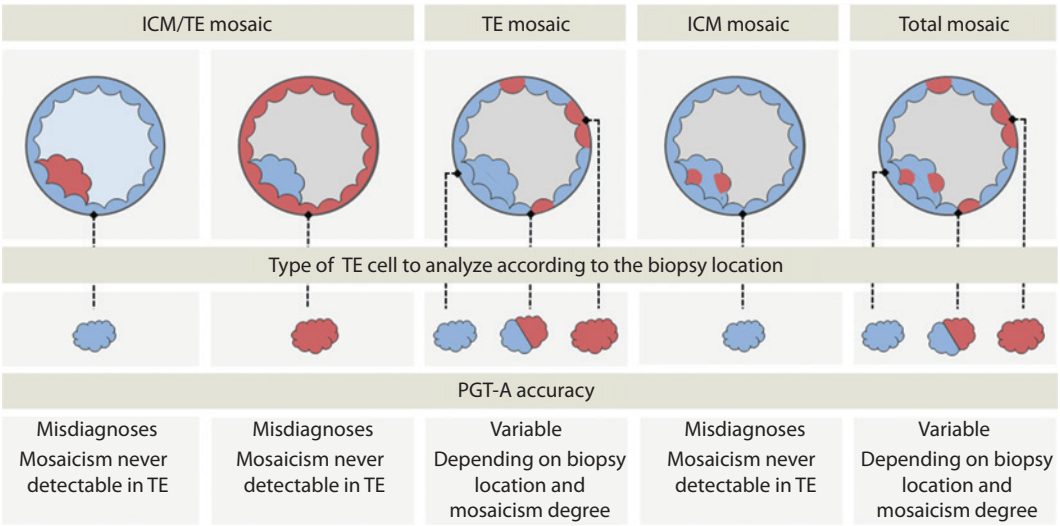

FIGURE 6.3 The accuracy of PGT-A in diagnosis of types of mosaicism.

Galiano [55] have discussed the need for balancing risks of discarding a competent embryo versus transferring an embryo that may ultimately have lower implantation potential, considering obstetrical and neonatal outcomes. Further research is needed to understand the relevance of mosaicism in the TE, as there are no studies of the effect of different percentages of aneuploid cells in the blastocyst and affected chromosomes. Vera-Rodriguez and Rubio [56] present a review of the topic, and Cinnuoglu, Fiorentino, and Harton [57] offer thoughts on future directions.

Common Indications of PGT-A in Couples with Normal Karyotypes

PGT-A was introduced in clinical routine practice to improve pregnancy rates in sub-fertile couples, based on the assumption that high rates of chromosomal aneuploidy—frequently found in cleavage-stage embryos and blastocysts of these couples—were responsible for low pregnancy rates after ART. Figure 6.4 shows aneuploidy rates according to maternal age for blastocyst biopsies with the newest NGS technology. Figure 6.5 shows the percentage of cases with at least one euploid embryo after blastocyst biopsy according to maternal age (Igenomix data obtained from 12,000 cases and more than 60,000 biopsied blastocysts).

The main goals for most indications are not only to increase implantation and pregnancy rates but also to decrease miscarriages, risk of aneuploid offspring, and time to conceive. More recently, cost efficiency per healthy baby at home has also been considered because with new PGT-A 3.0, blastocyst biopsy, and

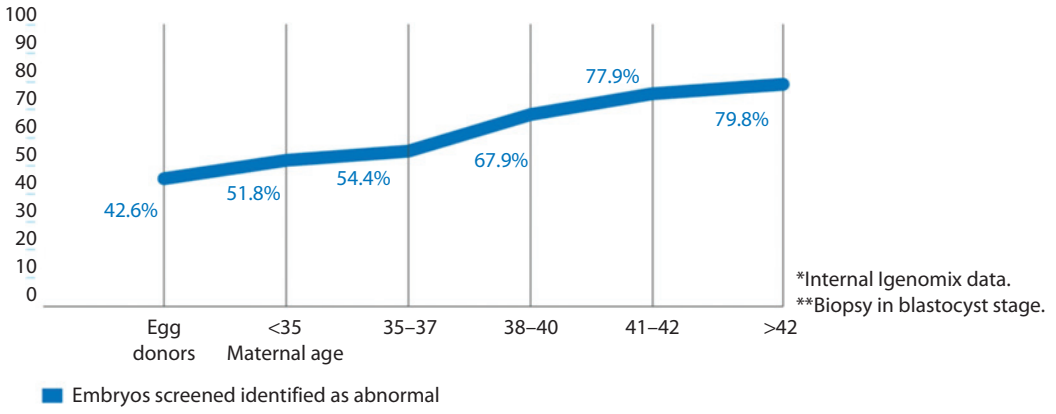

FIGURE 6.4 Incidence of aneuploidy according to maternal age.

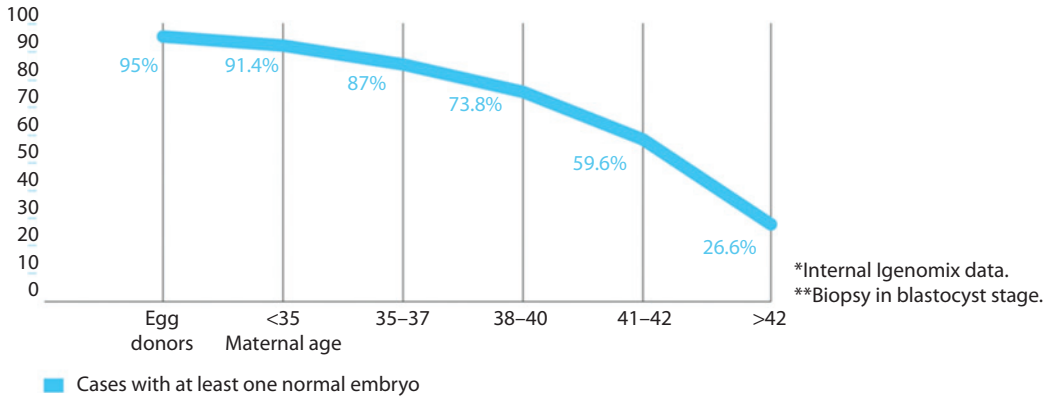

FIGURE 6.5 Chances of transfer according to maternal age.

NGS, PGT-A cost is no longer a limitation [19]. There are many factors that contribute to patient-perceived determinants when choosing to accept or decline PGT-A: cost, religion, ethical values, social and family support, provider influences, and past reproductive experience [58]. In light of these results, with the current trend toward single-embryo transfer (SET), it could be argued that failure to investigate chromosomal constitution of the preimplantation embryo to be transferred may raise ethical questions of its own [59].

Finally, with the growing popularity of blastocyst biopsy, blastocyst vitrification after TE biopsy, and deferred transfer, PGT-A fits well into this clinical scheme. A recent study comparing fresh blastocyst transfer and frozen cycles shows improved implantation rate per transferred embryo, although the difference is not significant (75% vs. 67%). However, ongoing pregnancy rates (80% vs. 61%) and live-birth rates (77% vs. 59%) are significantly higher with frozen samples compared to fresh transfer. Either transfer strategy can be a reasonable option, but there is a trend toward favoring deferred transfer [60]. Figure 6.6 shows clinical outcomes with PGT-A using blastocyst biopsy with deferred transfer (internal Igenomix data), compared to regular IVF/ICSI (www.sartcorsonline.com/2015).

The following are the most common current indications for PGT-A.

Advanced Maternal Age (AMA)

AMA is the most common indication for PGT-A. Maternal age is a major factor in the prevalence of aneuploidy [3]. A study performed in polar body biopsies reported that the rate of missegregation for most clinically relevant aneuploidies (chromosomes 13, 16, and 18) increases from 20% to 60% in women between the ages of 35 and 43 years [61]. Most clinical IVF groups have traditionally considered AMA to be any patient older than 37 years, although recently there is a move to lower the cut-off to 35 years.

Four RCTs have been published for AMA patients. Three indicate that PGT-A offers no benefit [62], but the fourth study describes lower miscarriage rates and increased delivery rates [63]. These studies have been criticized by several authors who argue that the study methodologies have some important pitfalls, including patient inclusion criteria, embryo biopsy procedures, embryo culture conditions, and type of genetic analyses performed [15,64,65].

However, our own experience differs from previously published studies. We conducted two prospective, randomized trials to evaluate the usefulness of PGT-A in AMA patients, the first using PGT-A-FISH in women 41–44 years of age. In this study, we observed a significant increase in live-birth rates in the PGT-A group compared to the conventional blastocyst transfer group (32.3% vs. 15.5%; $p = 0.0099$). We therefore conclude that classic PGT-A 1.0 is beneficial [18]. Despite these results, there remained a need for a technique to analyze all chromosomes while also producing reliable and accurate results in a short period of time. Therefore, a second study using aCGH in women 38–41 years of age confirmed higher live-birth rates using PGT-A compared to conventional morphological embryo selection per first transfer (52.9% vs. 24.2%; $p = 0.0002$), and per patient (36.0% vs. 21.9%; $p = 0.0309$). Also of note, PGT-A dramatically decreases miscarriage rates compared to controls (2.7% vs. 39.0%) [19].

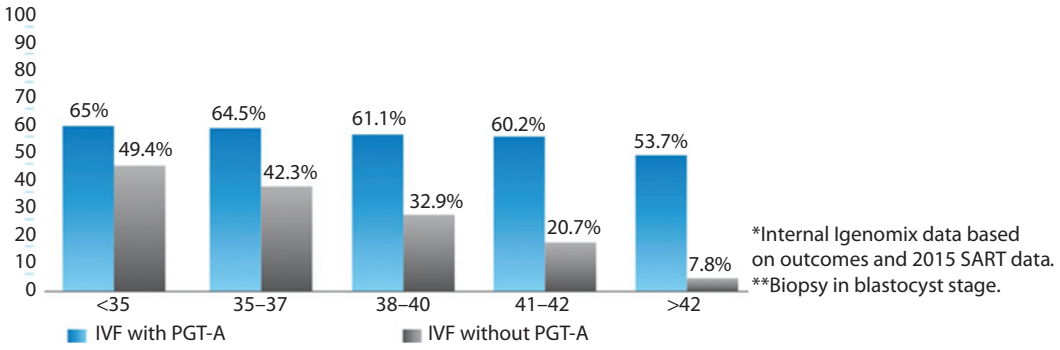

FIGURE 6.6 Ongoing pregnancy rate per embryo transfer.

Without the ability to screen for aneuploidy, AMA patients with a high percentage of aneuploid embryos may be subjected to multiple unsuccessful embryo transfers for months, some of which may end in distressing miscarriages with the associated medical risks. With the introduction of NGS, PGT-A cost is becoming increasingly affordable and enables embryo chromosome analysis in IVF at a lower cost [19,43].

Recurrent Miscarriage (RM)

The definition of RM varies by country but is generally considered the occurrence of two to three consecutive miscarriages with a gestational age up to 14 weeks. For PGT-A, other causes of miscarriage should be discarded before indicating this treatment, with a proper infertility work-up.

However, there is increasing evidence supporting the use of PGT-A. A study of prenatal diagnosis in 46,939 women published by Bianco et al. [66] confirms an increased risk of karyotypic abnormalities in conception products of idiopathic RM patients. The first evidence demonstrating that RM couples have an increased number of chromosomally abnormal embryos (50%–80%) was published by our group in 1998 [67]. Our studies demonstrate that, after PGT-A, couples who previously suffered aneuploid miscarriages have a significantly higher implantation rate and lower miscarriage rates. We also concluded that PGT-A should be recommended when RM is associated with a previous chromosomopathy and when there is a high incidence of chromosomal abnormalities in sperm [68].

In addition, a systematic review suggests that PGT-A may lower miscarriage rates [69]. More recent studies using aCGH on day 3 biopsies show high ongoing pregnancy rates in RM [16]. A retrospective case-control study reports PGT-A implantation rates of 52.63% compared to 19.15% in controls ($p = 0.001$) and an almost doubled ongoing pregnancy rate (61.54% vs. 32.49%; $p = 0.0001$) [17]. Another study comparing clinical outcome in PGT-A after day 3 or TE biopsies reports ongoing clinical outcomes of 50.4% and 63.6%, respectively [70].

Repetitive Implantation Failure (RIF)

RIF is defined as three or more failed IVF attempts or failed IVF treatments after cumulative transfer of >10 good-quality embryos. RIF-defining criteria are not homogenous, and an exhaustive and comprehensive definition has not yet been reached [18,71]. Therefore, RIF remains a challenge to clinicians because it can have multiple causes that are still poorly defined. Further, embryo and endometrial factors can play important roles in this condition [72,73].

One RCT in RIF patients concluded that there are no significant differences in clinical pregnancy rates with PGT-A-FISH compared to controls [74]. However, another study that analyzed a few more chromosomes shows a clear trend toward better live-birth rates with PGT-A-FISH (47.9% vs. 27.9%) [18].

Further, an aCGH study including 467 RIF couples shows that different factors affect clinical outcomes. In day 3 biopsies, pregnancy rates are 52.6% in patients <40 years old, compared to 41.5% in older patients. The best prognosis is observed in younger patients with a sperm concentration <10 million sperm/mL and with >15 mature (MII) oocytes. Number of previous failed cycles only increases the probability of couples producing embryos with a complex division pattern, but it does not impact their overall clinical implications. In a subset of patients with TE biopsies and deferred blastocyst transfer, pregnancy rate per transfer is 73.3% [75].

Severe Male Factor (MF) Infertility

An increased incidence of chromosome abnormalities has been reported in sperm samples of infertile men with normal FISH karyotypes [76]. Rubio et al. reported that oligozoospermia is associated with significant increases in sex chromosome disomy, chromosome 18 and 21 disomy, and percentage of diploid sperm, particularly in samples with markedly reduced sperm concentrations ($<5 \times 10^6$/mL spermatozoa). Such conditions might, in part, explain low implantation and high abortion rates observed in these patients.

Extreme teratozoospermia is also related to increased sperm aneuploidies [77]. Testicular sperm from non-obstructive azoospermia and from carriers of Y-microdeletions also show increased sperm aneuploidy, mostly for sex chromosomes [78,79]. Different types of sperm chromosomal aneuploidies are translated in the embryos, following a similar pattern, with increased trisomy for sex chromosomes in sperm samples with increased sex chromosome disomies, and higher triploidy rates in embryos from

sperm samples with increased diploidy rates [80]. However, conflicting publications have not clearly correlated sperm DNA fragmentation and paternal age with sperm aneuploidy [81–83].

PGT-A has been applied mainly in severe oligozoospermia, with day 3 biopsies showing >60% aneuploid embryos and 63% pregnancy rates [16]. Blastocyst biopsies have significantly greater sex chromosome abnormalities compared to embryos derived from normal semen samples. Further, aneuploidy rates in embryos derived from sperm with normal parameters are not significantly different whether ICSI or standard insemination is used to achieve fertilization. These results highlight severe MF infertility as a possible referral category for PGT-A [84].

Interim results of an RCT with day 3 biopsy and aCGH in couples with $<2 \times 10^6$ sperm show increased ongoing pregnancy and implantation rates, suggesting severe oligozoospermia as an indication for aneuploidy testing [85].

Previous Trisomic Pregnancy (PTP)

Some studies suggest that a PTP is associated with increased risk of another aneuploid conception. A study published in 2004 compared the rates of aneuploidy in preimplantation embryos from women with a history of a previous aneuploid conception among other groups. Using logistic regression to control for maternal age, the authors found the highest rate of detected aneuploidy in young women in this aneuploidy group. The study concluded that a history of trisomic pregnancy, whether it was a viable trisomy, is associated with increased risk of another aneuploid conception [86]. In 2009, De Souza and colleagues [87] used register data from Australian population-based birth defects to establish whether the risk of trisomies 13, 18, and 21 (Patau, Edwards, and Down syndromes, respectively) in a subsequent pregnancy was higher for women who have had a previous pregnancy with trisomy 13, 18, or 21. The relative risk of a trisomy 21 pregnancy following a previous trisomy 21 pregnancy is greater for women <35 years old at the previous pregnancy, as is the risk of the same trisomy and of a different trisomy subsequent to trisomy 13 or 18. Relative risk of a different trisomy subsequent to trisomy 21 is similar for women <35 and >35 years old at their previous pregnancy. The authors conclude that women who have had a previous trisomic pregnancy, particularly those <35 years old at the time, appear to have an increased risk of future trisomic pregnancies.

In relation to previous data, a more recent study stated that the incidence of chromosomal abnormalities in preimplantation embryos associated with a previous aneuploid miscarriage is significantly higher in individuals with a previous aneuploid conception [88]. In conclusion, the data suggest that using PGT-A can avoid recurrence of aneuploidies and could benefit this group of patients.

Good Prognosis Patients and SET

For patients with a good prognosis, TE biopsy using aCGH has high potential to increase overall pregnancy rates in IVF programs and to decrease multiple pregnancies when SET is performed. The first RCT comparing blastocyst-stage SET with and without aCGH in good prognosis patients shows an aneuploidy rate of 44.9% among biopsied blastocysts, with a significantly higher clinical pregnancy rate in the PGT-A group (70.9% vs. 45.8%; $p = 0.017$), and no twin pregnancies. This study reveals the limitations of SET when conventional morphology is used alone, even in patients without an increased risk for aneuploidy, because the aCGH group implanted with greater efficiency and yielded a lower miscarriage rate than those without aCGH [4].

Two other subsequent RCTs compared PGT-A with routine IVF techniques in good prognosis patients undergoing assisted reproduction techniques. In the first trial published in 2013 [23], mean female age was 35.1 ± 3.9 years in the PGT-A group and 34.5 ± 4.0 years in the control group, with antiMüllerian hormone (AMH) levels ≥1.2 ng/mL in both groups. In the PGT-A group, patients underwent euploid blastocyst SET, whereas the control group underwent blastocyst double embryo transfer (DET) after routine care for embryo selection. Clinical outcomes include a similar ongoing pregnancy rate between groups (60.7% after SET vs. 65.1% after untested DET; 95% CI: 0.7–1.2) and reduced risk of multiple gestations after SET (48%–0%). In the same year, Scott and colleagues [5] published another trial with infertile couples whose female partner or oocyte donor mean age was 32.2 ± 0.5 years in the PGT-A

study group and 32.43 ± 0.5 years in the control group. All participants had no more than one prior failed IVF attempt, had AMH levels of ≥1.2 ng/mL, and had normal uterine cavities. Delivery rates per cycle were significantly higher in the PGT-A group ($p = 0.01$). Collectively, these three RCTs show that use of PGT-A compared to embryo selection based on morphology criteria alone significantly improves clinical implantation rate in good prognosis patients undergoing assisted reproduction techniques. These findings are valid regardless of the technology used, such as aCGH or qPCR [89].

More recently, a single-center retrospective study assessed the use of PGT-A in donor egg FET cycles [90]. Blastocysts derived from donor eggs underwent TE biopsy and were tested for aneuploidy using aCGH or NGS. Data were analyzed separately for SET, DET, and for own uterus and gestational carrier (GC) uterus recipients. In DET, the PGT-A group has significantly higher live-birth implantation rates (number of babies born per embryo transferred), but not live-birth rates per transfer cycle. In SET, PGT-A has nominally, but not significantly, higher live-birth implantation rates and live births per cycle compared to controls. This study provides preliminary evidence that application of PGT-A may improve IVF outcomes using younger oocytes from an egg donation cycle. However, more studies taking into consideration clinical outcome per patient and per stimulation are needed to better address the benefits of euploid blastocyst transfer in ovum donation cycles.

PGT-SR in Carriers of Structural Chromosome Abnormalities

Balanced structural chromosome rearrangements are the most frequent chromosome abnormalities in the general population, with a prevalence of 0.4% in prenatal samples and 0.2% in newborns [91,92]. The most common structural chromosome rearrangements are translocations and inversions.

- *Translocations* are structural chromosome abnormalities that occur after a double break in two chromosomes and exchange of fragments between the two. Translocations can be reciprocal or Robertsonian translocations. Reciprocal translocations are produced by breakage and exchange of distal segments between non-homologous chromosomes. Robertsonian translocations arise by fusion of two acrocentric chromosomes (chromosomes 13, 14, 15, 21, or 22) and loss of their short arms.
- *Inversions* are structural chromosome abnormalities that occur after a double intrachromosomal break, 180° rotation of the fragment located between the two breakpoints, and subsequent reinsertion of the fragment into the chromosome. Inversions are classified according to relative position of the centromere with respect to the inverted fragment and can be pericentric or paracentric inversions. In pericentric inversions, the centromere is within the inverted fragment. In paracentric inversions, both breakpoints are in the same arm of the same chromosome.

When no loss of genetic material occurs, translocations and inversions are balanced and the heterozygous phenotype is normal. However, heterozygotic carriers of these types of structural chromosome rearrangements have an increased risk of fertility problems, recurrent miscarriages, and production of offspring with congenital abnormalities and mental retardation. These problems are mainly due to production of unbalanced gametes during meiosis because of abnormal segregations in translocation carriers or recombination events in inversion carriers [93–95]. Some authors have suggested the possibility that chromosomes involved in this type of rearrangement could interfere with correct segregation of other chromosomes by disrupting chromosome alignment on the spindle during meiosis I. This is known as *interchromosomal effect (ICE)* and was first described by Lejeune, who observed an increased rate of carriers of balanced reciprocal translocations among the parents of children with trisomy 21 [96]. Several published studies on ICE have reported controversial results from analysis of spermatozoa [97,98], oocytes [99,100], cleavage-stage embryos [101,102], and blastocysts derived from patients undergoing PGT-SR [103,104]. Some authors found that ICE [100,101] seemed dependent on rearranged fragment size, patient, and chromosome [22]. Other authors did not find ICE [23–26] or attributed increased aneuploidy rate to other factors, such as oligoasthenoteratozoospermia, which is frequently observed in these patients [105,106].

PGT-SR in these patients improves their reproductive expectations, reducing the time to achieve a successful live birth from 4 to 6 years to <4 months, and decreases incidence of miscarriage from >90%

to <15% in carriers [107,108]. Originally, a PGT-SR approach was taken for these cases based on FISH probes targeting chromosomes involved in the rearrangement. Recent optimization of technologies such as aCGH, SNP arrays, and NGS has allowed detection of not only chromosomal imbalances due to chromosomal rearrangements but also analysis of all chromosomes. Some authors have described pregnancy rates per oocyte retrieval and per embryo transfer of 16% and 27%, respectively, for carriers of structural chromosome abnormalities [109]. Other authors have described pregnancy rates per embryo transfer up to 70.6% with these new techniques. They also have described balanced aneuploid embryo rates of 43.3% [110]. The increase in pregnancy rates is most likely due to the ability to diagnose balanced aneuploid embryos that would be missed with use of FISH-PGT-SR alone.

Conclusions

While there has been much debate in the field surrounding genetic analysis of embryos during preimplantation, recent developments in both the IVF/embryology lab (routine growth of embryos to the blastocyst stage) and reproductive genetics lab (development of techniques to reliably assess 23 chromosome pairs in one test) have increased the efficiency of PGT-A technologies. Numerous recent studies have shown the benefits of testing embryos for common chromosomal abnormalities, including increased implantation and pregnancy rates per transfer, decreased miscarriage rates per patient, and faster time to pregnancy when compared to conventional embryo scoring by morphology alone. In addition, the usefulness of PGT-A to grade embryos ahead of SET is a great leap forward for IVF, allowing for safer pregnancies while maintaining high implantation and pregnancy rates across all patient populations. It should be made clear that screening embryos for common chromosome abnormalities does not make embryos better, although it does allow for a critical assessment of each embryo's chance of creating a normal live birth ahead of transfer, which can eliminate useless embryo transfers and allow for routine SET in IVF.

Figure 6.7 presents an algorithm summarizing indications and decision-making for PGT-A cycles.

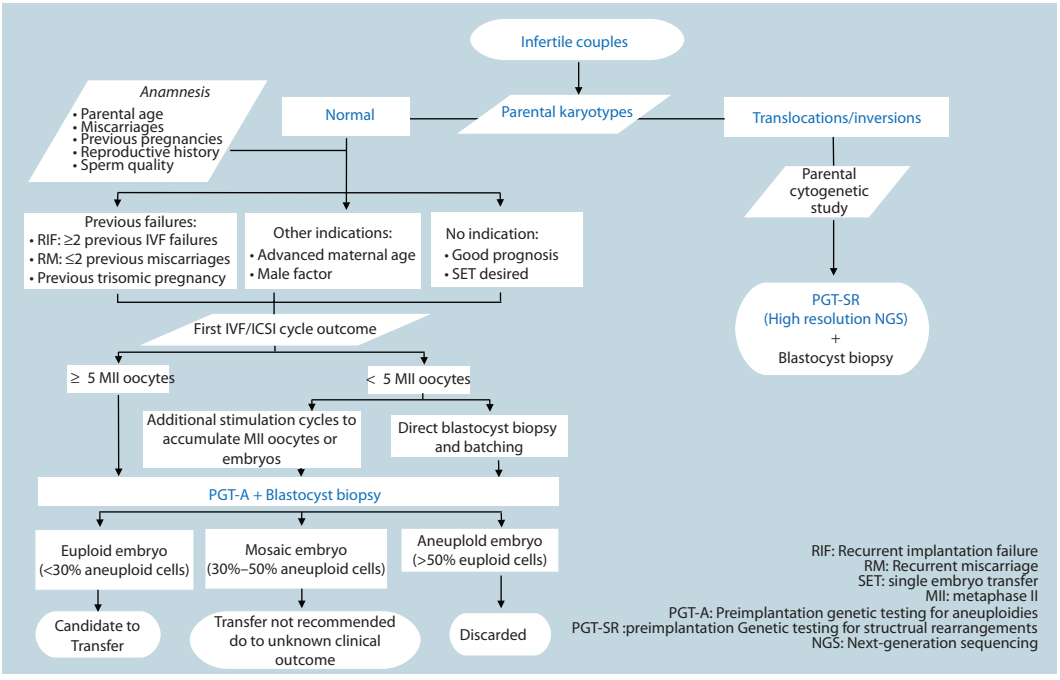

FIGURE 6.7 Algorithm for indications and decision-making for PGT-A cycles. MII, metaphase II; NGS, next-generation sequencing; PGT-A, preimplantation genetic diagnoses for aneuploidies; RIF, recurrent implantation failure; RM, recurrent miscarriage; SET, single-embryo transfer.

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7

Mitochondria and Embryo Viability

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CONTENTS

Introduction

Mitochondria play a critical role in the generation of metabolic energy in eukaryotic cells, using the process of oxidative phosphorylation to derive energy (ATP) from carbohydrates and fatty acids. Mitochondria contain their own DNA, which encodes tRNAs, rRNAs, and some mitochondrial proteins [1]. Ranging in size from 0.5 to 1.0 μm in diameter [2], these unique organelles have a double-membrane system consisting of inner and outer membranes separated by an intermembrane space [1]. The outer mitochondrial membrane encloses the matrix (internal space) and contains a large number of proteins that form channels allowing small molecules to pass. The inner mitochondrial membrane, which is folded into structures (cristae) that increase the surface area, is less permeable, blocking the movement of ions and other small molecules. Both the inner and outer membranes contain specific transport proteins that can move molecules by a passive or an active transport [2] (see Figure 7.1).

Origin of Mitochondria: Endosymbiotic Theory

New data are continuously shedding light on mitochondrial evolution. This concept involves two hypotheses on the origin of mitochondria. One proposes that the mitochondrion originated after the eukaryotic cell arose (endosymbiotic hypothesis); the other proposes that this organelle had its beginning at the same time as the cell (autogenous hypothesis). The endosymbiotic hypothesis hinges on the idea that mitochondria were originally prokaryotic cells capable of implementing oxidative mechanisms that later became endosymbionts. The autogenous hypothesis proposes that mitochondria split off from a portion of DNA in the nucleus of the eukaryotic cell [3]. The endosymbiotic hypothesis, initially proposed by

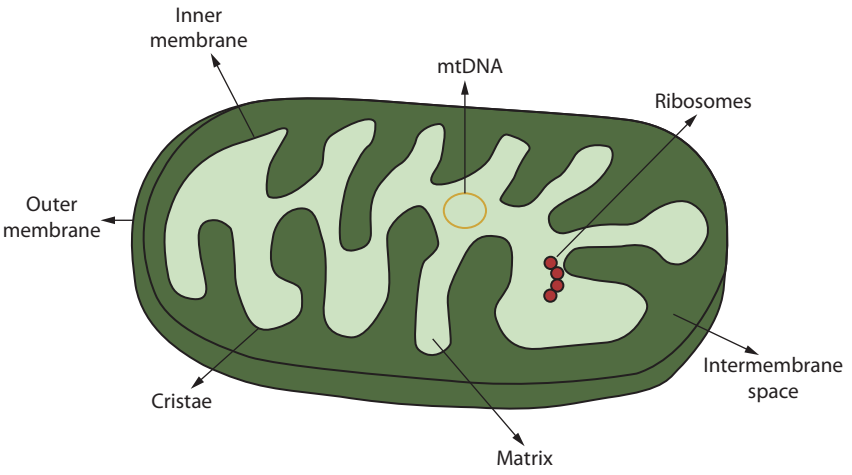

FIGURE 7.1. Diagram of a mitochondrion showing detail of the membranes and mitochondrial DNA.

Lynn Margulis, is the most widely accepted and exciting concept. It established the idea that mitochondria evolved from free-living bacteria via symbiosis within a eukaryotic host cell [4,5].

The oldest eukaryotic microfossils date back 1.45 billion years. This date corresponds to a time when the oceans were mostly anoxic because of the workings of marine H_2S-producing bacteria. Eukaryotes arose and diversified in an environment where anoxia was common [6]. The mitochondrial genome appears to have a monophyletic origin from α-Proteobacteria with Rickettsiales and several *Rickettsia*-like endosymbionts identified as the α-Proteobacterial order most closely related to mitochondria [3,7,8].

New lines of research suggest that the host that acquired the mitochondrion was a prokaryote. This view is linked to the idea that the ancestral mitochondrion was a facultative anaerobe, perhaps similar in physiology and lifestyle to modern Rhodobacteriales [6]. However, existing data demonstrate that the mitochondrial genome originated from the eubacterial, not the archaeal, domain of life (specifically α-Proteobacterial) [9,10], as indicated by phylogenetic analyses of both protein-coding genes and ribosomal RNA (rRNA) genes specified by mitochondrial DNA (mtDNA). In fact, *Rickettsia prowazekii*, the etiologic agent of epidemic typhus, transmitted in the feces of lice, stands out as the most genetically similar to mitochondria [9] (Figure 7.2).

The presence of mitochondria in the eukaryote continues to change the way we look at their origins, with new data and insights continually reshaping and refining our ideas; however, the endosymbiotic hypothesis plays a more central role [3,6].

Mitochondrial Functions: More Than ATP

The best understood mitochondrial functions include ATP production by oxidative phosphorylation, β-oxidation of fatty acids, and metabolism of amino acids and lipids [11]. In addition, multiple lines of evidence indicate that mitochondria have different and complex functions, such as participating in multiple cell signaling cascades. Proteins such as GTPases, kinases, and phosphatases are involved in bi-directional communication between the mitochondria and the rest of the cell, participating in the regulation of metabolism, cell-cycle control, development, antiviral responses, and cell death [12]. In addition, there is a dynamic change in the phosphorylation state of numerous proteins in response to calcium signaling [13]. Mitochondria respond to calcium both as a calcium buffer and as the propagator of intracellular calcium waves during muscle contraction and synaptic vesicle release [12]. Another example of the integration of mitochondria in signaling pathways is their role in the apoptotic cascade and cell death [14]; indeed, mitochondrial fragmentation and cristae remodeling are essential steps for cytochrome c release and cell death [12].

Further, mitochondrial dysfunction is linked to genomic instability and aberrant RNA and DNA synthesis because multiple enzymes responsible for metabolism of cytosolic ribonucleotides and deoxyribonucleotides are present in mitochondria [11]. For example, serine, a major source of one-carbon

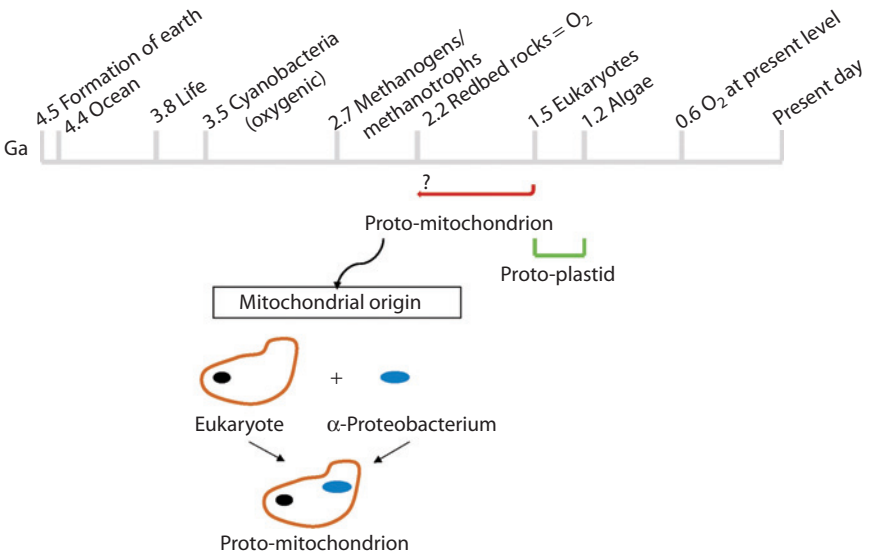

FIGURE 7.2 Timeline for the origin of life and major invasions giving rise to mitochondria and plastids. An α-Proteobacterium is shown in blue and eukaryotic cell in yellow. Ga, billion years ago.

units, is required for the synthesis of glycine, thymidylate, methionine, methylation reactions, and purine synthesis. It is converted to glycine, and a methyl group is transferred from serine to tetrahydrofolate (THF), yielding glycine and 5,10-methylene-THF [11]. In the mitochondria, 5,10-methylene-THF is converted into formate in a succession of enzymatic conversions beginning with conversion into 5-methyl-THF and afterward into 10-formyl-THF. Cytosolic 10-formyl-THF is a crucial one-carbon-unit donor for the de novo synthesis of purine nucleotides [11]. This process demonstrates the importance of mitochondria in DNA/RNA synthesis. Moreover, mutations affecting the activity of the electron transport chain can lead to a detrimental effect on cytosolic dNTP levels, causing instability in the nuclear genome [11].

Oxidative Stress

Reactive oxygen species (ROS) are highly reactive molecules that include diverse chemical species such as superoxide anion, hydroxyl radical, and hydrogen peroxide [15]. About 1%–2% of the molecular oxygen consumed during physiological respiration is converted into superoxide radicals [14]. Mitochondrial ROS are generated in the electron transport chain through one-electron carriers [15,16] interacting directly with mitochondrial proteins, lipids, and DNA. This results in lipid peroxidation, protein oxidation, and mitochondrial DNA (mtDNA) mutations [14,16]. Indeed, as the majority of ROS are products of mitochondrial respiration, mitochondria are the major targets for their damaging effects [14,15].

Oxidative damage modifies replication and transcription of mtDNA. This inhibits mitochondrial function, which enhances production of ROS. However, mitochondria have a defense system to detoxify ROS and repair ROS-induced damage [14]. Many studies have suggested that mtDNA is more susceptible to oxidative damage [15]. Further, oxidative stress plays an important role in aging [15], and the modulation of this system directly affects the susceptibility of cells to undergo apoptosis [14].

The study of how ROS influence the activation of the cell death program and other important pathways may continue to reveal mechanisms to be used for therapeutic intervention in human diseases.

Mitochondrial DNA: Characteristics

mtDNA is a covalently closed circular molecule [17] of 16.5 Kb that is located in the matrix. Its genetic code differs slightly from the universal one, in that AUA codes for methionine and not isoleucine and UGA codes for tryptophan and not STOP. Further, the AGA/AGG pair used for arginine does not code for termination in bovine mitochondria. Moreover, mtDNA has no introns [18] (Figure 7.3a). In humans,

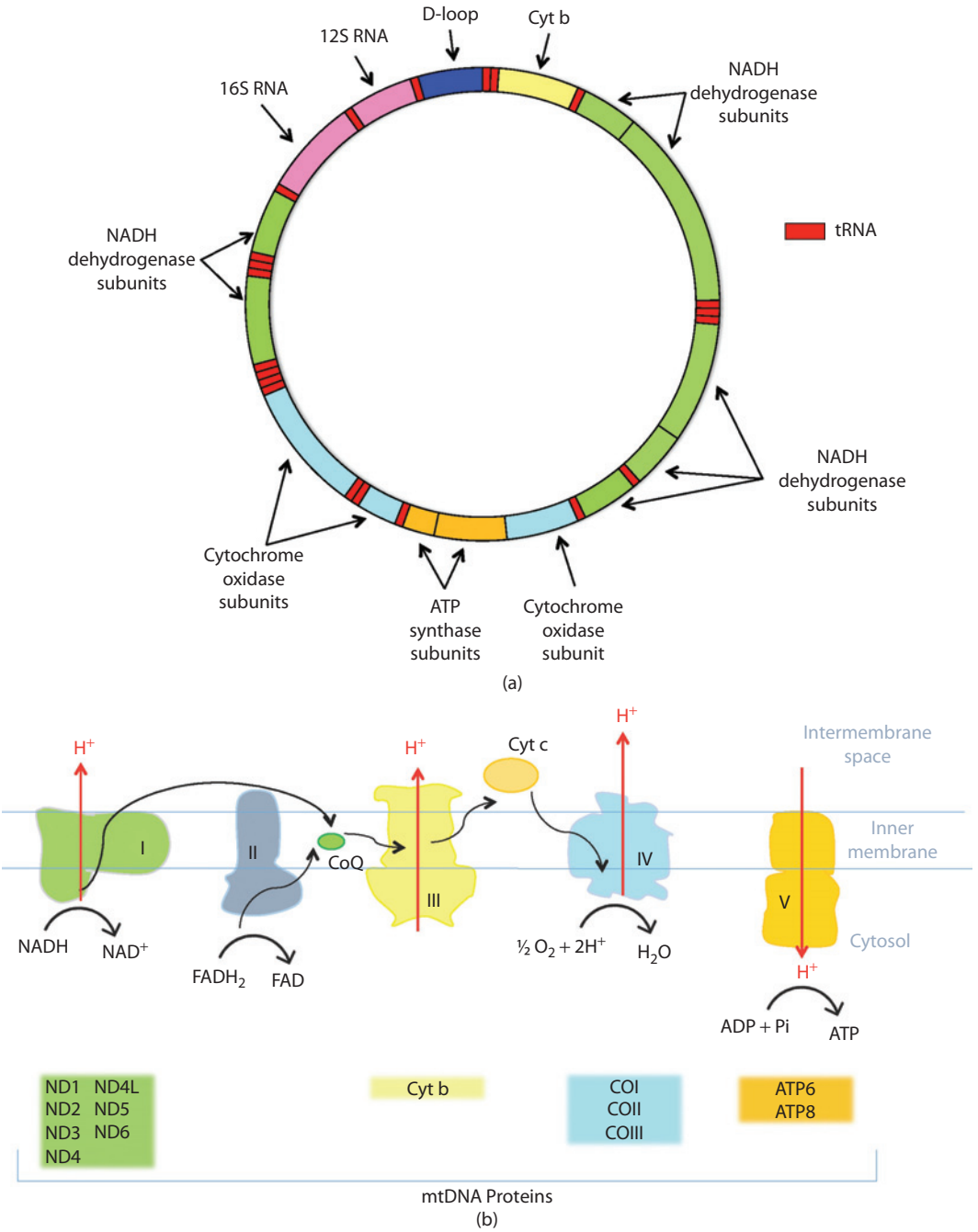

FIGURE 7.3 (a) Mitochondrial DNA representation. Mitochondrial DNA has a regulatory sequence, the D-loop (dark blue), and encodes 37 genes: 22 tRNAs (red), 2 rRNAs (pink), and 13 proteins. (b) The respiratory chain is located in the inner membrane, and it comprises five complexes. The 13 polypeptides encoded by mtDNA are located in the respiratory chain: 7 in complex I (green), 1 in complex III (yellow), 3 in complex IV (light blue), and 2 in complex V (orange). Complex II is entirely encoded by the nuclear genome (gray). Complexes I, III, and IV are H+ pumps that generate an electrochemical gradient across the membrane, where H+ is then pumped by complex V (ATP-synthase) to the matrix and ATP is produced. (Data from Mishra P and Chan DC, *Nat Rev Mol Cell Biol*, 15, 634–646, 2014.)

100–10,000 separate copies of mtDNA are usually present per cell. For instance, energy-intensive tissues such as cardiac and skeletal muscle contain between 2,000 and 10,000 copies of mtDNA per cell, with an average mtDNA copy number per mitochondrion of between 1,000 and 10,000 copies. Less energy-demanding tissues, such as the lungs, have an average of between 200 and 3,000 copies per cell and 50 and 300 per mitochondrion. Ovocyte mitochondrial content has been estimated between 100,000 and 640,000, and it is assumed that each mitochondrion contains one or two genomes (reviewed by Van Blerkom, [19]).

The mtDNA encodes 2 rRNAs (12S and 16S), 22 tRNAs, and 13 mRNAs [18]. It also has a regulatory sequence, involved in replication and transcription, known as the D-loop [18]. The proteins encoded by mtDNA are synthesized in the mitochondria. These proteins are essential subunits of the electron transport complexes of the electron transport chain (ETC): 7 subunits of complex I [20], 1 subunit of complex III [18], 3 subunits of complex IV [21], and 2 subunits of complex V [18]. If mtDNA genes are mutated, the functions of the ETC are affected; mutations in the nuclear genes encoding mitochondrial proteins can affect multiple mitochondrial functions [11]. It is important to highlight that mtDNA mutates 10 or more times faster than nuclear DNA due to its location near the ETC and the lack of protective histones [22]. As a result, mtDNA is more exposed to the deleterious effects of ROS (Figure 7.3b) [23].

Mitochondrial DNA: Inheritance and Bottleneck Selection

Human mtDNA is inherited maternally [24]. Although a zygote receives both maternal and paternal mtDNA at fertilization, the paternal mtDNA is specifically targeted for degradation and removed from the cytoplasm of the zygote during embryogenesis [25,26].

The number of mitochondria present in a metaphase II oocyte [19] represents only a small fraction of the maternal mtDNA pool due to a genetic bottleneck that occurs during oogenesis [27]. In the primordial germ cells there is a large population of mtDNA that represents the maternal mtDNA pool. This is the starting point of the genetic bottleneck. During development of the germline, the maternal mitochondrial pool is sub-sampled to a relatively small number [24], resulting in only a small fraction of mtDNA being represented in the mature egg [27]. In this way, the diversity of mtDNA is limited in each oocyte, and homoplasmy is promoted. However, within a cohort of oocytes there are differences because the maternal mtDNA is randomly segregated [27]; indeed, in mtDNA diseases the level of mutant mtDNA differs between oocytes from the same patient [28]. After the bottleneck, during oocyte maturation, there is an increase in mitochondrial content and mtDNA copy number [27].

Mitochondrial Replication and mtDNA Replication

mtDNA replication is mediated by several nuclear-encoded transcription and replication factors. These factors, along with mtDNA, form the mitochondrial nucleoid [29], which is responsible for the packaging, transcription, and replication of the mitochondrial genome [30]. The nucleoid proteins comprise mitochondrial-specific polymerase gamma (which contains two subunits, POLGA and POLGB), mitochondrial RNA polymerase (mtRNA pol), mitochondrial transcription factor A (TFAM), mitochondrial transcription factor B (TFBM), mitochondrial single-stranded DNA-binding protein (mtSSB), helicase (Twinkle), key transcriptions factors (TFB1M, TFB2M), and mTERF. All of these are located in the central region of the nucleoid; in contrast, ATAD3 is arranged peripherally and acts as the backbone of the nucleoid (reviewed by St. John, [29]). Table 7.1 summarizes the functions of mitochondrial nucleoid components.

TFAM initiates replication; its binding to mtDNA induces structural changes that result in exposure of the promoter region. Next, mtRNA polymerase synthesizes an RNA that becomes a primer used by POLGA to initiate mtDNA replication. This process is supported by POLGB, which stabilizes POLGA and increases efficiency, and mtSSB and Twinkle, which mediate mtDNA unwinding. The timing of

TABLE 7.1

Functions of Mitochondrial Nucleoid Components

Nucleoid Protein	Function
POLGA	mtDNA polymerase, catalytic subunit
POLGB	mtDNA polymerase, accessory subunit; forms heterotrimer 2:1 with POLGA
mtRNA pol	Transcription of mtDNA that generates RNA primer to mtDNA replication
TFAM	Transcription factor, starts replication
TFBM	Forms heterodimer with RNA polymerase, allowing specific transcription initiation
mTERF	Termination of transcription
mtSSB	Stabilizes mtDNA and stimulates Twinkle activity
Twinkle	mtDNA unwinding
ATAD3	Backbone to the nucleoid

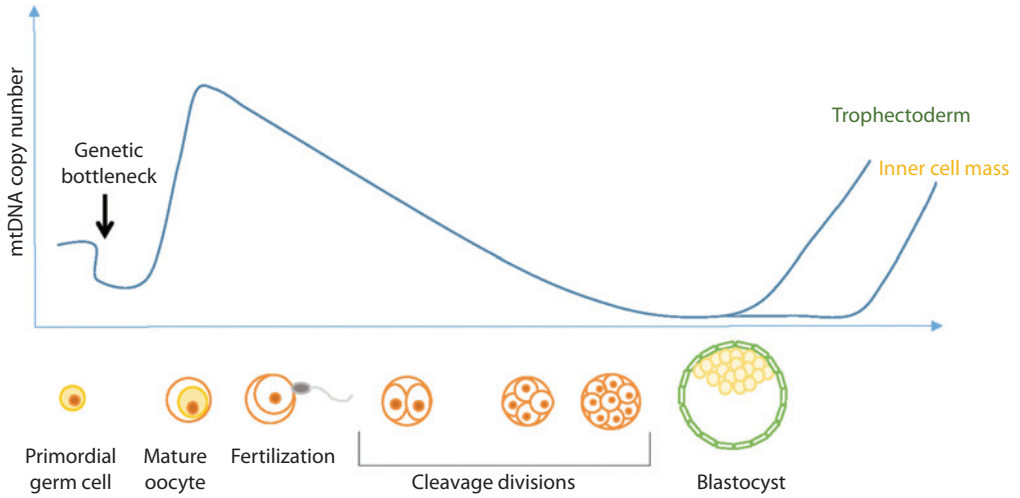

FIGURE 7.4 Representation of changes in mtDNA copy number. During oogenesis, a genetic bottleneck occurs and the number of mtDNA molecules is reduced. After that, the remaining small fraction of mtDNA is amplified and the mature oocyte has more mtDNA, but it represents only a part of the initial maternal pool. From this time, no further mtDNA replication takes place until the blastocyst stage. Thus, the number of mtDNA molecules is being reduced with each cell division. At the blastocyst stage, mtDNA replication is resumed, and it is thought that it starts first in the trophectoderm, then in the inner cell mass.

mitochondrial genome replication differs from the organelle's replication and is controlled by signals modulated by cellular energetic demand.

Studies in mouse models indicate that, in normal situations, no further mtDNA replication takes place between the mtDNA amplification in the fertilized ovum and the blastocyst stage: the total amount of mtDNA remains stable from the one-cell ovum through the early blastocyst stage [31]. Therefore, during cleavage divisions, the amount of mtDNA remains stable and mtDNA is presumably reduced with each cell division. As a result, the number of mtDNA molecules per cell is continually diluted. At the blastocyst stage mtDNA replication is resumed [32,33]; it is believed to begin first in the trophectoderm (TE), then in the inner cell mass (ICM). In a porcine model, an increased level of mitochondrial-specific polymerase is observed in the outer edges of embryos at the morula stage, in the region of cells that likely become TE. However, no increase is seen in the inner embryo, representing future ICM cells [33] (Figure 7.4). Quantitative analysis of ATP production in mouse embryos also supports the idea that approximately 80% of ATP is generated in TE [34].

Early Mitochondrial Number and Morphology Changes

Mitochondria undergo morphological changes during embryo development. Unlike differentiated cells, oocyte and fertilized eggs have structurally undeveloped mitochondria. Mitochondria of fetal oogonia are elongated, their matrix is dense, and they have tubular cristae. Those from oocytes of primordial follicles are rounded, their matrix is less dense, and their membranes are arranged in fewer cristae. During oocyte growth and maturation, mitochondria are predominantly spherical to oval with a diameter ≤1 μm and are characterized by an increasingly dense matrix [35,36]. Their appearance makes them seem inactive; however, some are actively generating ATP by oxidative phosphorylation [19]. The structure remains unchanged until about the 8-cell stage, when a decrease in matrix density occurs progressively. During blastocyst differentiation, expansion, and hatching, mitochondria become progressively more elongated, show a lighter matrix, and have more numerous cristae, a clear sign of increased metabolic activity [35,36].

Early Embryo Development Mitochondrial Metabolism

Early embryos exhibit changes in their metabolism throughout development. Initial cleavage divisions take place under the control of maternal mRNA. During that time, the embryo has a metabolic preference for pyruvate. By the time of embryonic genome activation, at about the 8-cell stage in humans, anabolic conditions switch to catabolic metabolism and glucose is the main energy source. The early embryo preference for pyruvate shows the importance of mitochondrial metabolism during this time [37] (Figure 7.5).

Pyruvate and glucose uptake have been measured in human preimplantation embryos to confirm metabolic changes. An increasing pyruvate uptake is observed from day 2.5 until day 4.5, when pyruvate consumption starts to lessen [38]. On the other hand, glucose uptake occurs throughout, increasing on successive days of development [39]. However, the change is small from day 2.5 to 4.5, but increases in magnitude on day 5.5, at the blastocyst stage, as is expected [38].

The relationship between glucose and pyruvate uptake and embryo development has also been studied to uncover any relationship between metabolism and embryo quality. Embryos that develop successfully have higher pyruvate uptake than those that arrest at cleavage stages. Differences in glucose uptake

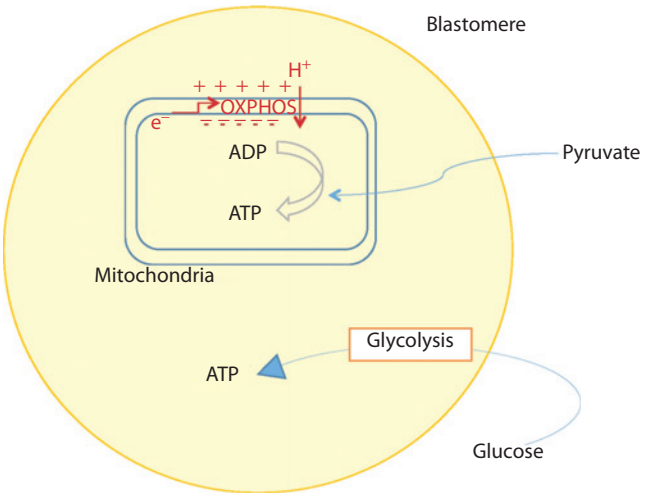

FIGURE 7.5 Blastomere energy production. Early embryo development requires energy, i.e., ATP, which can be produced by two possible mechanisms: glycolysis, in which glucose is the substrate, and oxidative phosphorylation, using aerobic substrates such as pyruvate. During the first stages of development, mitochondria are the energy producers by OXPHOS; at the blastocyst stage, there is a shift to ATP generation by glycolysis.

occur in the final upsurge in consumption. At that point, embryos that are going to arrest fail to show the final upsurge in glucose consumption [38]. Further, embryos that form high-quality blastocysts have higher glucose consumption on days 5 and 6 than those of bad quality, but they exhibit no difference pyruvate uptake. This is in line with the established metabolism, and indicates that glucose is the more important energy source for human blastocysts, allowing for aerobic and anaerobic glycolysis [39].

Mitochondrial Function in the Early Embryo

Oxidative Stress and Early Embryo Development

Notably, mitochondria produce ROS during oxidative phosphorylation. These ROS can induce oxidative stress if they are overproduced. Oxidative stress in an embryo can lead to apoptosis and embryo fragmentation during early development. Logically, then, the embryonic DNA should be protected from mitochondrial ROS, and this likely represents one of the consequences of little mitochondrial activity in the embryo.

Mitochondria have a crucial role in apoptosis during early development; pro-apoptotic factors such as cytochrome c and apoptosis-inducing factor are released from the intermembrane space into the cytosol and trigger caspase activation. In mouse zygotes undergoing in vitro culture, treatment with hydrogen peroxide (H_2O_2) induces cell death. Mitochondria are clearly involved in oxidative stress-induced cell death by release of mitochondrial pro-apoptotic factors [40]. Oxidative stress is also associated with the fragmentation rate of human embryos. H_2O_2 production is significantly higher in fragmented embryos compared to non-fragmented ones, and is correlated with apoptosis and DNA fragmentation [41]. However, the in vivo environment differs from the in vitro environment used in the studies mentioned above. The main difference is the presence of defense mechanisms against ROS [41,42]. Antioxidants are present in the cumulus cells [43], follicular fluid, ovarian tissue, fallopian tubes, and endometrium. They neutralize oxidative stress and protect the oocyte and embryo by preventing ROS formation, intercepting ROS, and promoting DNA repair [44]. A study in which ROS levels were measured on the first day of in vitro culture after conventional in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) showed that, in both types of cycles, lower pregnancy rates are related to high ROS levels. However, increased ROS levels are associated with low blastocyst rates, low fertilization rates, low cleavage rates, and high embryonic fragmentation only in ICSI cycles. This finding can be explained by the fact that, in conventional IVF but not ICSI-only, cumulus cells are present and they may have antioxidant activity [44].

Mitochondrial Ca^{2+} Signaling and Fertilization

Fusion of egg and sperm leads to metaphase II oocyte activation, resumption and completion of meiosis, extrusion of the second polar body, and pronuclear formation. This activation produces cortical granule exocytosis, zona pellucida and plasma membrane remodeling, and initiation of maternal mRNA translation. Intracellular Ca^{2+} signaling has a well-defined role during fertilization. Sperm-egg fusion leads to an increase in cytosolic Ca^{2+} levels, followed by a series of oscillations in Ca^{2+} levels [45]. In the mouse, sperm-triggered Ca^{2+} waves produced in the fertilized egg stimulate mitochondrial oxidative phosphorylation. Moreover, mitochondrial oxidative phosphorylation is necessary to maintain those Ca^{2+} oscillations. When a mouse egg is cultured in a medium without the substrates needed by mitochondria, it can be fertilized but the Ca^{2+} waves are inhibited [46].

As fertilization requires ATP, it could be thought that ATP levels will decrease during the process. However, after fertilization in the mouse, no decrease in ATP levels is observed. This indicates that the energy demand is matched by energy supplies. Ca^{2+} signaling produced by the entrance of the spermatozoa to the oocyte activates ATP production in mitochondria; thus, the energy required for this process is supplied [45], and low levels of oxidative phosphorylation are maintained when energy is not needed [47]. In summary, the Ca^{2+} increase produced by fertilization acts as a bridge between cell energy requirements and energy producers, i.e., mitochondria. This triggers the production of energy necessary

to undergo subsequent developmental stages, and when no energy is needed low levels of oxidative phosphorylation are maintained. In turn, mitochondria are essential to maintaining Ca^{2+} oscillations that are needed to complete meiosis and start embryo development [48].

Mitochondria as a Stress Sensor

Mitochondria are able to sense internal or environmental changes, such as diet and toxic substances. They signal stress by membrane depolarization, alterations in adenine nucleotide levels, ROS production, Ca^{2+} fluxes, permeability transition pore opening, and perhaps secretion of proteins/peptides [49]. They also regulate bioenergetic, thermogenic, oxidative, and/or apoptotic responses so they can reestablish homeostasis.

Acute exposure to stress mediators is associated with increases in mitochondrial biogenesis and changes of activity in the respiratory chain complexes, controlled production of ROS, thermogenesis, and apoptosis. In a chronic stress situation, the damage can exceed mitochondrial responses and cause abnormal mitochondrial biogenesis, respiratory chain dysfunction, decreased ATP production, increased ROS generation, lipid peroxidation, mitochondrial and nuclear DNA damage, and increased cell apoptosis [50] (Figure 7.6).

The "quiet embryo hypothesis" suggests that, by minimizing oxygen consumption (quiet metabolism) from the zygote to morula stage, the embryo limits the formation of ROS and there is less damage to the genome, transcriptome, and proteome. In contrast, the "noisier" the embryo, the greater the level of damage and demand for nutrients and energy [51,52]. The embryo is likely dependent on energy accumulation during oocyte maturation, and only in a stress situation (reduced metabolic fuel) does the cellular machinery react to increase mtDNA copy number in an effort to produce more mitochondria. Thus, mitochondrial dysfunction is associated with mitochondrial hyperproliferation [51,53].

Mitochondrial biogenesis is mainly activated by increased energetic demands. It requires the regulation of numerous processes such as mtDNA synthesis, import and synthesis of nuclear-encoded proteins, and the assembly of proteins from nuclear and mitochondrial DNA. This regulation involves a

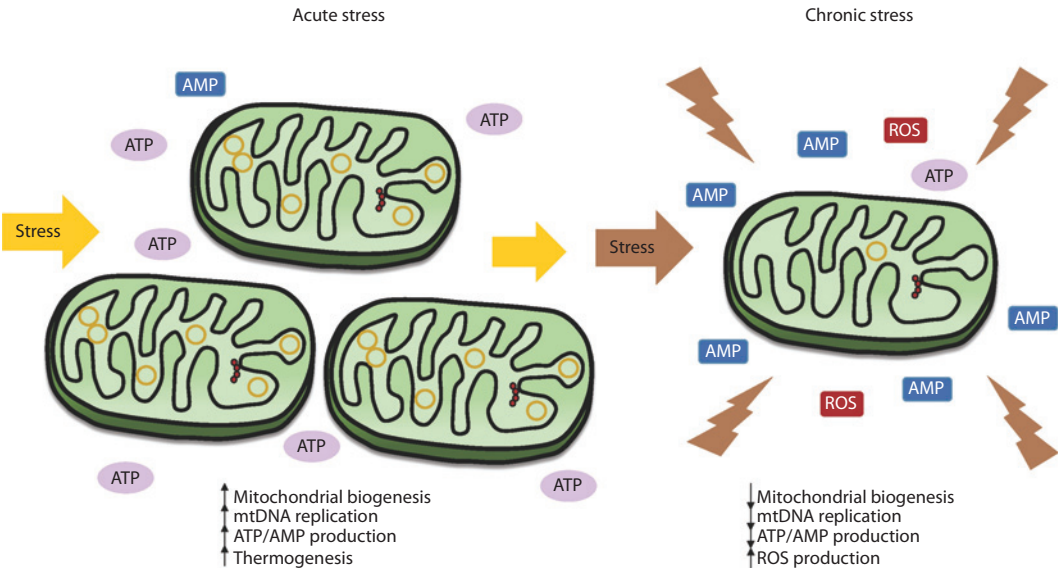

FIGURE 7.6 The mitochondrial stress response under acute or chronic stress. Acute stress is associated with increases in mitochondrial biogenesis and the enzymatic activity of selected subunits of the respiratory chain complexes, to meet the increased energy demands of the cell. Prolonged stress to mitochondrial homeostasis can exceed mitochondrial reserves and lead to abnormally decreased mitochondrial biogenesis, respiratory chain dysfunction, decreased ATP production, increased ROS generation, and mitochondrial DNA damage.

set of nuclear transcription factors and coactivators, including PPAR coactivator 1a (PGC-1a) as the principal member. Increased energetic demands increase the cellular adenosine monophosphate (AMP)/ATP ratio, which is sensed by AMP-activated protein kinase (AMPK). AMPK phosphorylates and activates PGC-1a, inducing the maturation of mitochondria and increasing mtDNA copy number and cristae density [53].

An increase in mtDNA copy number in embryos would be symptomatic of metabolic stress. This stress could be related to intrinsic factors during oocyte maturation or could be in response to impaired respiratory capacities due to mtDNA mutations [53,54].

Oxidative stress is also implicated in the shortening of telomeres that contribute to aneuploidy [53]. Because of the relationships between mtDNA content, implantation, female age, and embryo chromosomal status, mtDNA quantification could represent a new biomarker: the mtDNA copy number in the embryo is an index of energetic stress and thus could be used to predict embryo implantation capacity [23,51,54].

Mitochondria in Embryonic Implantation and Development

An important contributor to embryo viability is an adequate energy supply. This energy is provided by the accumulated mitochondria present in the oocyte, and only in cases of reduced metabolic fuel is there an increased mtDNA copy number [53,54].

Because mtDNA content remains stable during the first days of embryonic development, as shown in the mouse, the total amount of mtDNA must be split among cells during embryo division. On day 6 of development, each embryo cell should contain very few copies of mtDNA [22,54] until mtDNA replication resumes after implantation [55]. Disrupting the mouse gene for mitochondrial transcription factor A (low mitochondrial number in oocytes) does not affect fertilization or early embryonic development because the embryo continues with implantation and gastrulation. However, these embryos die by embryonic day (E) 10.5, indicating that the replication of mitochondria is not initiated until well after implantation [19,53,56]. The ability of embryos to implant suggests that oxidative phosphorylation is not required at this stage, or that the maternal contribution of functional mitochondria is sufficient [56].

During the cleavage and early blastocyst phases the mitochondria exhibit morphological changes. During cleavage (8–16 cells) in mouse, rabbit, and human embryos, mitochondrial geometry varies from spherical to elliptical and the cristae become more numerous. Finally, in the expanded blastocyst stage, particularly trophectodermal cells, mitochondrial organization and morphology are similar to the ones in differentiated cells, with many well-formed cristae [19]. Studies have suggested that mitochondria complete this maturation process after the embryo has undergone the first cellular differentiation into trophectoderm (TE) and inner cell mass (ICM) [23,57].

As described earlier, the TE of blastocysts starts replicating mtDNA first; this is believed to be because of its prior differentiation and loss of pluripotency to form the placenta of the fetus. Thus, the TE accumulates mtDNA to provide sufficient ATP to mediate the process of implantation. In contrast, the ICM maintains its pluripotency until later stages of development, so mtDNA replication begins later in these cells, reducing their mtDNA copy number [23,57].

In the cleavage stage, the oocyte mitochondria are dispersed into blastomeres and there is little, if any, replication of them. There is no significant mtDNA replication between fertilization and the blastocyst stage and the majority of the mtDNA during the cleavage stage is derived from the oocyte [23]. The low number of mtDNA copies in the ICM is maintained until organogenesis, which suggests that mtDNA replication is limited to pluripotent cells [57]. Later, each cell type has specific requirements for ATP production through oxidative phosphorylation. Neurons, cardiomyocytes, and muscle cells, for example, need many mtDNA copies; in contrast, endothelial cells (which utilize glycolysis rather than oxidative phosphorylation) need fewer copies of mtDNA [57].

Currently, there remain many different interactions during the maternal-embryonic transition that need to be understood as the full functionality of the embryonic genome emerges [58]. However, the correct function of mitochondria and the mitochondrial genome during early stages of embryonic development is critical because the essential processes related to metabolism, synthesis, cell division, and

differentiation require significant quantities of energy [23]. New research has demonstrated that high mtDNA copy number in euploid embryos is indicative of lower embryo viability in terms of implantation potential [54], and aneuploid blastocysts contain significantly greater amounts of mtDNA. Further, increases in mtDNA content are associated with embryo loss [51].

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8

Preimplantation Genetic Testing for Monogenic/Single Gene Defects

Ana Cervero, Jose Antonio Martínez-Conejero, Lucía Sanz-Salvador, Claudia Gil-Sanchís, Maribel Sánchez-Piris, and Alan Thornhill

CONTENTS

Introduction

Preimplantation genetic testing (PGT) is an alternative to prenatal diagnosis for those couples with known risk of transmitting a genetic condition to their offspring. Oocytes and/or preimplantation embryos obtained by in vitro fertilization (IVF) are analyzed and only those embryos free of the disorder under study are transferred to the uterus to achieve pregnancy. The main advantages of PGT are (1) to circumvent invasive prenatal diagnosis, which is frequently followed by the difficult decision of pregnancy termination if results are unfavorable, and (2) to try to increase the likelihood of having an unaffected pregnancy by selecting from multiple embryos.

Early research in PGT was pioneered by Edwards and Gardner [1], who, in 1968, biopsied rabbit blastocysts to determine gender. The first successful application of PGT in humans was performed in 1990 by Handyside and colleagues [2], who carried out sexing of embryos by polymerase chain reaction (PCR) to avoid males affected with an X-linked disorder. Gender was determined in single blastomeres by PCR using primers for amplifying Y-chromosome-specific DNA sequences and those embryos identified as female were selectively transferred to the uterus [2]. Later, successful PGT was reported for cystic fibrosis [3], based on the amplification of a DNA fragment containing the causative mutation and its detection by fragment analysis [3].

PGT indications have been expanded and PGT is now performed in laboratories worldwide. The precise number of PGT cycles that have been performed to date can only be estimated. The European Society for Human Reproduction and Embryology (ESHRE) PGD consortium has collected data on

PGT cycles since 1999, showing thousands of PGT cycles performed allowing the birth of hundreds of healthy children. The last such analysis between January and December 2010 reported a total of 5,732 cycles of IVF cycles with PGT for monogenic/single gene defects (PGT-M), for strutural rearrangements (PGT-SR) or for aneuploidies (PGT-A). Of these, 2753 (48%) were carried out for PGT-M and PGT-SR purposes, in which 1,574 cycles were performed for single-gene disorders, including human leucocyte antigen (HLA) typing [4]. Although the ESHRE data represent only a partial record of the PGT cases conducted worldwide, they are indicative of the general trend in the field.

PGT involves a multidisciplinary team and requires close collaboration between the assisted reproduction unit and the genetic laboratory, which can either be co-located within the same institute or geographically separate. In this latter situation, an effective transportation and logistics operation must be in place; IVF treatment (controlled ovarian stimulation, oocyte retrieval and in vitro fertilization, embryo culture, and embryo transfer) is carried out in an assisted reproduction unit and only the biopsied embryonic samples are transported to the genetic unit where the PGT analysis is performed.

Regarding clinical results of PGT, the last data collection carried out for the ESHRE consortium showed that 81% of PGT-M cycles resulted in an embryo transfer. The clinical pregnancy rate obtained for the cycles was 28% per oocyte retrieval and 36% per embryo transfer, giving an overall implantation rate of 27%. Finally, the delivery rate was 24% per oocyte retrieval and 31% per embryo transfer and the miscarriage rate was 10% [4]. However, it is important to note that there may be significant differences in the clinical results between the different IVF centers.

PGT is regulated or even prohibited in many countries based on national or local laws. In many countries, PGT is limited to serious conditions with a high likelihood of transmission. Sometimes, especially for adult onset disorders, HLA matching, or a previously untested disorder, it is necessary to apply to a specific national committee to get the approval for performing the PGT [5].

Indications for PGT-M

PGT-M is recommended when couples are at risk of transmitting a known genetic abnormality to their children and therefore, its indications are similar to conventional prenatal diagnosis. PGT-M has been used mainly to diagnose and prevent well-defined autosomal recessive, autosomal dominant, or X-linked single-gene disorders, although most single-gene disorders that can be diagnosed can be also identified in the embryo. The conditions for which PGT-M has been applied increase annually. Other uses for PGT-M include gender selection, compatible HLA typing (aiming for a "savior sibling"), the identification of hereditary cancers with variable penetrance (e.g., BRCA 1,2 status), and late-onset genetic diseases, all of which have been considered controversial.

Single-Gene Disorders

According to the latest ESHRE PGD consortium data, the most common indications for autosomal recessive disorders are cystic fibrosis, spinal muscular atrophy, and hemoglobinopathies [4], which involve the presence of two mutated copies from each healthy carrier parent.

For the autosomal dominant conditions, one mutated copy of the gene is enough for a person to be affected. Myotonic dystrophy type 1, neurofibromatosis, and Huntington's disease are the most frequently requested indications [4]. In some cases, an affected person inherits the condition from an affected parent. In others, the condition may result from a new mutation in the gene and occur in people with no history of the disorder in their family. When the patient has a de novo mutation it is necessary to identify the molecular variant causing the disease. Once the mutation has been characterized, this variant can be analyzed in the embryo.

X-linked recessive disorders are mainly transmitted by healthy carrier mothers to their sons. In this case, an affected male will not have a risk of affected offspring; all his daughters will be obligate carriers but none of his sons will be affected. PGT for the X-linked disorders is mainly carried out for Duchenne's muscular dystrophy, hemophilia, and fragile X syndrome [4]. Although initially sexing with FISH was widely applied for X-linked disorders in order to select female embryos for transfer [6], specific

diagnosis of the molecular defect has important advantages and has replaced FISH. First, it facilitates the identification and subsequent transfer of those healthy male embryos that by FISH diagnosis would be discarded. Second, female carriers may be identified who can be excluded from transfer or not, according to patient wishes or center policy. Indeed, this is significant for those X-linked dominant disorders (e.g., fragile X syndrome) where it is possible that carrier females may manifest symptoms of the disease.

Cancer Predisposition and Late-Onset Diseases

PGT is also offered to those couples where one partner carriers a mutation predisposing to cancer or other late-onset disease [7]. For cancer predisposition syndromes that are not fully penetrant and for which some form of therapeutic measures may be available, prenatal diagnosis and termination of pregnancy remain controversial, and PGT-M appears as an attractive option, preventing the difficult decision of termination of an established pregnancy. In contrast, the use of PGT for diseases that will not develop until adulthood or for mutations that only confer a heightened risk raises issues of how to weigh the possible benefits of PGT for the future child against the risks of PGT and IVF for the patient. Despite these ethical and legal issues [8], the number of PGT-M cycles reported for this type of condition is increasing [4], and the procedure has already been carried out for several diseases, including the common syndromes of genetic predisposition to colon and breast cancer [7,9].

For some adult-onset conditions (e.g., Huntington's disease, HD), patients do not want to perform the genetic study because they do not want to know their genetic status in advance, but they want to make sure that their children do not have the mutation. Unlike prenatal diagnosis, PGT for HD can keep the status of the carrier of the mutation blinded [10], either via non-disclosure or the less ethically troublesome exclusion tests. In the former, the mutation is analyzed but the results are not revealed to the patient. This approach has several practical and ethical issues. It is essential not to give any details of the IVF cycle in order to avoid a potential clue about the patient´s carrier status. Moreover, a mock transfer could be necessary if there are not any embryos for transfer so that the patients don't guess they are carriers. In the exclusion test, those embryos inheriting the haplotype coming from the affected grandparent are ruled out for the transfer. The drawback in this case is that unaffected embryos could be rejected if the patient is not a carrier.

HLA Matching

Another controversial but relatively well-established indication is HLA matching in which PGT is employed to conceive a child (with or without a specific genetic risk themselves) who may donate compatible cord blood or hematopoietic stem cells for transplantation to save an affected sibling [11]. Hematopoietic stem cell transplantation (HSCT) from an HLA-identical donor is the best therapeutic option for genetic diseases affecting the hematopoietic and/or immune system in children (e.g., β-thalassemia, Fanconi anaemia), and can also be an effective therapeutic option for acquired diseases (e.g., leukemia, acquired medullary aplasia) [12,13]. The frequent unavailability of HLA-identical donors for affected children within the corresponding families or in HSC banks has made the combination of IVF with HLA-typing for the selection of HLA-identical embryos a realistic therapeutic approach for such families.

PGT for HLA typing alone is performed for acquired diseases, such as severe aplastic anemia or leukemias, or can be performed in conjunction with a single-gene disorder, in order to select an embryo free of the inherited condition and HLA-matched to an existing affected child [11–13]. This approach was applied for Fanconi anemia for the first time in 2001 [11] and since then has been performed for a number of different diseases affecting the hematopoietic system. Worldwide, current HLA testing on preimplantation embryos is usually performed using short tandem repeat markers (STRs), since multiple STRs throughout the HLA region allow 100% accuracy HLA typing and detect possible recombination events [14,15].

It should be noted that the clinical results for this indication are worse than those obtained for the others, mainly due to the low number of embryos available for transfer. In the standard case, where PGT-M/HLA testing will be used to select embryos that are both free of a specific disease and an HLA match, the chance of an embryo being both healthy and a suitable match is only 18.75% in the case of autosomal recessive conditions, such as beta thalassemia. The other important limitation is that the majority of patients requesting preimplantation HLA typing are of advanced reproductive age, so the outcome of

the procedure has limited success, with many patients requiring two or more attempts before they become pregnant and deliver an HLA identical offspring. Therefore, patients should have realistic expectations for the overall success of this approach, and should be informed about the possible risks and complications. Since the development of HLA testing, establishing pregnancy, and pregnancy itself are time consuming, the diseased child will sometimes die before the HSCT becomes available [16]. Moreover, as indicated by Kahraman et al. [16], the stem-cell dose obtained from umbilical cord blood is frequently insufficient, and extra time is needed for the child to gain sufficient weight to be able to donate his or her bone marrow cells. All of these limitations might increase the time it will take for the sick sibling to undergo the transplant, aside from the fact that 9 months are required for delivery of a successfully implanted embryo.

Despite ethical objections, including the instrumentalization of the future child (the new child is considered by certain people to be an instrument to cure another child), the results show that this clinical procedure is an option, with documented positive outcomes, for couples with affected children requiring HLA-compatible stem cell transplantation [14–16].

It is important to note that, as with some other PGT indications, the law regulating the PGT for HLA matching depends on the country and, in some countries, HLA-PGT cases have to be approved on a case-by-case basis by a national committee after evaluating the clinical and therapeutic characteristics and weighing carefully the potential risks and benefits to all those involved [5].

Isoimmunization

Hemolytic disease of the newborn, also known as erythroblastosis fetalis, isoimmunization, or blood group incompatibility, occurs when fetal red blood cells, which possess an antigen that the mother lacks, cross the placenta into the maternal circulation, where they stimulate antibody production. ABO incompatibility is the most common cause of hemolytic disease of the newborn, followed by the Rhesus and Kell systems [17].

Although the incidence of severe RhD alloimmunization has decreased with prophylactic anti-D immunoglobulin administration during and after pregnancy, sensitization still occurs in a small group of women. In such women, Rh disease will continue to be a significant problem and for their babies who may be affected. PGT may be utilized to avoid materno-fetal blood group incompatibility in an RhD-sensitized woman. PGT can also be indicated in women who are Rh negative and are highly sensitized with antibodies against Rh factor. If Rh genotyping in the male shows that he is heterozygous, it is feasible to perform a PGT to avoid possible erythroblastosis fetalis and intrauterine blood exchange transfusion [18]. In the same way, PGT can also be used in women sensitized by other blood factors, such as the Kell antigen or other antigens present on the platelet surface.

Diagnostic Methods

PCR and Fragment Analysis

Multiplex PCR using targeted primers designed specifically for the mutation of interest combined with primers for closely linked STR markers has been traditionally the gold standard to perform the PGT-M [15]. During pre-PGT workup, the analysis of polymorphic markers in DNA samples from patients and other relatives identifies which alleles are expected in the embryos, and the specific marker alleles which co-segregate with the mutation. This combined approach improves accuracy, minimizing potential errors caused by undetected allele drop out (ADO) or contamination [19]. ADO refers to the amplification failure (or extreme preferential non-amplification) of one of the two alleles, making a heterozygous locus appear homozygous, and potentially leading to misdiagnosis.

Genotyping of the amplified products can be performed by means of different strategies, with minisequencing the most frequently used method for the detection of point mutations [20]. In the minisequencing technique, a primer extension reaction is performed, allowing rapid and accurate detection of point mutations. The minisequencing primer is designed to anneal one base before the target site, and it can only be elongated with one specific dideoxynucleotide. The four different dideoxynucleotides are labeled with different fluorochromes, and the products can be analyzed on an automated DNA sequencing

system. Other strategies such as amplification refractory mutation system [21], restriction enzyme digestion [22], and real-time PCR [23] have been also applied in PGT. Small deletions and duplications can also be detected by sizing of PCR products from specific regions containing the mutation under study.

The use of multiplex PCR for linkage markers alone (so-called preimplantation genetic haplotyping or PGH) has become widespread in PGT [15,24] and HLA typing [25]. The main advantage is that such protocols can be used for several couples, independent of the mutation they carry, thus saving time and resources in pre-PGT workups. However, the ability to use such indirect testing depends on the availability of appropriate family samples to determine the "at-risk haplotype." In cases where no such samples are available or in de novo mutation cases, it is necessary to identify the disease-causing mutation and analyze it directly in the embryos.

WGA Amplification

The creation of a robust and accurate multiplex protocol requires careful design and optimization and validation before its clinical use. As a result, investment of time and resources is needed. In recent years, the use of whole genome amplification (WGA) has become widespread and has proved to be a practical and efficient alternative to performing PGT [26]. WGA amplifies the entire genome, producing enough amplified DNA for multiple downstream applications. Many different standard PCR assays may be performed for haplotyping and the direct analysis of mutations in the case of monogenic diseases, avoiding the necessity of optimizing of multiplex PCR protocols [27]. Moreover, WGA allows combining the PGT-M or HLA typing with array comparative genomic hybridization (aCGH) or next-generation sequencing (NGS) for the detection of chromosomal imbalances using the same sample with the aim of improving clinical PGT-M results [28]. Finally, WGA facilitates repeat testing of samples for use in proficiency testing, validation, or in the event of run failure.

Despite the large quantities of amplified DNA produced, it is well established that WGA methods yield relatively high ADO rates overall when a single blastomere is biopsied [29]. This problem can be circumvented with the application of enough linked markers to avoid misdiagnosis and/or reduced with the use of trophectoderm biopsies instead of single cells because the former provides multiple cells in the biopsy sample, which is known to result in lower ADO rates [28].

Karyomapping

Karyomapping was recently developed and commercialized, providing a comprehensive, robust, off-the-shelf method for linkage-based diagnosis of almost any single-gene disorder [30]. Karyomapping uses a high-density single-nucleotide polymorphism (SNP) array to accurately identify DNA haplotypes in samples even when the starting DNA is minimal. By genotyping the parents at several hundred thousand SNP sites throughout the genome, a dense set of informative SNP markers are identified for each of the four parental chromosomes [30]. The phase of the alleles for each informative SNP locus along each chromosome and linkage of the risk alleles with the parental chromosomes can then be established by reference to the genotype of a relative of known disease status. The parental origin of each chromosome in the embryo is then ascertained by comparison with the genotype of the reference [30]. The principal advantage of this platform is that it is applicable to almost any familial single-gene disorder, or any combination of loci, within the chromosome regions covered by informative SNP loci, eliminating the need for developing patient- or disease-specific tests [30]. The main disadvantages of the karyomapping approach are that diagnosis is challenging when insufficient informative SNP markers are available (e.g., in some telomeric genes) or when pseudogenes are involved. Moreover, it cannot be used on its own for de novo mutation cases or when other tested family members are not available to provide samples or are not informative owing to recombination [30]. In such cases, direct mutation testing from at least one embryo is necessary to establish phase [31].

Currently, it is possible to determine both monogenic diagnosis and aneuploidy detection (plus HLA haplotyping) by PGT using the same WGA product [32]. For this reason, a single assay using the same platform to detect both chromosomal and monogenic disorders simultaneously is desirable. Karyomapping is such a method because it defines unique sets of SNP markers for each of the four parental chromosomes [31], allowing

accurate identification of the region of interest containing the mutation and simultaneous high-resolution molecular cytogenetic analysis. Meiotic trisomies can be identified by the presence of both haplotypes from one parent in segments of the chromosome, resulting from the inheritance of two chromosomes with different patterns of recombination, in combination with a single haplotype from the other parent. Moreover, monosomies or deletions can be identified by the absence of one of the parental haplotypes [30,31].

Several studies have reported the clinical use of karyomapping, showing the simultaneous detection of monogenic and chromosomal disorders [33,34]. However, this approach has several limitations (as described above) that need to be overcome as more carrier screening tests are performed that identify co-carriage of mutations in the same gene among couples with no family history or affected children—the historic source of referrals for PGT-M. At present karyomapping is not commercially validated for aneuplody screening and does not readily detect mitotic trisomies or simple copy number variation. Nonetheless, use of karyomapping, for the most part, represents a significant advance over the current gold standard for PGT and will be a powerful tool to investigate parental origin and phase of origin of meiotic chromosome errors.

Next-Generation Sequencing

NGS provides high throughput and base pair resolution data, providing the analysis of multiple genetic loci and samples from different couples simultaneously. Moreover, NGS, as with karyomapping above, allows the combined evaluation of aneuploidy and single gene disorders from the same biopsy using a single platform.

Several studies have been published showing the possibility of using NGS to test single cells [35,36]. In 2013, Treff et al. published a specific protocol to test DNA from a trophectoderm biopsy with NGS that was consistent with two conventional methodologies of PGT-M [37]. However, the major concern relating to NGS technology is that an insufficient sequencing depth may result in a false positive or failure to identify a mutation (false negative) due to the presence of sequencing artifacts and ADO, respectively. Moreover, NGS has technical limitations in testing for dynamic mutations. Therefore, further studies are needed to evaluate this technology before its routine clinical use in PGT-M.

Combined PGT-M and PGT-A

Extensive evidence has revealed a high incidence of chromosomal abnormalities in human embryos obtained by assisted reproduction techniques, leading to miscarriages and implantation failures [38,39]. Couples presenting for PGT to avoid transmission of single-gene disorders are not without these same problems despite the fact that they are often categorized as “fertile.” For this reason, they can also benefit from the simultaneous analysis of the disease and the presence of aneuploidies, selecting for transfer those PGT “unaffected” and simultaneously euploid embryos.

Several studies have shown significant improvement in rates of pregnancy and live births following testing for aneuploidy in patients undergoing IVF for infertility [39–41]. The use of WGA product provides a straightforward solution for performing any required test in the same biopsy material, allowing the simultaneous analysis of PGT-M and PGT-A. Using this approach, several studies have reported the clinical use of simultaneous detection of monogenic and chromosomal disorders using different technologies [28,33,37,42]. One of the first articles was published by Rechitsky et al. where the simultaneous detection of cytogenetic disorders and cystic fibrosis was described [28]. In 2015, the first systematic study of PGT-M combined with aneuploidy screening was published, demonstrating an increase in the pregnancy rate from 45.4% in the conventional PGT-M to 68.5% with combined aneuploidy screening and threefold miscarried reduction (5.5% vs. 15%) [42].

Our data indicate that 43.6% of the embryos diagnosed at the blastocyst stage as normal for the genetic disease have some chromosome abnormalities that can give rise to miscarriage or implantation failure [43]. Specifically, 16.3% of those PGT-M normal embryos exhibited trisomies or monosomy X that could lead to miscarriage if transferred. A further 20.2% of PGT-M normal embryos were carrying some monosomies that could result in implantation failure. Therefore, these results, in line with other studies, indicate that the combination of accurate PGT-M and detection of chromosome aneuploidy may improve implantation and rates of healthy live births.

Limitations and Barriers to Access

Not everyone who wishes to have PGT-M can have it. It is essential that the disorder that is intended to be diagnosed has a comprehensive and accurate genetic characterization to at least identify the gene responsible for the condition. Some conditions, including autism and some immunological disorders, where the causes remain unidentified, are not suitable for PGT-M.

As PGT-M involves both an IVF procedure and a selection procedure based on genetic testing, it is extremely important to predict the number of unaffected embryos expected for transfer prior to starting the procedure. This number will depend on the embryo quality and the theoretical risk according to the genetic disorder (e.g., recessive, dominant, sex-linked). The embryo's potential to implant depends mainly on the woman's age and the absence of additional factors that facilitate the production of incompetent gametes in men or women. It is well known that chromosome aneuploidy is the major cause of IVF failure and miscarriage. To benefit patients, a sufficient number of embryos should be analyzed to obtain non-affected embryos for transfer. Recent advances in vitrification procedures have made it feasible to batch oocytes or embryos to reach a minimum number of embryos for analysis [44]. Table 8.1 shows the rates of transferable embryos considering the inheritance patterns and the potential presence of aneuploidies.

One final significant point regarding PGT-M concerns patient and clinician expectations. PGT-M provides no guarantee of a completely healthy baby. Rather, PGT-M minimizes the risk for the disease the couple has a high risk of transmitting to their offspring. However, there remains a small risk of misdiagnosis due to mosaicism or technical limitations, and therefore couples should always be offered the possibility of prenatal diagnosis to confirm results. Moreover, couples sometimes assume that they will have a normal healthy child following successful PGT for a specific disorder, forgetting that a more cryptic disorder may be present, which was not what was causing the disease identified previously in the family. Furthermore, no other genes or mutations even within the same gene are routinely analyzed and, if chromosomes are not analyzed, an aneuploid but genetically unaffected embryo may be transferred. The entire procedure should always be explained in detail before the treatment to ensure that couples are informed about the potential risks and limitations.

Conclusions

PGT-M can be used to screen embryos for almost any kind of genetic disorder in which the genetic cause is characterized, increasing the number of the indications and the total number of PGT-M cases year by year. With improving technologies, best practice guidelines, and the adoption of external quality assessment programs and laboratory accreditation [45–47], PGT-M analysis has reached a high level of accuracy and has enabled the possibility of performing multiple diagnoses from the same sample. Diagnosis of a monogenic disease can be combined with HLA typing and/or with the detection of chromosomal abnormalities, allowing the process to improve reproductive outcomes (see Figure 8.1). The explosion of preconception

TABLE 8.1

Rates of Transferable Embryos Considering the Inheritance Patterns and the Potential Presence of Aneuploidies

Indication	Unaffected Embryos	PGS Normal Embryos[a]	Transferable Embryos
Autosomal dominant	1/2	1/2	1/4
Autosomal recessive	3/4	1/2	3/8
X-linked recessive	3/4	1/2	3/8
X-linked dominant	1/2	1/2	1/4
HLA matching	1/4	1/2	1/8
HLA + AD	1/8	1/2	1/16
HLA + AR	3/16	1/2	3/32
HLA + X-linked	3/16	1/2	3/32

[a] According to Igenomix internal data, on average, 50% of blastocysts are expected to be PGT-A abnormal.

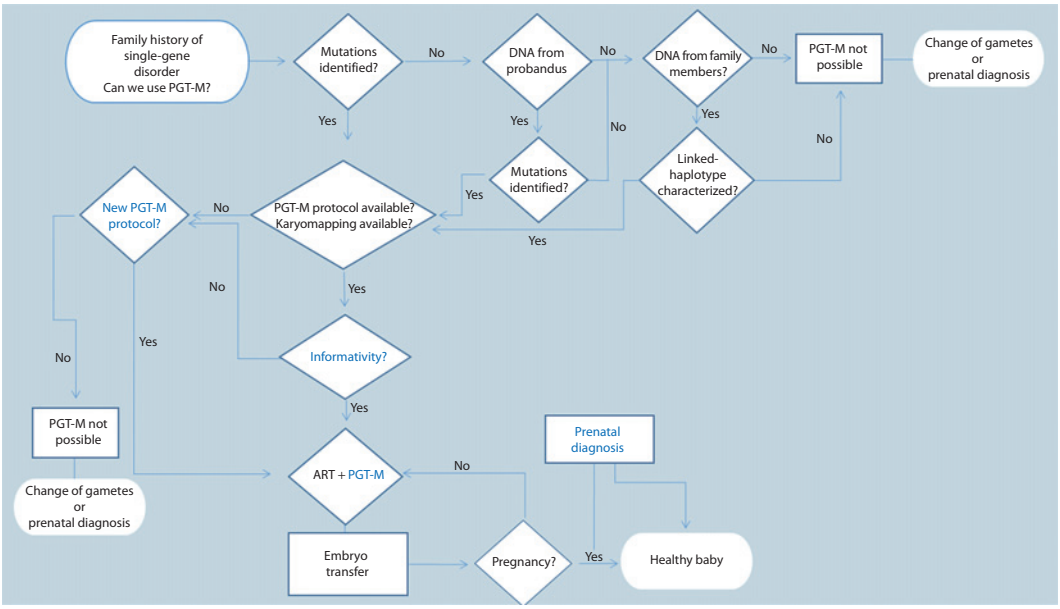

FIGURE 8.1 Algorithm for PGT-M. PGT-M, preimplantation gentic testing for monogenic/single gene defects; ART, assited reproductive technology.

carrier screening for couples with no family history of specific genetic disease as a result of best practice guidelines, reduced cost, and improved access to pan-ethnic expanded carrier screening panels as well as government funding for PGT in many countries is certain to increase further the number of PGT-M cases.

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9

Molecular Diagnosis of Endometrial Receptivity

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CONTENTS

Introduction

Endometrial receptivity is the period of time during which the uterine lining is receptive to implantation of a fertilized embryo. Determining this window of implantation (WOI) in a patient is necessary to synchronize embryo transfer during the optimal receptive period, a strategy known as personalized embryo transfer (pET).

The concept of endometrial receptivity and the existence of a WOI for the implantation of human embryos was first suggested by Hertig and Rock in 1956 [1]. Then, in the 1990s, the clinical WOI was demonstrated, using ovum donation, as the limited period of time during which the embryo must be transferred back to the mother [2]. Further work by Wilcox et al. in 1999 [3] determined that the human embryo implants 8 to 10 days after ovulation. The methods they used to determine ovulation were never officially adopted; however, the clinical community has accepted their assertion that the endometrium in all patients becomes receptive during that time. In addition, implantation has been believed to be equally successful over these 3 days, regardless of individual variations or hormonal treatment received (this is observed to occur within natural cycles, controlled ovarian stimulation, and hormonal replacement cycles).

A fair number of methods have been proposed for classifying endometrial status. The Noyes criteria [4], based on the histological features of the different compartments of the endometrium across the menstrual cycle, reflect the differentiation of the endometrium each day of the luteal phase. However, the accuracy and functional relevance of these criteria as a predictor of endometrial receptivity have been questioned in randomized studies [5,6], and they are no longer in clinical use. Several single markers have been suggested as predictors of receptivity, though none has demonstrated consistent clinical applicability [7]. More recently, the status of human endometrium has been more objectively classified by using transcriptomic profiling throughout the menstrual cycle [8,9], as well as during the window

of receptivity [10]. These pioneering diagnostic techniques, in conjunction with accumulated evidence that the endometrial molecular profile is unique during the WOI, prompted us to translate the molecular expression profile of the endometrium as it relates to endometrial function using transcriptomics.

Endometrial Receptivity Analysis (ERA)

Our group identified the transcriptomic signature of endometrial receptivity, characterized by the expression of 238 genes unique to the WOI. This led to the creation of the endometrial receptivity analysis (ERA). ERA is now performed using next-generation sequencing (NGS) coupled with a computational predictor and an algorithm able to identify the receptivity of an endometrial sample. The assay then provides the personalized WOI (pWOI) of a patient independent of endometrial histology.

To perform ERA, messenger RNA (mRNA) is extracted from an endometrial biopsy, analyzed by NGS, and evaluated by a computational predictor. The ERA predictor classifies the sample as receptive or non-receptive (this can be pre-receptive or post-receptive). A non-receptive result reflects the displacement of the WOI since the endometrium may require different timing of progesterone (P) administration than the standard 5 days to reach receptivity. A displaced WOI can be confirmed by analyzing a second endometrial biopsy performed during the subsequent cycle at the specific day suggested by the first ERA result.

Standard Protocol to Obtain the Endometrial Sample

To perform an ERA test, a small endometrial biopsy must be taken from the uterine fundus using a pipelle catheter (Cornier Devices, CCD Laboratories, Paris, France) or similar as a routine procedure. This requires 30–50 mg or approximately 1/5 of the pipelle. If the inside of the uterus is not accessible with the pipelle, the biopsy can be taken with the same transfer catheter through syringe aspiration.

Based on personal and/or clinical reasons, the endometrial biopsy can be obtained during either a natural or hormone replacement therapy (HRT) cycle. If it is collected during a natural cycle, for the sake of consistency, it is recommended that ovulation be triggered by hCG once the follicle reaches 18 mm (hCG+0), and then the endometrial biopsy should be taken 7 days later on hCG+7 (Figure 9.1a).

It is recommended that a dose of 400 mg/day of progesterone (P) be administered in two doses of 200 mg, one in the morning and one in the afternoon. If the biopsy is taken at hCG+7 in the afternoon, the administration of P should start at hCG+2 at night; if the biopsy is taken at hCG+7 in the morning, the administration of P should start at hCG+2 in the morning.

An HRT cycle is the preferred choice due to its simplicity, consistency, and low cost involving hormonal treatment with estrogen and progesterone. The classic endometrium build-up preparation protocol begins with estradiol valerate at a dose of 6 mg/day or estradiol hemihydrate patches delivering 150 μg every 48 hours between the first and third day of menstruation if an ultrasound reveals ovaries without functional follicles. Between days 7 and 10 of HRT priming, if ultrasound assessment reveals a trilaminar endometrium >6.5 mm and endogenous progesterone <1 ng/ml, progesterone administration is begun. Vaginal micronized progesterone (or similar) should be administrated at a dose of 400 mg/12 h for 5 complete days (120 h). The day on which the progesterone treatment begins is referred to as P+0 and the biopsy must be taken on day P+5, 5 days after progesterone administration or after approximately 120 +/– 3 hours (Figure 9.1b).

The endometrial biopsy must be transferred immediately to a cryotube that contains 1.5 mL of RNA-later (Sigma-Aldrich, St. Louis, MO), a solution that keeps RNA from degrading during shipment to the laboratory. The recommended amount of endometrial tissue is approximately 30–50 mg, which is equal to 1/3 of the volume of the cryotube and must be shaken vigorously for a few seconds so that the RNA-later fully penetrates the sample. The cryotube containing the sample must be kept inside a refrigerator (never a freezer) at 4°C for at least 4 h until shipment, which can be at room temperature (<35°C).

The reproducibility of this test has been demonstrated using second endometrial biopsies obtained from the same women under the same conditions as in the first study cycle. These second biopsies are obtained in a subsequent cycle between 29 and 40 months after the first [11] and show no variations between cycles. Furthermore, several patients have undergone successful embryo transfers consistently

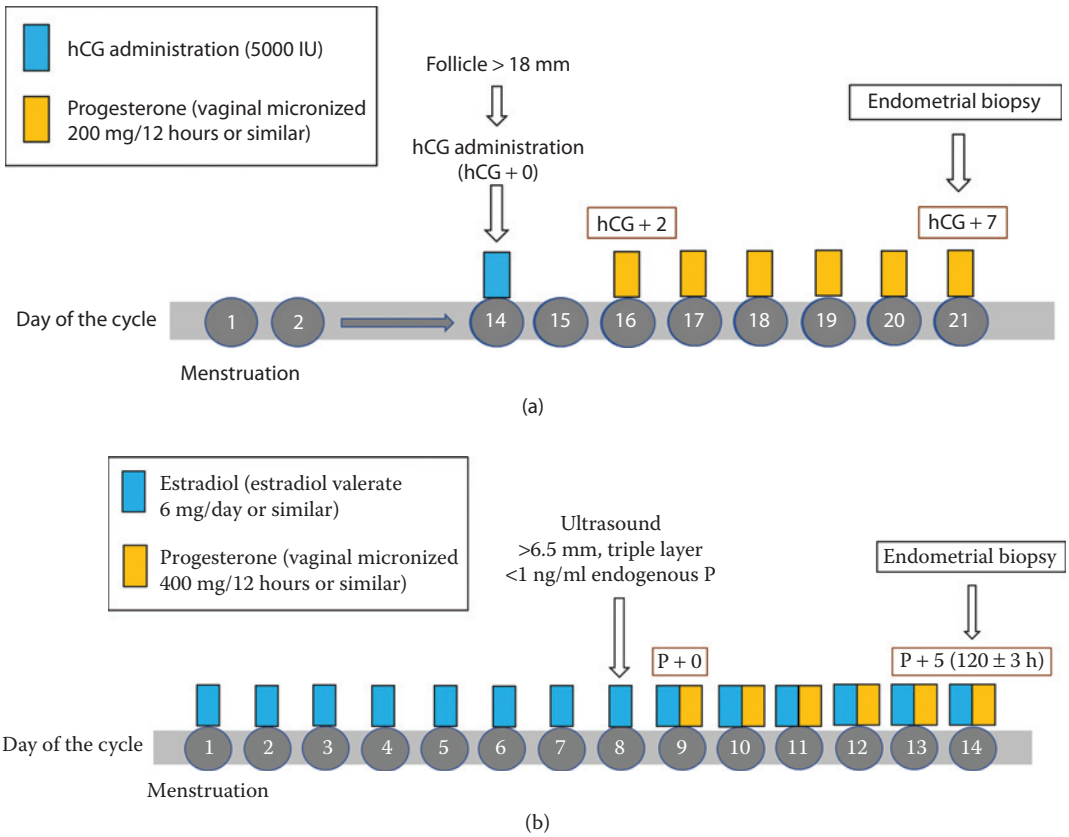

FIGURE 9.1 (a) The endometrial sample for an ERA test in a natural cycle must be taken 7 days after hCG injection. (b) The endometrial sample for an ERA test in an HRT cycle must be obtained after 5 days (120 h) of progesterone administration.

in the same pWOI that was detected up to 2 years prior and resulted in a second live birth, supporting the idea that ERA can be used successfully in reproductive medicine to assess the endometrium by its transcriptomic signature and that the pWOI is maintained over the course of several years.

Interpretation of ERA Results

1. *Receptive:* A receptive endometrial profile is divided into three sub-signatures: optimal receptive, early receptive, and late receptive.

   - An optimal receptive profile indicates an optimally receptive endometrium. In this case, it is recommended to proceed with the embryo transfer in the same type of cycle and on the same day in which the endometrial biopsy was performed.
   - An early receptive profile indicates that the endometrium is entering the receptive phase but needs an additional 12 h of P administration in an HRT cycle to acquire an optimally receptive profile.
   - A late receptive profile indicates that P administration should be reduced by 12 h in a further cycle to achieve optimal receptivity.

The early and late receptive profiles are considered transitional profiles and it is recommended that personalized embryo transfer be performed after following the indicated treatment with P (+/–12 h) without need of further verification.

2. *Non-receptive:* Our algorithm revealed that the gene expression profile in a non-receptive endometrium is usually due to a physiological displacement of the WOI. In addition to a proliferative profile, which generally indicates that the endometrium has not been exposed to endogenous or exogenous progesterone, a non-receptive patient can also show a pre-receptive or a post-receptive transcriptomic profile.
   - A pre-receptive diagnosis indicates that the transcriptional activation necessary to achieve receptivity has not yet occurred. The patient needs 1 or 2 more days of progesterone administration from the day of cycle in which the biopsy was taken to reach the receptive state.
   - A post-receptive diagnosis indicates that the endometrium has already passed the ideal window for embryo implantation in the day of the cycle when the biospy was performed, so 1 or 2 days less of P administration is required to achieve receptive status.

Whether to take a new endometrial biopsy following the P timing indicated by the ERA report will vary if it is necessary to validate the displacement and to guide the pET. The embryo at the blastocyst stage must be transferred under exactly the same conditions as when the receptive result was obtained, that is, under the same type of cycle conditions and on the same day in which the biopsy was performed.

Figure 9.2 shows the algorithm followed to identify the endometrial receptivity by the ERA test.

Clinical Data

The original design of the ERA test was based on microarray data analyzing 238 genes related to endometrial receptivity [12]. Following the accumulation of data after 6 years from the analysis of more than 20,000 transcriptomic profiles, algorithms have been developed to provide a new computational predictor based on NGS technology. The new ERA predictor defines a shorter, optimal WOI frame. To define this receptivity signature, the training of the new predictor was performed by selecting well-defined and curated endometrial profiles. Only receptive profiles from patients who were receptive and became pregnant in this cycle were used. For the non-receptive stages, training was performed using only samples in which receptivity was reached after following the specific recommendation associated with that profile. This technique has been refined and improved such that the predictor potency provides more detailed

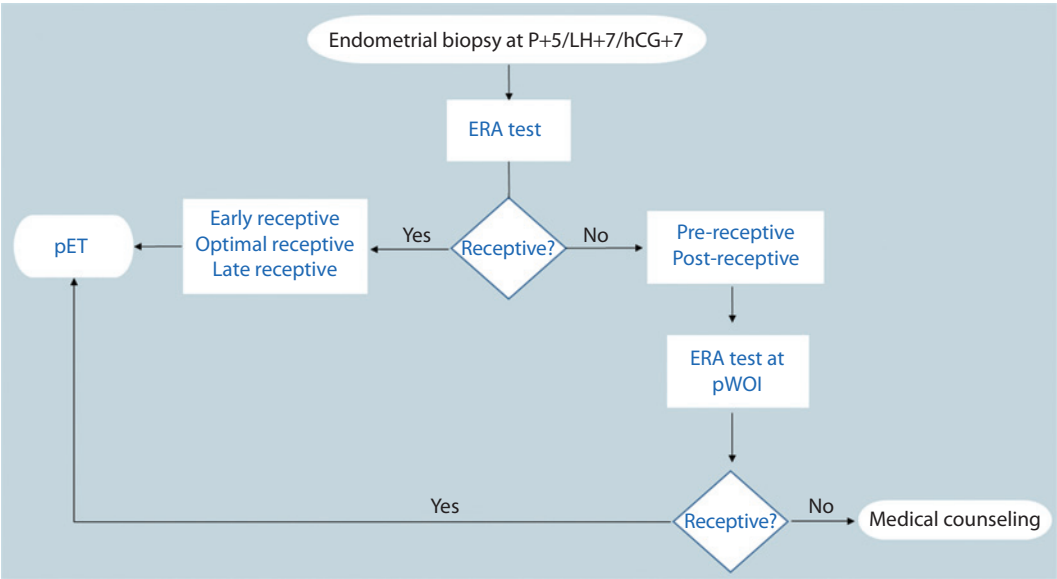

FIGURE 9.2 Algorithm to identify the receptivity of an endometrial sample. ERA, endometrial receptivity analysis; pET, personalized embryo transfer; pWOI, personalized window of implantation.

TABLE 9.1

Clinical Outcome and Efficiency of Embryo Transfer According to ERA Status

Clinical Outcome	NR (52)	R (205)
IR first attempt	13% (12/90)	45% (161/355)
IR total attempts	10% (17/174)	41% (182/441)
PR first attempt	23% (12/52)	60% (123/205)
PR total attempts	17% (17/100)	55% (140/253)
OPR first attempt	0% (0/12)	74% (91/123)
OPR total attempts	0% (0/100)	74% (103/140)
Clinical efficiency	Positive (52)	Negative (205)
True	40	123
False	12	82
Sensitivity (TP/TP+FN)	0.33	
Specificity (TN/TN+FP)	0.91	
PPV (TP/TP+FP)	0.77	
NPV (TN/TN+FN)	0.60	

Note: IR, implantation rate; NPV, negative predictive value; NR, non-receptive; OPR, ongoing pregnancy rate; PPV, positive predictive value; PR, pregnancy rate; R, receptive.

insights into the use of gene signature profiles for patient stratification. In fact, we have observed a logical evolution between receptive and non-receptive profiles from 71.7% to 56% of cycles, taking into account the transition profiles in patients undergoing HRT.

The clinical efficiency of pET has also been assessed according to its specificity and sensitivity. Following a similar protocol as the pilot study [13], the clinical outcome of pET was analyzed in a group of 205 receptive patients and compared to frozen embryo transfer (FET) on a day after the determination of non-receptive status in 52 patients. Differences in implantation rate (IR) and pregnancy rate (PR) between both groups was highly similar to those found in previous research, obtaining a 23% PR and 13% IR in FET versus 60% and 45% when pET was performed. To calculate specificity and sensitivity, the "positive" condition was considered to be non-receptive and the "negative" to be receptive; pregnancy achievement was the gold standard. After proper analysis, a specificity of 0.91 and a sensitivity of 0.33 were obtained due to the multifactorial character of the implantation process. The positive predictive value obtained was 0.77 while the negative predictive value was 0.60 [14]. Closer clinical outcome results were obtained after increasing the number of patients to 400 receptive and 100 non-receptive cases: 20% PR and 12% IR was observed in FET versus 58% and 45% when pET was performed. Data obtained from this study are shown in Table 9.1.

The Assessment of the Endometrial Factor under Different Conditions

Recurrent Implantation Failure

Repeated implantation failure (RIF) in otherwise healthy women presents an intriguing clinical quandary in reproductive medicine that remains poorly characterized [15,16]. The direct consequence of our inability to understand the etiology of RIF has led to the introduction of numerous empirical, and thus far ineffective, adjuvant interventions that are costly, inefficient, and frustrating for our patients.

Various definitions of RIF exist, but one expert proposed that pathologic implantation failure be defined as the failure of three IVF cycles in which one or two high-grade quality embryos were transferred to the patient in each cycle [16], or after two failures in oocyte donor recipients.

For academic purposes, the causes of RIF can be grouped into several categories, the first of which includes pathological alterations of the endometrial cavity such as hyperplasia, submucous myomas or endometrial polyps, endometritis, and synechiae (which can be found in 18%–27% of cases) [17]. Other

categories include hydrosalpinx [18], either acting through a direct embryo-toxic effect or adversely affecting endometrial receptivity [19], an increased incidence of embryonic chromosomal abnormalities [20,21], obesity [22], and lifestyle or other factors such as hereditary or acquired thrombophilias [23]. An immunological factor has been used unsuccessfully to explain and treat this condition [24].

In clinical practice, if diagnosed, all the pathological issues indicated above can and must be corrected to obtain a successful pregnancy. Ultimately, we will face an intriguing situation involving two necessary collaborators: the embryo and the endometrium. It is obviously critical to ensure the adequacy of the embryo and endometrium individually, but it is of paramount importance to determine the ideal timing and synchronization of their pairing. Timing is everything in life: the first major milestone is at fertilization and the second at conception [25].

The initial proof of concept that RIF is not an endometrial dysfunction that will stigmatize a patient forever, but rather a desynchronization between embryo and endometrium, was presented in a prospective study demonstrating that the WOI was displaced in 25.9% of RIF patients versus 12% in control non-RIF patients [26]. The identification of the personalized WOI (pWOI) in RIF patients has led to a new and interesting finding: one in four RIF patients has a displaced or asynchronous WOI and our computational predictor classifies them as non-receptive endometrium that is either pre- (84%) or post-receptive (16%), which is further verified by a second ERA test. Taking this forward, we translate these genomic results to the clinic by transferring embryos according to the pWOI of the patient, providing a personalized embryo transfer (pET) resulting in 50.0% PR and 38.5% IR, similar to controls. These results suggest that rescue of non-receptive RIF patients by pET results in normalized pregnancy and implantation rates [26]. This initial study has been further validated by the report of a clinical case of successful personalized embryo transfer after seven previous IVF failed attempts (four with her own oocytes and three with oocyte donation) [13]. This case report was complemented by a pilot study of 17 patients undergoing oocyte donation who suffered multiple failed implantations with routine embryo transfer but were subsequently treated with pET after determining their pWOI, resulting in normalization of their reproductive outcome [13]. Given these results, we must pose the question of whether RIF of endometrial origin is a "disease" or simply results from our inadequate timing of ET when the individual woman's endometrium is receptive.

Obesity

Despite the fact that many obese women (BMI >30 kg/m^2) conceive naturally, several implications concerning reproduction have been recently published. Rich-Edwards [27] reported that the risk of suffering from anovulation in obese woman is three times more than in non-obese woman. Thus, the time to conception is twofold longer in overweight women (BMI >25 kg/m^2) even if they are ovulatory [28]. Several publications have stated this public health concern has implications for assisted reproduction and affirmed that implantation, clinical pregnancy, and live birth rates are lower in obese woman, whereas this trait improves miscarriage rates [29–32].

Nevertheless, it has not been determined if the impact of obesity in negative pregnancy outcomes is due to factors that affect the endometrium, the conceptus, or both. While some early publications defend the notion that BMI does not exert a negative effect on endometrial receptivity in donor oocyte recipients or in blastocyst or day 3 embryo transfers [33–35], the trend is to identify a reduction in implantation rates among obese women [36–38]. However, the molecular mechanism connecting obesity to reduced fertility remains poorly understood.

To evaluate how increased BMI could affect the receptive profile, our group carried out a prospective cohort study using ERA to determine the incidence of non-receptive endometrium in an overweight/obese population. Ninety-one infertile patients included in all BMI categories according to the WHO obesity classification system [39] underwent an endometrial biopsy at P+5 in HRT cycles. Although not statistically significant, it was observed that the incidence of a non-receptive endometrium was higher in the obese and morbidly obese patients (22.5% and 37%, respectively) compared to normal-weight and overweight patients (9.1% and 7.7%, respectively). In addition, a significant endometrial gene expression alteration during the optimal WOI in obese subjects was recently reported, highlighting the idea that energetic metabolism is also important in determining the WOI and that obesity poses an increased risk

of a displaced WOI [40]. Since overweight patients seem to present more complications before achieving implantation success, ERA is indicated at their first fertility clinic appointment.

Endometriosis

Endometriosis is an estrogen-dependent disorder affecting an estimated 10% of women of reproductive age [41], and the prevalence in women experiencing pain or infertility reaches up to 50% [42]. Endometriosis is defined by the presence of endometrial tissue outside the uterus; symptoms may range from practically none to chronic pelvic pain, dysmenorrhea, and cyclic urinary or bowel complaints. It has an impact on women's physical, mental, and social well-being and has been commonly related to infertility even though the relationship between the two is controversial.

The effects of endometriosis on ovarian reserve and the quality of retrieved oocytes seems obvious. However, lower implantation rates raise the question of whether this is due to poor embryo quality or number or whether it compromises endometrial receptivity as well.

Aiming to elucidate the main cause of compromised reproductive outcomes in endometriosis patients, several studies based on oocyte donation programs have been carried out using two different approaches: by a prospective study splitting oocytes from the same donor between patients with and without endometriosis [43] and by comparing indications for oocyte donation in a retrospective study of oocyte donation cycles with discordant outcomes [44,45]. Our group has also analyzed the origin of donated oocytes comparing women who received fresh oocytes from healthy women with those receiving oocytes from women with endometriosis [46,47].

Several studies have reported a huge number of endometrial markers in women with endometriosis [41,48–51] without elucidating if it is caused by endometriosis or by a molecular epigenetic phenomenon. Furthermore, altered endometrial gene expression in patients with endometriosis has been related to impaired embryo implantation [51,52]. Nevertheless, several publications have demonstrated that endometriosis is not detrimental to embryo implantation in ovum recipients [43,53].

A prospective functional study of the transcriptomic signature of endometrial gene expression during the WOI in endometriosis and healthy patients was conducted using ERA [54]. Non-differentially expressed genes (DEG) were found among the different endometriosis stages (minimum, mild, moderate, and severe). Furthermore, clustering analysis shows that gene expression was linked more closely to the day when the biopsy was performed than to the stage of endometriosis. Interestingly, only 13 DEG were found in women with and without endometriosis on day 18 compared to days 19–20 of the cycle, indicating that, according to ERA diagnosis, the transcriptomic signature during the WOI is similar in infertile patients regardless of whether endometriosis is present.

After years of experience and research in which several implicated factors have been analyzed, we conclude that the primary limiting factor of fertility in women with endometriosis is the oocyte itself, although a receptive endometrium is also required. Evidence shows that in patients with endometriosis, healthy donated oocytes contribute to pregnancy with similar chances as a healthy woman. However, endometriotic oocytes have poorer reproductive outcomes even in a non-endometriotic endometrium.

ERA in the Diagnostic Work-Up of the Infertile Couple

An international randomized controlled study is under way to perform endometrial assessment during fertility screening at the beginning of reproductive care (The ERA Test as a Diagnostic Guide for Personalized Embryo Transfer; ClinicalTrials.gov Identifier NCT01954758). Interim results were published in the American Society of Reproductive Medicine (ASRM) 2016 Scientific Congress [55]. Results show that 14% of patients have a displaced WOI whose correction would likely result in an effective cost-benefit strategy at the first clinical appointment. The study consists of three arms comparing fresh embryo transfer under stimulation protocol, frozen embryo transfer at P+5 in HRT cycles, and pET guided by ERA with frozen embryos in HRT cycles. At the midpoint of recruitment, results show significant differences between PR for pET arm (85.7%) versus fresh ET (61.7%) and deferred ET (60.8%).

Although not yet significant, there are also differences in IR (47.8% for pET, 35.3% for fresh ET, and 41.4% for deferred ET) and in ongoing pregnancy rate per ET (55.1% for pET, 43.3% for fresh ET, and 44.6% for deferred ET).

Limitations

Transcriptomic analysis uses mRNA, a highly sensitive genetic material that can be degraded if strict considerations are not applied (ranges of temperature for shipment and storage, use of RNAse inhibitors, and sterile conditions among others). Furthermore, endometrial biopsies could present difficulties during collection. For these reasons, 5% of the samples received are not suitable to process because it is not possible to obtain enough RNA or it is degraded. Currently, our group is validating a non-invasive test to make it easier for clinicians to obtain samples and avoid unnecessary pain caused to the patients when the biopsy is taken by endometrial aspiration.

An ERA test involves replicating the type of cycle in which the subsequent embryo transfer is going to be performed. Since ERA has only been tested during HRT and natural cycles, it is not possible to extrapolate to controlled ovarian stimulation cycles. The protocol currently limits embryo transfer to the same cycle in which the biopsy is taken and it is only possible to perform pET with frozen embryos from the same patient or fresh embryos in ovum donation cycles.

ERA assessment only accounts for the assessment of the endometrium at the transcriptional level. However, other possible changes may be present, such as an altered uterine microbiome, which may impair the clinical results of an otherwise receptive endometrium. Not only synchrony, but also a chromosomally normal embryo, is needed for successful implantation.

Conclusions

Synchrony between the major players in the implantation process has been proved to be one of the critical factors contributing to the success of assisted reproductive treatment. The embryo must be viable, but even with chromosomally normal embryos, human embryo implantation has a low level of efficiency, reaching only 50%–65% implantation in a variety of endometrial thicknesses and patterns [56]. The maternal endometrium is clearly an important limiting factor and should be incorporated into the calculus of assisted reproductive treatments.

The ERA test covers a relevant gap in the evaluation of infertile couples that has previously not been addressed. Currently, 600 clinics from 50 different countries worldwide use ERA for the evaluation of endometrial receptivity in their patients. The personalized WOI of more than 20,000 patients have been diagnosed since the first test was performed in 2010. Now, we face the challenges of assessing the cost effectiveness of this diagnostic test as a routine checkpoint during infertility work-ups and the transition to a non-invasive ERA test that analyzes endometrial fluid in the same cycle in which the embryo transfer is performed.

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10

Chromosome Abnormalities in Human Pregnancy Wastage: A Review of Cytogenetic and Molecular Analyses

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CONTENTS

Introduction

Pregnancy loss occurs with astonishing frequency in our species. From studies involving couples attending assisted reproduction facilities, it is clear that a large proportion of conceptuses perish in the earliest stages of pregnancy. However, even among those pregnancies that survive to the time of clinical recognition (about 6–8 weeks gestation), at least 15%–20% terminate as spontaneous abortions (SABs). In this review we focus on the contribution of chromosome abnormalities to SABs, summarizing methodologies used to detect them and factors contributing to their occurrence.

Analysis of Spontaneous Abortions: Technical Considerations

Collection of Tissue Samples

Tissue samples from spontaneous abortions must be of inner cell mass or fetally derived extra embryonic origin to be of use in assessing the reason for the abortion. Indeed, as discussed in more detail below, contaminating maternal tissue is one of the most common, yet underappreciated, reasons for failure to identify the cause of the pregnancy loss (Figure 10.1a). The ideal tissue sample is a complete conceptus

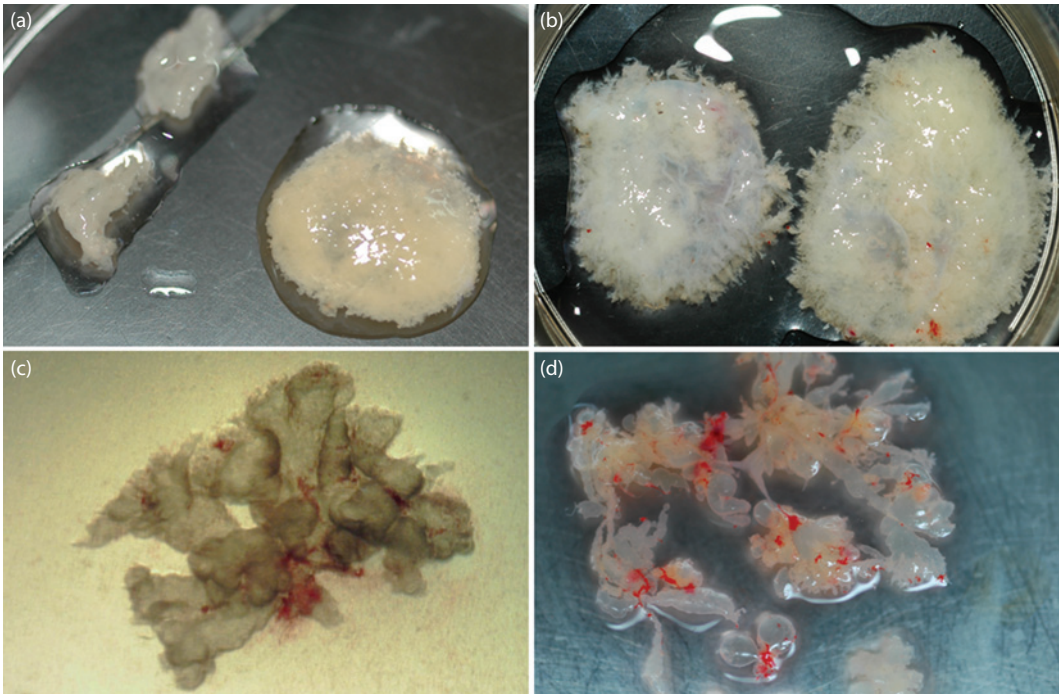


FIGURE 10.1 Tissue samples from SABs. Samples of SAB tissues forwarded for cytogenetic studies. (a) A typical sample containing maternal decidua (left) and a small conceptus (right). The maternal tissue is thick and rubbery, while the fetally derived tissue, including overlying villous material, has more delicate membranes. (b) Failed twin pregnancy, with two intact sacs that are discrepant in size. (c) Fragments of villi obtained by dissection of a sample containing no discernible fetal material. (d) Villus morphology is variable and may be related to the specific chromosomal error. This sample shows swollen, hydropic villi (with a grape-like appearance), typical of triploidy of paternal origin.

from an early loss (Figure 10.1b). This is not feasible in many instances, and fragments of fetal membranes with attached chorionic villi or simply villus fronds provide the next best options (Figure 10.1c and d). Such fragments can be obtained from samples where tissue from the conceptus has disintegrated or never formed. Later gestational age conceptions (post-12 weeks) may be represented by tissue from the extra-embryonic membranes, umbilical cord, placenta, or fetus proper. Importantly, the time between fetal demise and sample collection will affect tissue viability differentially. The embryo/fetus seems to be the first to die, followed by the umbilical cord, placenta, and finally the extra-embryonic membranes. This last tissue is therefore the most reliable in providing a fetal result.

Methodological Approaches

Historically, SAB samples have been analyzed using conventional cytogenetic methodology. This involves tissue culture, slide preparation, banding, and microscopic analysis of metaphase spreads to obtain a karyotype. However, as molecular techniques have evolved, so too has the study of SABs. Fluorescence in situ hybridization (FISH) can be applied in instances in which no dividing cells are present, or when the morphology of the metaphases is too poor for identification of individual chromosomes/chromosome regions [1–3]. In its initial application, FISH also required tissue culture and slide preparation. However, as probe sets became more sophisticated and covered wider parts of the genome, comparative genomic hybridization (CGH) approaches became available [4–7]. This technique still requires the traditional steps from culture to slide preparation, although the fluorescence signals can then be measured computationally. The subsequent application of proteases to disaggregate tissues has allowed laboratories to apply fluorescence techniques to the primary sample, thus removing the need for tissue culture. More recently, molecular approaches have further advanced, with the introduction of whole genome microarray technology [8,9].

Each of the approaches described above has advantages and limitations (e.g., [3,10,11]). Traditional cytogenetics is hampered by tissue culture failure, maternal tissue contamination, and maternal overgrowth [12–15]. Indeed, successful tissue culture results have ranged from 37% [16] to 95% [17] in different published series. Speed of analysis, the ability to analyze all samples, and elimination of time-consuming tissue culture are advantages of molecular tools over classical cytogenetics. However, molecular methods are also hampered by maternal tissue contamination [3,18–20], although the newer techniques may utilize SNPs to differentiate maternal from fetal tissue [9,19]. In addition, array-based techniques may be hampered by poor-quality DNA samples [10,20] and they rely on the premise that the fetal material contains an unbalanced genome. Thus, conditions such as triploidy, tetraploidy, and balanced structural rearrangements pose problems for array-based approaches [18,19,21], suggesting the utility of a combined approach that utilizes both array-based methods and either FISH or flow cytometry [20,22].

The Contribution of Chromosome Abnormalities to Spontaneous Abortions: Results of 50 Years of Studies

Early studies conducted in the 1960s and 1970s demonstrated that a small, but clinically important, proportion of newborn individuals had recognizable numerical or structural chromosome abnormalities. Indeed, in a review of studies of more than 50,000 consecutive newborns, Hook and Hamerton [23] reported abnormalities in approximately 1/200 individuals, with sex chromosome trisomies (47,XXX, 47,XXY and 47,XYY) and trisomy 21 the most common specific abnormalities. Similar studies of late fetal wastage (i.e., stillbirths) indicated a much higher level of chromosome abnormality but, in general, the types of abnormalities were in keeping with those identified in the newborn series (e.g., [24]).

These results prompted investigators to ask whether the other common category of clinically recognized pregnancy—spontaneous abortions (i.e., fetal losses occurring between about 6–8 to 20 weeks gestation)—might also include cases of chromosome abnormalities. Spearheaded by initial studies of Carr and colleagues (e.g., [25]), it soon became clear that a large proportion of SABs were attributable to chromosome abnormalities, and that the types of abnormalities were much more varied than those identified in stillbirths or newborn individuals. Tables 10.1 through 10.4 provide a summary of representative analyses of SABs; a description of cytogenetic studies and their results are provided in Tables 10.1 and 10.2 and more recent molecular and molecular cytogenetic studies are reviewed in Tables 10.3 and 10.4. Several important conclusions are clear from these analyses.

Maternal Contamination Complicates Interpretation of SAB Data

For most purposes, karyotypic analysis provides an unambiguous approach to the identification of numerical or major structural abnormalities. However, in the instance of SABs, fetally derived tissue may be scant, unviable, or difficult to culture. Coupled with the presence of maternally derived placental material, artifactual 46,XX results are a common complication of SAB studies. The consequences of maternal contamination are readily apparent from the results of cytogenetic studies of SABs (Table 10.2). For example, the sex ratio among euploid samples (i.e., 46,XY:46,XX) varies by an order of magnitude among the different studies, from nearly 3:1 to 0.3:1, presumably reflecting differences in the likelihood of including maternal tissue samples. Accordingly, the overall proportion of chromosome abnormalities is also remarkably variable among the different studies, with the lowest chromosome abnormality rates typically tracking with the lowest sex ratios.

Notably, the largest study to use a single-nucleotide polymorphism (SNP)–based approach (9; Table 10.4) removed from consideration 528 46,XX cases in which the genotypic information matched that of the mother, effectively raising the sex ratio from approximately 0.4 to nearly 0.9. Similarly, in a recent SNP analysis by Lathi et al. [19], in which results on specific categories of chromosome abnormalities were not detailed, 269 of 456 46,XX results were judged to reflect maternal contamination. Clearly, continued use of SNP-based analyses will eliminate much of the confusion created by inclusion of spurious 46,XX results.

TABLE 10.1

Summary of Methodology, Dates of Study, and Study Population Characteristics for Representative Cytogenetic Studies of SABs[a]

Study	Approach	Years	Mean Maternal Age (in years)	Gestational Age Range (in weeks)	No. Cases
Creasy et al. [26]	Cultured tissue; G- and Q-banding	1971–1974	—	8–38	941
Lauritsen [27]	Cultured tissue; aceto-orcein, Q-banding	1971–1973	—	<16	255
Byrne et al. [28]	Cultured tissue; G-banding	1977–1981	—	—	1356
Andrews et al. [29]	Cultured tissue; G- and Q-banding	—	25.9	9–28	154
Eiben et al. [30]	Direct preparations; G-, Q-, and C-silver staining	—	30.1	6–24	140
Ohno et al. [14]	Direct preparations; G-, Q-, and C-banding	—	30.8	6–16	144
Menasha et al. [31]					
Period A	Cultured tissue; non-specified banding	1990–1997	35.6	—	717
Period B	Cultured tissue, direct preparations; non-specified banding	1998–2002	36.6	—	1203
Cheng et al. [32]	Cultured tissue; G-banding	1995–2013	32.4	—	223
Choi et al. [33]	Cultured tissue; G-banding	2000–2013	30.3	<10–20	164
Hardy et al. [34]					
Hawaii	Cultured tissue; non-banded and Q-banding	1976–1985	28.1	2–32	2899
Emory	Cultured tissue; Q-banding	1989–1992	32.0	—	1365
CWRU	Cultured tissue; Q-banding	1993–1997	30.5	—	883
Perth-1	Cultured tissue; G-banding	1996–2007	34.8	<12	1188
Perth-2	Cultured tissue; G-banding	2008–2015	35.1	<12	1984

[a] For both the cytogenetic and molecular/molecular cytogenetic studies, we restricted our analysis to studies with relatively large study populations (i.e., >100 cases). Studies were excluded from consideration if they primarily involved recurrent miscarriages or were restricted to assisted reproductive technology (ART)–derived pregnancies. For all studies, we attempted to provide information only when it was clearly reported in the manuscript; however, in some instances in which the karyotypes were unclear, we excluded the case or assigned it to the most likely karyotypic category.

TABLE 10.2

Summary of Karyotypic Results for Representative Cytogenetic Studies of SABs

Study	Normal (XY:XX)	Sex Chromosome Monosomy	Trisomy	Triploidy	Tetraploidy	Structural Abnormality	Others	% Chromosomally Abnormal
Creasy et al. [26]	654 (1.27)	68	152	38	12	10	7	69.5
Lauritsen [27]	115 (0.92)	40	65	14	12	4	5	54.9
Byrne et al. [28]	816	86	301	85	28	17	23	39.8
Andrews et al. [29]	125 (1.23)	8	15	3	1	1	1	18.8
Eiben et al. [30]	72 (0.76)	5	43	10	1	2	7	48.6
Ohno et al. [14]	44 (0.83)	7	69	9	1	6	8	69.4
Menasha et al. [31]								
Period A	410 (0.33)	42	208	32	6	15	4	42.8
Period B	411 (0.71)	54	572	91	18	31	26	65.8
Cheng et al. [32]	98 (0.44)	16	73	8	6	9	13	56.1
Choi et al. [33]	81	12	53	6		6	6	50.6
Hardy et al. [34]								
Hawaii	1433 (0.87)	263	844	180	66	69	44	50.6
Emory	530 (0.66)	96	529	87	38	45	40	61.2
CWRU	499 (0.66)	40	239	59	13	21	12	43.5
Perth-1	330 (1.60)	89	584	80	30	33	42	72.2
Perth-2	413 (2.93)	157	1048	167	49	58	92	79.2

TABLE 10.3

Summary of Methodology, Dates of Study, and Characteristics of Study Populations for Representative Molecular or Molecular Cytogenetic Studies of SABs

Study	Approach	Years	Maternal Age Mean	Gestational Ages	Cases
Zhang et al. [35]	Cultured tissue; G-banding, PCR-based microsatellite genotyping, array CGH	2006–2007	—	—	115
Gao et al. [22]	Cultured tissue; G-banding, arrayCGH, FISH, QF-PCR	—	32.0	<12	100
Jenderny [8]	Cultured tissue; G-banding, QF-PCR	2002–2013	—	7–34	398
Levy et al. [9]	DNA from fetally derived tissue; SNP-based chromosomal microarray	2010–2012	36.2	3–20	1861

TABLE 10.4

Summary of Karyotypic Results for Representative Molecular or Molecular Cytogenetic Studies of SABs

Study	Normal (XY:XX)	Sex Chromosome Monosomy	Trisomy	Triploidy	Tetraploidy	Structural Abnormality	Others	% Chromosomally Abnormal
Zhang et al. [35]	45	7	46	4	6	2	5	60.9
Gao et al. [22]	39 (2.08)	4	49	4	0	3	1	61.0
Jenderny [8]	153 (0.94)	17	141	32	8	9	38	61.6
Levy et al. [9]	755 (0.86)	53	794	114	4	38	90	59.4

Numerical Chromosome Abnormalities Are the Leading Cause of SABs

The above concerns notwithstanding, the results of both the cytogenetic (Table 10.2) and molecular/molecular cytogenetic studies (Table 10.4) make it clear that chromosome abnormalities are the primary cause of SABs. Indeed, of the overall total of 16,090 abortuses reported in Tables 10.1 through 10.4, 9,054 (56.3%) were chromosomally abnormal.

Intriguingly, by comparison with liveborn or stillborn conceptuses, SABs are characterized by a wide variety of chromosome abnormalities, the majority of which involve single missing or extra whole chromosomes (i.e., aneuploidy). The single most common specific abnormality, sex chromosome monosomy (45,X), accounts for 6.6% of the cases reported in Tables 10.1 through 10.4. Trisomies are the most common class of abnormality, identified in 36.2% of abortuses. Trisomies for all chromosomes have been identified, although a few (e.g., trisomies 1 and 19) are extremely rare. Conversely, other trisomies are quite common, i.e., trisomy 16 is the most commonly identified trisomy and, together with trisomies 15, 21, and 22, these four conditions account for well over one-half of all single trisomies. Importantly, these results are in relatively good agreement with recent studies of human preimplantation embryos from assisted reproduction technology (ART)–derived pregnancies. For example, similar to SABs, chromosomes 15–22 are more likely to be present in aneuploid state in preimplantation embryos than are larger chromosomes (e.g., [36,37]). Thus, while it is clear that selection eliminates a large number of aneuploid conceptuses between the time of conception and the time of clinical recognition, it also seems likely that certain chromosomes are more likely than others to undergo meiotic mis-segregation.

In addition to aneuploid abnormalities, two classes of polyploids are common in SABs: triploids, accounting for 6.4% of cases and tetraploids, occurring in 1.9% of SABs. Surprisingly, structural abnormalities—which are nearly as common as numerical abnormalities in the newborn series [23]—are uncommon in SABs, accounting for only 2.4% of the cases reported in Tables 10.1 through 10.4. Further, the initial application of molecular analytic approaches, which should make it possible to identify subtle structural abnormalities, appears not to have substantially increased the frequency of such abnormalities in SABs (e.g., 9).

Maternal Age Is the Primary Determinant of Chromosome Abnormality Rate in SABs

Surprisingly, there is considerable variation in the rates of chromosome abnormality among different studies of SABs, even after taking into account apparent differences in levels of maternal contamination. In general, abnormality levels appear to have increased over time, with studies conducted in the 1970s–1980s typically reporting rates of 40%–50% (e.g., [26,28,29,38–42], while several recent studies have reported rates of 60% or more (e.g., [9,19,22,31,33,43–46]).

While the reasons for these differences are not known, they might reflect real changes in the likelihood of conceiving chromosomally abnormal fetuses (e.g., because of changing environmental conditions) or they might simply be attributable to changes in population demographics over time. To discriminate between these possibilities, we recently compared results among five different cytogenetic studies of

SABs in which we participated: one study was conducted in Honolulu, HI in the 1970s and 1980s (by T.H.), one in Atlanta, GA in the 1980s and 1990s (by T.H.), one in Cleveland, OH in the 1990s (by T.H.), and two in Perth, Australia from the 1990s to the present (by K.H.). The results were instructive [34, 47]. Consistent with expectations, we saw an increase in the rate of chromosome abnormality over time, with the earliest studies having abnormality rates of approximately 40%–50%, and the rates in the more recent ones exceeding 70% (Table 10.2). However, analyses of racial/ethnic background revealed no important effect, nor was among-study variation in gestational age a factor. Indeed, an examination of the types of chromosome abnormalities showed that the only class of abnormality that varied among the studies was trisomy, and that the differing levels of trisomy could be completely explained by maternal age, i.e., in the earliest study (conducted in Hawaii) the mean maternal age was 28.1 years, while in the most recent study (conducted in Perth), the comparable mean was 35.1 years. Taken together, our results suggest that the primary variable affecting the rate of chromosome abnormality is maternal age, and that this exerts its effect by varying the likelihood of mis-segregation during maternal meiosis. This is not to say that other factors, including environmental contaminants, have no role in mediating chromosome abnormality rates, only that the magnitude of any such effect is minimal by comparison with that exerted by maternal age.

How Do the Abnormalities Originate?

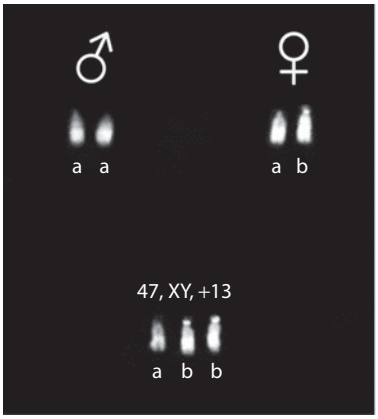
For many human genetic disorders, the low frequency of the abnormality complicates attempts to directly study the origin of the underlying mutation(s); instead, alternative approaches (e.g., in vitro analyses or generation of animal models) are needed. However, this is not the case for numerical chromosome abnormalities. For reasons that we do not understand, the rates of numerical abnormalities in humans are at least an order of magnitude higher than in other mammalian species that have been appropriately studied, meaning that ample material is available for analysis. Given the incidence and variety of these abnormalities in spontaneously aborted fetuses, SABs have been especially useful for investigating the origin of the abnormalities. These studies have relied on inheritance of polymorphisms (Figure 10.2 [48]) to address three questions about the origin of the abnormalities:

- In which parent did the abnormality originate, i.e., is the extra or missing chromosome(s) maternal or paternal in origin?
- At what stage of development did the abnormality originate, i.e., at meiosis I or meiosis II, at the time of fertilization, or in the early cleavage divisions?
- Was abnormal recombination a contributor to the abnormality? That is, by examining polymorphic markers along the chromosome, the recombinational "history" of a chromosome can be re-created and compared between chromosomally normal and abnormal progeny. This approach has been used extensively to investigate the role that abnormal recombination plays in the generation of trisomic SABs.

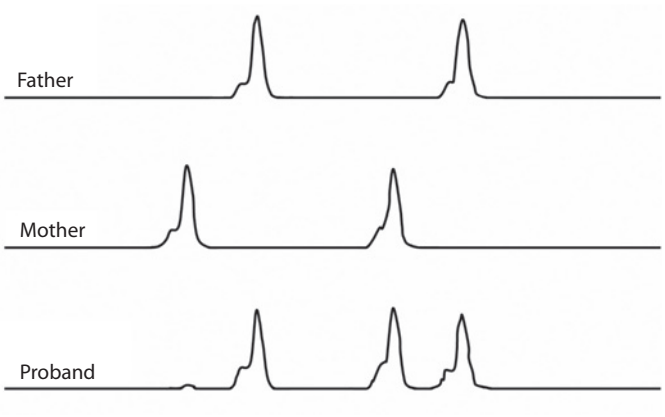
The utility of polymorphism analysis in studying the different classes of numerical abnormality is summarized below, and in Figures 10.2 and 10.3.

Sex Chromosome Monosomy

Unlike abnormalities in which additional chromosome material is present in the conceptus, the 45,X condition involves a missing chromosome, either an X or a Y chromosome. Thus, it is only possible to specify the parental origin of the X chromosome that is available and, by exclusion, the parental origin of the sex chromosome that is missing. Such studies have been conducted both in SABs and in liveborn Turner syndrome individuals, with similar conclusions (e.g., [49–51]). Specifically, among approximately 100 apparently non-mosaic 45,X cases, some 70% had a single, maternally derived X chromosome and the remainder were X^{pat} cases. There was no obvious difference in results between the spontaneously aborted and liveborn cases, indicating that the parental origin of the single X does not affect survival.



(a)



(b)

Locus	Proband	Father	Mother	MAT/PAT	R/N
Centromere					
D14S742	ABC	AC	BC		N
MYH7	ABC	AB	AC		N
D14S581	AAB	AB	AB		-
D14S615	ABC	AC	BC		N
D14S49	AAB	AB	AC		R
D14S1432	ACC	AB	AC	MAT	R
D14S587	ABC	BB	AC	MAT	N
D14S1429	BCC	AC	BC		N
D14S588	ABB	BB	AB		N
D14S43	ABC	AA	BC	MAT	N
D14S1433	ABC	AB	BC		N
D14S617	ABB	BC	AB		N
D14S611	AAB	AA	AB		N
D14S1426	BCD	AB	CD	MAT	N
D14S1007	ABC	AC	BC		N

(c)

FIGURE 10.2 Polymorphism analysis of human chromosome abnormalities. Several different approaches have been used to investigate the origin of chromosome abnormalities. Early efforts made use of blood group markers (e.g., from Sanger, R. et al., *J Med Genet*, 8, 417–426, 1971) but these were gradually replaced by chromosome heteromorphisms, variable markers detectable by conventional cytogenetic methodology and occurring at pericentromeric regions of 8 of the 23 autosomes. (a) An example of Q-banding chromosome heteromorphism analysis, indicating a maternal origin for a case of trisomy 13. With the demonstration of sequence-based variants, DNA polymorphism analysis (initially involving RFLPs, and subsequently, mini-satellites, microsatellites, and most recently, SNPs) became the standard technique for analysis of the origin of chromosome abnormalities. (b) A microsatellite-based analysis of trisomy 14, indicating a paternally derived additional chromosome. In addition to assessing parental origin, DNA polymorphisms have been used extensively to determine the meiotic/mitotic stage of origin of trisomies, as well as the amount of recombination that occurred between the nondisjunctional homologs. (c) An example of this type of analysis, as applied to a case of trisomy 14. Multiple polymorphic sites along chromosome 14 were examined, with four providing evidence of a maternal origin. Subsequent analysis focuses on whether, at individual polymorphic sites, the trisomic conceptus received both maternal alleles (non-reduced, or "N") or two copies of the same maternal allele (reduced, or "R"). If pericentromeric sites are N, this is consistent with a meiosis I error and if R, a meiosis II error; thus this case is of meiosis I origin. Finally, switches between N and R at adjacent loci indicate points of crossing-over that occurred in the maternal meiosis that generated the additional chromosome; in this case, two cross-overs occurred between the chromosome 14 homologs in the trisomy-generating meiosis.

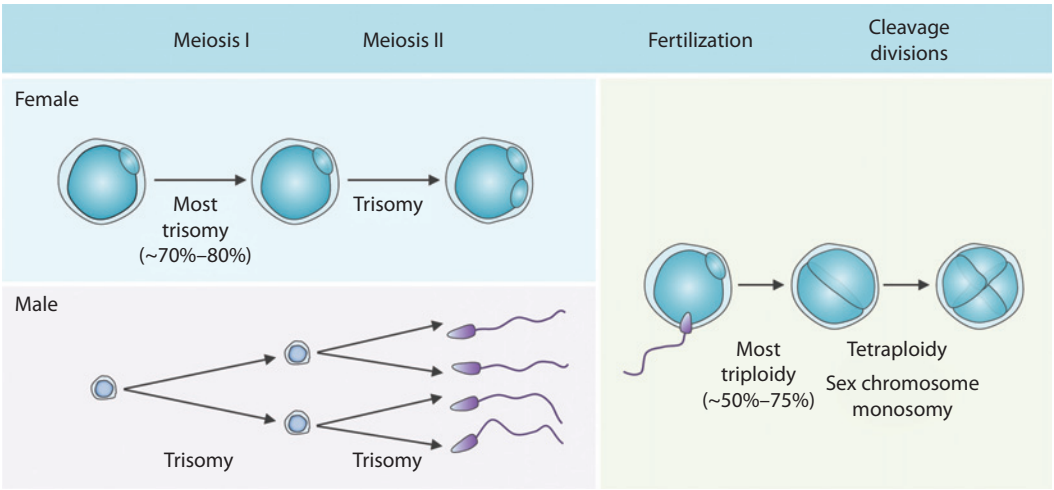


FIGURE 10.3 Summary of studies investigating the origin of chromosomally abnormal SABs. The results of analyses of numerical chromosome abnormalities indicate that errors can occur at any of the possible meiotic stages (maternal meiosis I and II, paternal meiosis I and II), as well as at the time of fertilization and during the first few cleavage divisions. However, against this background, certain timepoints appear to be especially vulnerable. Perhaps most importantly, abnormalities at maternal meiosis I are the leading cause of trisomy and arguably the most common cause of spontaneous abortion. Additionally, fertilization by two sperm appears to be the leading cause of triploidy, while errors in early mitotic cell divisions are likely causes of tetraploidy and sex chromosome monosomy.

However, mosaicism for a second normal or structurally rearranged sex chromosome is much more common in liveborn Turner syndrome individuals than in aborted 45,X conceptuses, indicating an association with survival to term [52,53]. Additionally, studies of liveborn 45,X individuals suggest phenotypic variation between 45,X^{pat} and 45,X^{mat} cases (e.g., [54]); thus, while there does not appear to be a correlation between parent of origin of the single X and survival to term, subtler phenotypic effects apparently are affected by origin of the X.

While we can obtain information on the parental origin of sex chromosome monosomy, the absence of the "offending" chromosome prevents us studying the mechanism or stage of origin of the error. Nevertheless, the high frequency of the 45,X condition by comparison with sex chromosome trisomies, which are thought to originate from meiotic nondisjunctional errors [49], suggest that 45,Xs arise from a different mechanism. Random loss of a sex chromosome, presumably at an early mitotic division, is an attractive possibility, since this would generate an apparent bias toward X^{mat} cases. That is, assuming equal likelihood of loss of the X or Y chromosome in early stage 46,XX and 46,XY embryos, we would expect to generate 45,Y conceptuses (presumably early lethals) and 45,X conceptuses, two-thirds of which would carry a single, maternally derived X chromosome.

Trisomy

Over the past 25 years, the parent and meiotic stage of origin of human trisomies have been extensively investigated. Because of its clinical importance, trisomy 21 has received the most attention, with nearly 1,000 cases having been analyzed (e.g., [55,56]). From these studies two aspects of chromosome 21 mis-segregation are now clear. First, the vast majority of trisomies 21 involve errors at maternal meiosis I, e.g., in the largest series of cases, Sherman et al. [57] observed that approximately 90% of cases were maternally derived, with 70% of these attributable to meiosis I errors and the remainder ascribed to meiosis II errors [58]. Second, abnormal meiotic recombination is an important contributor to trisomy 21. Indeed, the overwhelming majority of trisomy 21 cases appear

to be linked to one of three types of susceptible crossover configurations: failure of recombination with maternal meiosis I errors, extremely distal crossovers with maternal meiosis I errors, and extremely proximal crossovers with apparent maternal meiosis II errors. Since meiotic recombination occurs in the fetal ovary in mammalian females, this means that a proportion of human oocytes are predisposed to mis-segregate right from the beginning of meiosis. However, intriguingly, the relationship between these recombination risk factors and the only other known etiological agent for trisomy 21—advancing maternal age—is still unclear. Oliver et al. [59] reported an increase in telomeric exchanges in meiosis I cases involving young women, an increase in pericentromeric exchanges among meiosis II cases involving older women, and no clear linear association between maternal age and cases involving failure to recombine. Thus, the effect of recombination on trisomy is likely complex, presumably reflecting the fact that there are multiple maternal age-dependent and age-independent routes to trisomy 21.

How do the results for trisomy 21 compare with those for other trisomies? Given the complexity of errors involving chromosome 21, it is perhaps not surprising that other trisomies exhibit similarities and differences. For example, like trisomy 21, maternal meiotic errors predominate for most trisomies, and errors of recombination, especially absence of crossovers, have been observed for all trisomies that have been appropriately studied (e.g., [60]). However, there are differences in the details. For example, trisomy 18 typically involves a maternal error at maternal meiosis II, not meiosis I [61]; trisomy 16, the most common trisomy, is associated with telomeric exchanges but rarely, if ever, with failure to recombine [62]; and the 47,XXY condition is as likely to be paternal as maternal in origin [63]. Thus, while we have a long way to go in our understanding of the causes of human nondisjunction, one fact is clear: should we ever get to the point where we think about preventing the occurrence of meiotic mis-segregation, we will need many "magic bullets" to accomplish the task.

Triploidy

Triploidy can result from a diploid contribution from the father (diandry), either from fertilization by two sperm (dispermy) or by a diploid sperm; alternatively, triploidy may be maternal in origin (digyny), because of failure of either of two meiotic divisions. DNA polymorphism analyses indicate that dispermy is the most common mode of origin, accounting for over two-thirds of cases [64,65]. Of the remaining maternally derived cases, errors at meiosis II appear to be the most common source of the additional haploid set [66] although errors at meiosis I and, intriguingly, "dieggy" (i.e., fusion of two oocytes), have also been reported [63].

Unlike most other numerical abnormalities, the parental origin of triploidy has a profound effect on phenotype. Specifically, diandric triploids typically abort between 10 to 20 weeks of gestation, with limited development of fetal structures but good development of the extra-embryonic membranes and villi [67]; indeed, diandric triploids are frequently diagnosed as partial hydatidiform moles (for discussion of moles, see [68]). In contrast, there appear to be two general categories of maternally derived triploids. The more common category aborts very early in pregnancy but a subset of cases is associated with good fetal development and with abortion late in gestation; indeed, the extremely small proportion of cases of triploidy that survive to term are thought to be maternal in origin. Thus, triploidy provides an important example of the existence of imprinted loci, although the phenotypic contribution of the specific loci is not known.

Tetraploidy

Very little effort has been directed at studying the origin of tetraploidy. However, virtually all tetraploid SABs have 92,XXYY or 92,XXXX karyotypes, consistent with doubling of 46,XY or 46,XX fertilized eggs, and early chromosome heteromorphism studies indicated they consisted of two duplicated sets of chromosomes (e.g., [69]). Thus, it seems likely that most tetraploids arise from a failure of the chromosomes to divide at the first or a very early embryonic cell division.

Overview: Where Do We Go from Here?

Fifty years of studies of SABs have provided us with a wealth of knowledge about the incidence and origin of human chromosome abnormalities. It is now clear that numerical chromosome abnormalities are the leading cause of pregnancy failure in our species; indeed, it could be argued that, cumulatively, they represent the most common category of de novo mutation in humans. The parent and stage of origin of these abnormalities is complex, with errors identified at all possible stages of meiosis, at fertilization, and likely during the early mitotic divisions (Figure 10.3). However, against this background it is also clear that maternal meiotic errors are especially common, and represent the most important source of SAB-causing mutations. These occur in high frequencies in all human populations that have been appropriately studied and are presumably under selective pressure, although the evolutionary benefit remains obscure.

Given the extensive data available on the incidence and types of chromosome abnormalities in SABs, is there a compelling reason to continue these analyses? Obviously, the information is clinically warranted, since it may provide the reason for the demise of a wanted pregnancy, as well as often being of prognostic value. However, from a research prospective, this is clearly a field that is "mature," and it might be argued that there is little left to learn from it. We think this conclusion is incorrect, for two reasons. First, most of our information on SABs has come from conventional cytogenetics and, while this is suitable for detection of numerical abnormalities and large structural abnormalities, the level of resolution is insufficient to detect small, but potentially SAB-associated structural rearrangements. The advent of array- and sequence-based approaches now provides the opportunity to ask whether such abnormalities contribute to SABs, and we suggest that this is an important area of future clinical research. Second, SABs provide a valuable resource for examining the origin of chromosome abnormalities in natural conceptions, and provide an important comparison group for ART-associated pregnancies. We have already seen the utility of such comparisons, e.g., in studies comparing the types and frequencies of different aneuploidy conditions in ART-derived preimplantation embryos versus those associated with SABs (e.g., [37]). Recent "MeioMapping" studies [70] demonstrate that it will also be possible to compare the mechanism(s) of origin of specific types of chromosome abnormalities between these categories of pregnancies. Thus, while studies of SABs may no longer be innovative or groundbreaking, if properly orchestrated, they still have much to teach us.

Acknowledgment

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11

Products of Conception: Current Methodologies and Clinical Applications

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CONTENTS

Introduction

Despite medical advances, miscarriage is the most common complication observed during the first trimester of pregnancy, with approximately 15%–25% of clinically recognized pregnancies lost. The primary cause of early pregnancy loss is the presence of a chromosomal abnormality that is incompatible with life; these are found in up to 50% of first trimester losses [1]. However, miscarriage and perinatal loss is an etiologically heterogeneous condition for which the causes are difficult to elucidate. In fact, 25%–50% of all miscarriages reported remain unexplained. Well-established causes include paternal or de novo chromosomal aberrations [2], antiphospholipid syndrome [3], some inherited thrombophilias, such as Factor V Leiden and prothrombin G20210A gene mutation [4,5], congenital or acquired uterine anomalies [6], and endocrine, autoimmune, or alloimmune disturbances [7] as well as unhealthy lifestyle habits (e.g., smoking, obesity, or psychological stress) [8,9]. Thus, recurrent miscarriage can result from many factors. Once uterine malformations, endocrinology pathologies, and antiphospholipid syndrome have been ruled out, parental karyotype assessment should be considered, especially if some of the miscarriages suffered by the couple were karyotypically abnormal [10].

The analysis of products of conception (POC) makes it possible to diagnose cytogenetic anomalies that could explain a pregnancy loss. Defining the POC karyotype has an impact on couples' reproductive future because it distinguishes between non-hereditary and hereditary chromosomal abnormalities (i.e., reciprocal translocation carriers) and detects gestational trophoblastic diseases such as hydatidiform mole formation to assist with post-loss diagnosis and follow-up. POC analysis also benefits women experiencing recurrent pregnancy loss (RPL). RPL is defined as two or more consecutive pregnancy losses in the first or early second trimester [2]. Although the overall incidence of RPL is low and is estimated to occur in less than 5% of pregnant women [11], an abnormal embryonic karyotype is the most common cause of recurrent miscarriage [12].

In summary, POC analysis is essential to determining the cause of sporadic and recurrent pregnancy loss and assists in the estimation of recurrence risk in future pregnancies, thus improving the chances of subsequently producing a healthy full-term pregnancy.

Methodology for POC Analysis

Tissue Collection

Once a pregnancy loss has occurred, a sample of fetal tissue can be recovered by surgical methods, such as dilation and curettage (D&C) or uterine aspiration. These methods present a higher risk of maternal cell contamination (MCC) due to the maternal and fetal tissue mixing during the procedure. A hysteroembryoscopy (that allows for direct and selective embryo and chorion biopsy) performed before curettage diminishes the likelihood of MCC and avoids the risk of misdiagnosis [13]. However, hysteroembryoscopy requires a specialist in the technology and this approach is not current practice in the obstetrics field. In this section, we present an alternative to detect the presence of MCC in POC recovered by D&C or aspiration paired with molecular diagnosis of aneuploidies.

Molecular Approaches to Chromosomal Analysis

Since 2008, emerging technologies have been used as part of a robust and accurate diagnostic approach to assess for aneuploidy of any of the 24 chromosomes: the 22 autosomes and the sexual chromosomes [14].

Techniques such as chromosomal array comparative genome hybridization (aCGH), next-generation sequencing (NGS), BACs on Beads (BOBs), fluorescence in situ hybridization (FISH), multiplex ligation-dependent probe amplification (MLPA), and quantitative fluorescent polymerase chain reaction (QF-PCR) have overcome disadvantages related to conventional cytogenetic techniques in the study of POC after a miscarriage, including poor chromosome preparations or failure in culture [15].

Due to advances in recent decades, POC chromosomal assessment is now performed using molecular genetics rather than traditional cytogenetic methods (Giemsa staining of metaphase spreads). Molecular methods are more reliable and do not require a previous cell culture, avoiding the possibility of cell culture failure and increasing the rate of informative results. Cell culture failure is an inherent problem in traditional cytogenetic methods. But cell culture failure is not the only problem. Cytogenetic studies cannot distinguish between a normal female result coming from the fetus and a normal female result because of MCC. There is also a high percentage of non-informative test due to potential tissue degradation with standard karyotyping. With molecular techniques, the turnaround time is much lower than with conventional karyotype. Finally, the resolution could be higher with molecular studies. Table 11.1 shows the comparison of conventional karyotype with molecular analysis.

Several groups have applied different molecular approaches as technology evolves, including aCGH or, most recently, NGS.

Comparative Genomic Hybridization (CGH) Arrays

To perform aCGH analysis, first a DNA extraction must be performed from each type of POC tissue. A small portion of tissue is mechanically dilacerated using a scalpel blade. Then DNA extraction is

performed followed by the labeling of POC DNA samples and control DNA with Cy3 and Cy5 fluorophores. Labeled mixes can be combined and hybridized in commercial platforms such as 24sure BAC arrays (Illumina Inc., San Diego, CA) for 6–12 h. Each probe is specific to a different chromosomal region and occupies a discrete spot on the slide. The different spots are distributed with 1 Mb distance. Chromosomal losses or gains are revealed by the color adopted by each spot after hybridization. Fluorescence intensity is detected using a laser scanner and a specific software [14] (Figure 11.1).

TABLE 11.1

Reasons to Use Molecular Approach Instead of Conventional Karyotype for POC Assessment of Chromosomal Abnormalities

Conventional Karyotype	Molecular Studies
Requires in vitro cell culture	Does not require in vitro cell culture
42% of the tests performed are not informative due to tissue degradation	Results obtained in more than 98.6% of the tests performed
33.3% are false negatives due to maternal contamination	Rules out false negatives caused by MCC[a]
Results provided in 2–4 weeks	Results obtained in 1 week
Low-resolution analysis	Higher resolution than conventional karyotype

[a] MCC, maternal cell contamination

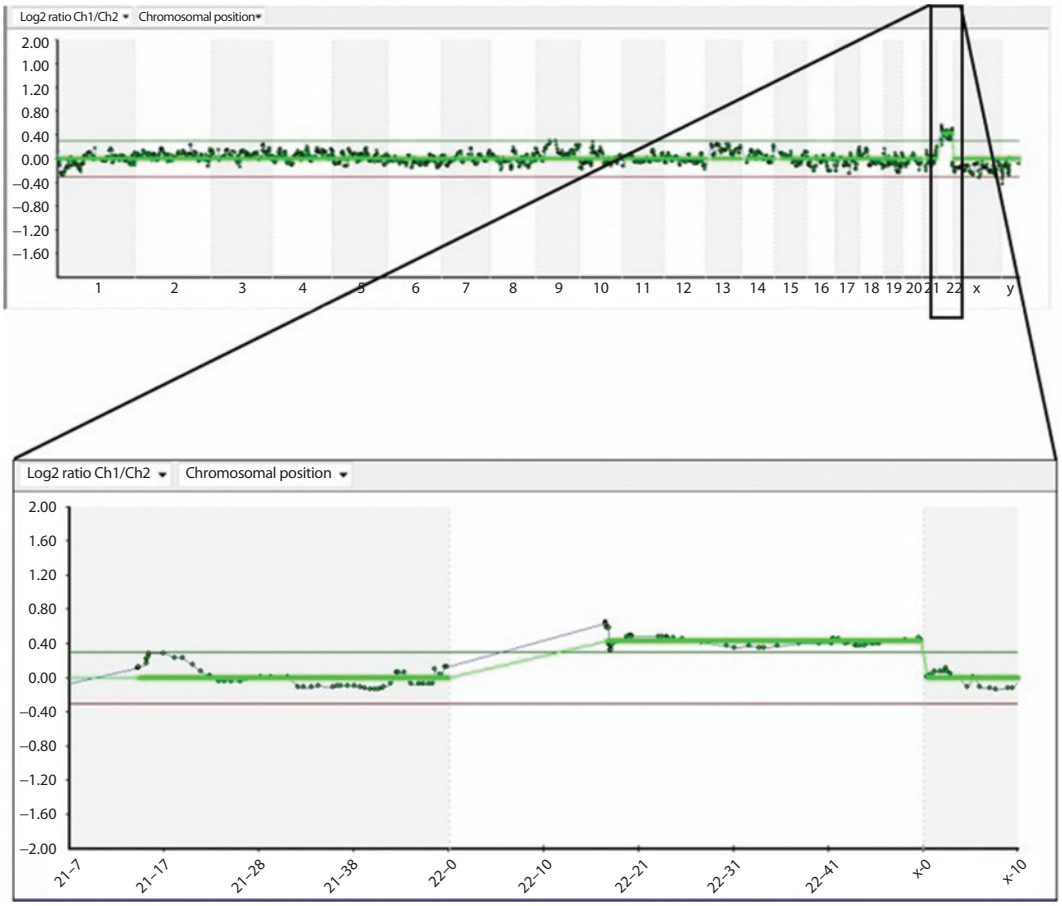


FIGURE 11.1 aCGH technology. Example of an abnormal female fetus with a chromosome 22 trisomy, compared to female reference DNA.

Single-Nucleotide Polymorphism (SNP) Array

To perform the SNP array the tissue sample was rinsed with phosphate-buffered saline (PBS) and separated from maternal decidua and blood using standardized techniques. Next, the tissue was again rinsed in sterile PBS and dissected into Tic Tac–sized villi/fetal samples (~3 mm^3) and placed in 1.5-mL microfuge tubes. The DNA was then extracted from the villi and the maternal blood sample using a QIAamp Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany). Purified DNA was run on Illumina Cyto 12 SNP microarrays per standard protocol [16].

Next-Generation Sequencing (NGS)

This is currently the most innovative technology. Different commercial platforms have kits developed for aneuploidy, mosaicism, and segmental aneuploidies detection.

- *Ion Torrent Technology.* Briefly, DNA is amplified, purified, and quantified (a process known as library construction). Then a clonal amplification (templated preparation) is performed, followed by template enrichment. Finally, the template is loaded onto the sequencing machine. The Ion Personal Genome Machine (PGM™) sequencer sequentially floods the chip with one nucleotide after another. If a nucleotide complements the sequence of the DNA molecule in a particular microwell, it will be incorporated and hydrogen ions will be released. The pH of the solution changes in that well and is detected by the ion sensor, essentially going directly from chemical information to digital information (Thermo Fisher Scientific, Waltham, MA). A specific software aligns the sequence reads to reference human genomes, which are classified in 2-Mb bins. Differences in copy numbers for each bin are represented in a plot. With this technology single and multiple whole chromosome aneuploidies have been detected in POCs, as well as small deletions/duplications and mosaicism (Ion Reporter 5.0) (Figure 11.2).
- *Illumina Technology.* From amplified and quantified WGA products, the NGS libraries are prepared using a VeriSeq PGS-MiSeq kit. The libraries are purified and then normalized using Solid Phase Reversible Immobilization paramagnetic bead-based technology (AMPure XP beads; Beckman Coulter, Brea, CA) and the VeriSeq PGS bead-based sample normalization kit, respectively. Dual index 36 base pair read (1 × 36 DI) sequencing was performed according to the VeriSeq PGS recipe (Rev. O), using a MiSeq Reagent Kit v3-PGS (Illumina). The software to perform onboard secondary data analysis using the VeriSeq PGS workflow was MiSeq Reporter (Illumina). BlueFuse Multi Software, version 4.2 (Illumina), was used to analyze sequencing data generated by the MiSeq instrument and to report results (Figure 11.3) [17,18].

Complementary Studies to Rule Out MCC and Triploidy

MCC is one of the major sources of misdiagnosis or non-informative results when testing POCs. A retrospective study showed that over half of the normal 46,XX results in miscarriage specimens were due to MCC [16]. Errors like this may lead to misdiagnosis and/or inappropriate counseling recommendations. Having an accurate result is the only way that the physician and medical team can give appropriate reproductive counseling to the couple, when a pregnancy loss occurs after a natural conception or after assisted reproduction techniques (ARTs).

To rule out the risk of misdiagnosis due to MCC, a complementary strategy performing STRs (short tandem repeats), such as NGS for aneuploidy detection, can be run in parallel to the molecular method. DNA from POC samples and gestational carrier DNA are extracted using a QIAamp® DNA Mini and Blood Kit (Qiagen). An AmpFlSTR Identifier Plus Kit PCR Protocol (amp16str) (Thermo Fisher Scientific) is used to run the PCR and the electrophoresis to detect or rule out MCC (Figure 11.4a, b) and triploidy (Figure 11.5).

Multisampling is a good strategy to follow to diminish to the greatest extent possible the likelihood of obtaining MCC as a result of the analysis. In our laboratories, multisampling comprises three dissections of the POC instead of only one. This action makes it possible that, for example, after performing STRs

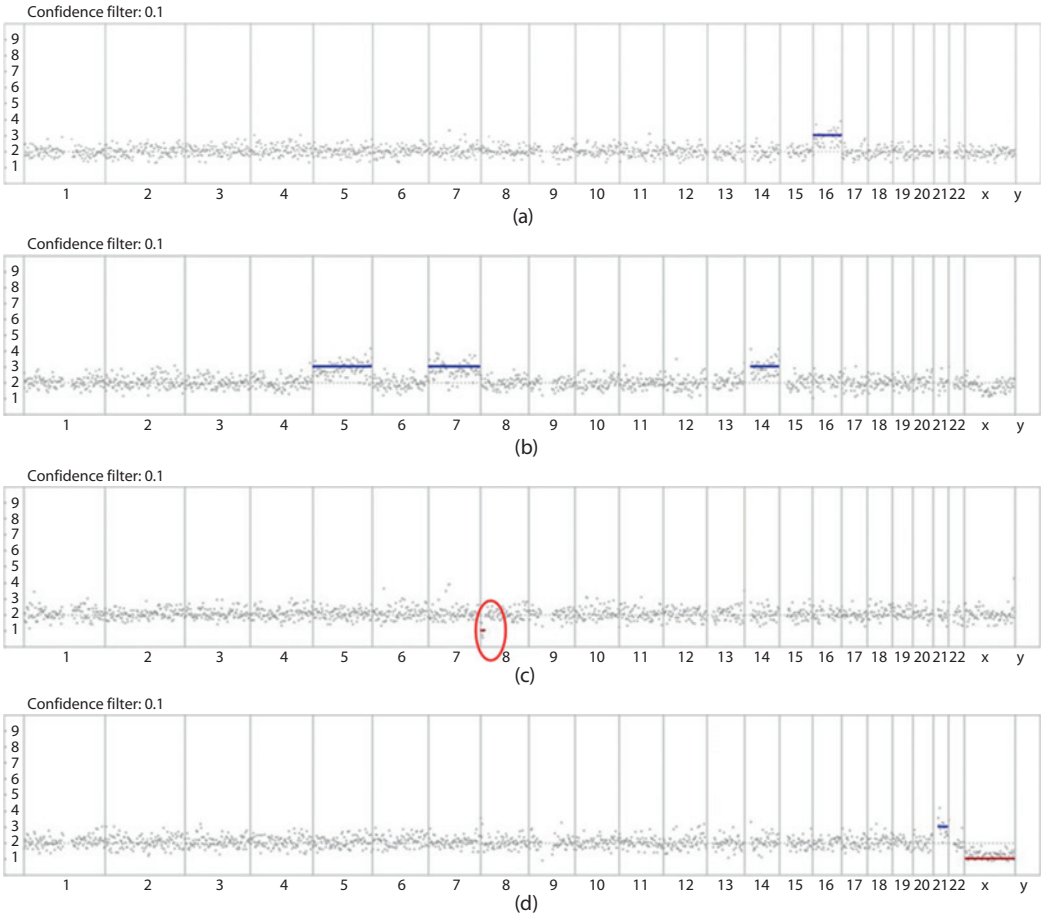
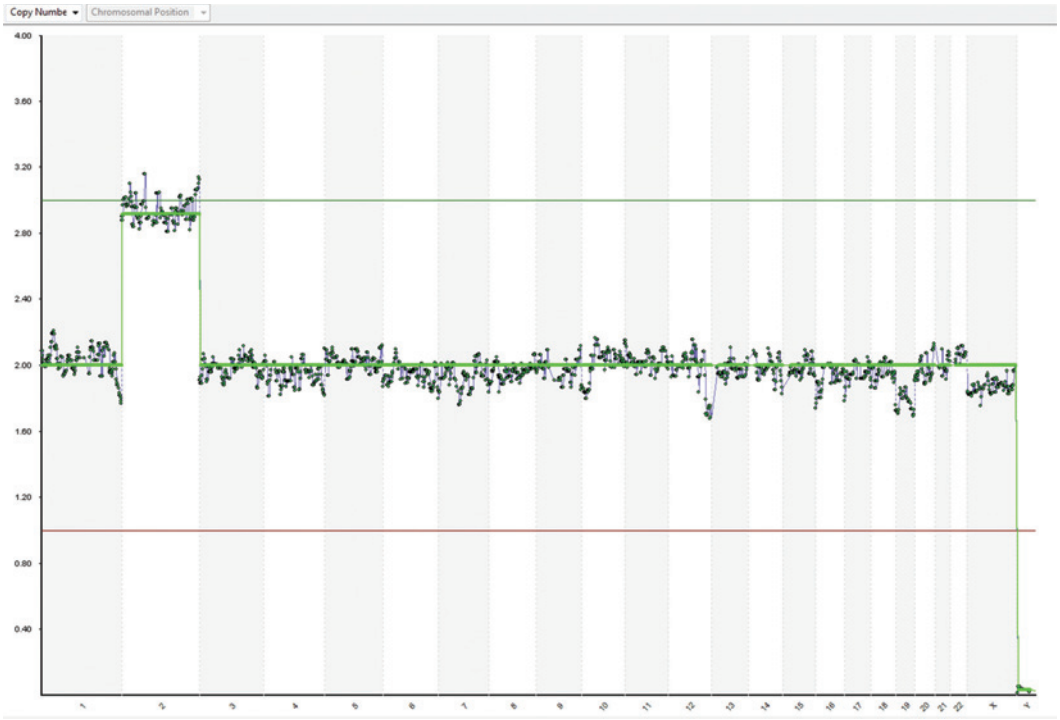


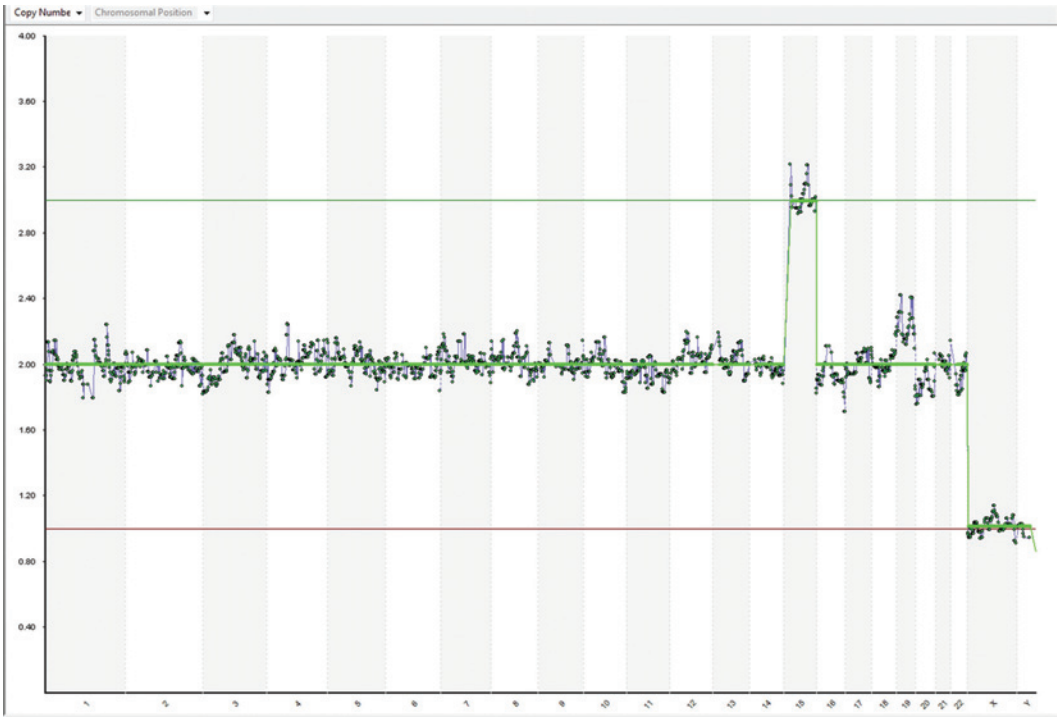
FIGURE 11.2 NGS technology (Life Technologies). (a) Abnormal female fetus. Trisomy 16. (b) Abnormal female fetus. Trisomies 5, 7, and 14 (anembryonic). (c) Abnormal female fetus. Partial deletion on p arm of chromosome 8. (d) Abnormal female fetus. Trisomy 21 and sexual monosomy.

analysis, sample #1 and sample #2 are MCC (pertain to the mother or gestational carrier) but sample #3 pertains to the fetus. Sample #3 would be the one to run on the sequencer for chromosomal analysis and would provide accurate results. In the scenario where only one dissection is performed, if you take sample #1, the final result would be MCC, with non-informative results or a false normal female result if STRs were not performed.

Using this approach, NGS/aCGH for aneuploidy detection plus STRs, we have already analyzed more than 600 POC cases. The average maternal age was 36.5 ± 4.3 years old, and mean gestational age at which the spontaneous miscarriage occurred was 7.8 ± 1.5 weeks. The percentage of POC samples with non-conclusive results after the first dissection was 30.0%, mostly due to MCC. After a total of three dissections from different locations in the piece of tissue, we reduced the non-informativity rate due to MCC to 18.7%. We were able to provide results (either normal or abnormal) in 81.3% of the cases. A statistically significant increase in detection rate was obtained with the multi-sampling strategy ($p = 0.001$, Fisher's exact test). Notably, in this study three dissections were done per POC sample to establish a lower rate of MCC. We also performed an internal study, taking six or nine samples when the first three showed MCC. In our results, if the first three dissections showed MCC, a larger number of dissections did not change the results. Therefore, when the first three dissections result in MCC the recommendation would be not to perform more dissections and provide results as a MCC. We found chromosomal abnormalities in up to 53.3% of the cases. As expected, trisomies were the most common abnormality found (79.9%), with 10% of the cases with double trisomy. Within the trisomies,



(a)



(b)

FIGURE 11.3 NGS technology (Illumina). (a) Abnormal female fetus. Trisomy 2. (b) Abnormal male fetus. Trisomy 15.

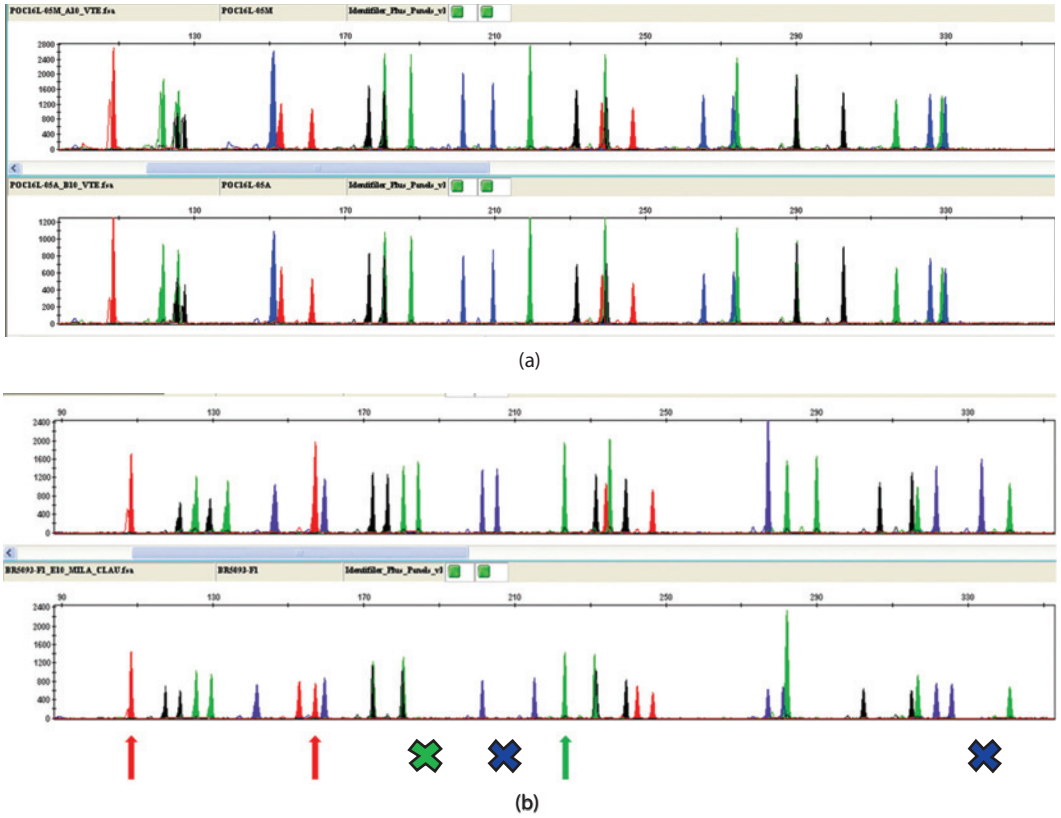


FIGURE 11.4 (a) MCC (maternal cell contamination). Top: DNA from mother or gestational carrier. Bottom: DNA from fetus. All of the alleles are coincident between mother and fetus. This means that the sample that is supposed to be fetus is not from the fetus but is actually from the mother resulting in MCC. (b) No MCC (maternal cell contamination has been ruled out). Top: DNA from mother or gestational carrier. Bottom: DNA from fetus. Some of the alleles are coincident between mother and fetus (arrows). Some of the alleles are present in the mother but not in the fetus (crosses). This means that the sample that is supposed to be fetus is real fetus and not contaminated. The sample is determined to be either normal or abnormal after running the aneuploidy study.

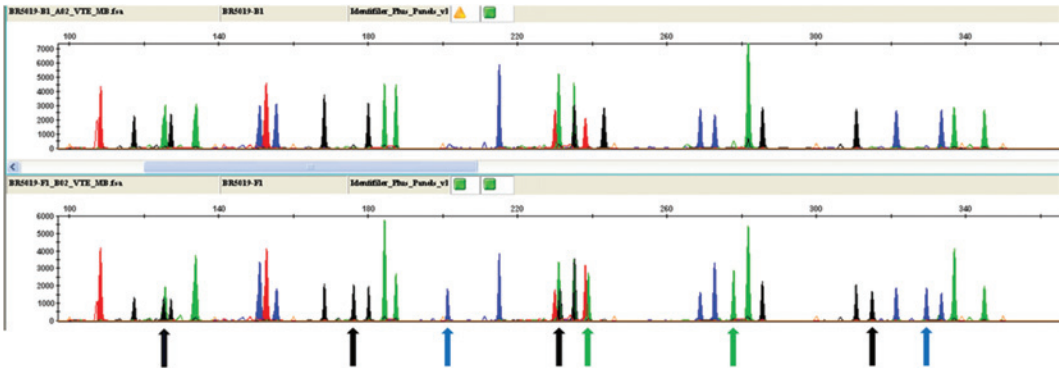


FIGURE 11.5 Polyploidy (Triploidy). Top: DNA from mother or gestational carrier. Bottom: DNA from fetus. Some extra peaks are observed on the fetus profile (arrows) and not in the maternal profile. This means that the miscarriage was the result of a polyploid (triploid in this case) fetus.

chromosomes 15, 16, and 22 were the most prevalent with 11.1%, 26.7%, and 17.8%, respectively. Aside from trisomies, deletion/duplication (del/dup) was found in 4.2% of the cases, involving chromosomes 1, 7, 8, 10, and 20. Monosomy 45,X was found in more than 11% of the cases. Interestingly, we found monosomy 21 in 1% of the cases, and this was the only autosomal monosomy found. Table 11.2 shows the detailed results.

Indications

While it would be useful to analyze every miscarriage, we recommend that the focus remain on those experiencing recurrent miscarriages as well as infertile patients undergoing ARTs.

Couples with Recurrent Miscarriage

Several studies have shown that in couples with previous miscarriages or aneuploid conceptions due to both sex chromosomes and autosomes the risk of fetal aneuploidy increases. Women who have had a previous trisomic pregnancy, particularly those <35 years old, appear to be at increased risk for subsequent trisomic pregnancies. The relative risk of trisomy 21 subsequent to trisomy 21 is greater for women <35 years old at the previous pregnancy [19,20]. Implantation and pregnancy rates decreased and miscarriage rates increase as the number of previous spontaneous abortions was higher [21].

Infertile Couples Undergoing ARTs

In the general population, spontaneous abortion accounts for no less than 15%–20% of all clinically recognized pregnancies during the first trimester. The incidence of chromosomal abnormalities in spontaneous pregnancy loss is approximately 50% [1].

Nevertheless, several studies have found that patients undergoing ARTs present higher rates of chromosomally abnormal products of conception compared to age-matched controls [22]. Some studies have reported chromosomal abnormality rates in POCs from infertile patients ranging between 63% and 76%, with higher than 50% estimated in the general population [23].

Additional studies suggest not only that chromosomal abnormalities are higher in infertile populations undergoing ARTs, but that the percentage and type of chromosomal aberrations vary depending on the

TABLE 11.2

Results of STRs + 24 Chromosome Analysis (aCGH and NGS) on POC Samples and Percentage of Specific Abnormalities Related to the Total Abnormalities Found

	Number of Cases	(%)
Number of cases processed	605	—
Maternal age (SD)	36.5 (4.3)	—
Gestational weeks (SD)	7.8 (1.5)	—
MCC[a]	113	18.7
Informative results	486	81.3
Normal results	227	46.7
Abnormal results	259	53.3
Trisomies	207	79.9
Monosomy 21	2	0.8
Sex chromosome monosomy	30	11.6
Del/Dup[b]	11	4.2
Triploidy	9	3.5

[a] MCC, maternal cell contamination

[b] Del/Dup, deletion (loss of small part of the chromosome)/duplication (gain of small part of the chromosome)

type of ART used [24]. Martinez et al. reported an increased percentage of chromosomal abnormalities in miscarriages from an IUI cycle compared to those from an IVF cycle, with the ARTs with the lower chromosomal abnormality rate in POCs those after IVF treatment in patients in an oocyte donation program [24]. They also reported that the types of chromosomal aberrations differ in the general population and infertile patients. Monosomy X presented with double the incidence in the group receiving ART compared to the spontaneous abortion group, while polyploidy presented with double the incidence in the spontaneous abortion group.

Couples with Severe Male Factor

Campos-Galindo et al. published in 2015 [25] that couples from ART with own oocytes in which the male had sperm concentration less than 5 million/mL produced 75% of abnormal results. Those with a concentration greater than 5 million/mL produced 51% of abnormal results. Males with oligoasthenozoospermia, low motility, and/or high FSH concentrations showed frequent synaptic abnormalities, which led to the production of aneuploid and/or diploid sperm. The risk of recurrent abortion increases with the presence of chromosome abnormalities in sperm [26,27]. This means that the male factor should be considered an important factor after a spontaneous abortion.

Couples with Environmental Exposure to Endocrine Disruptors

Many chemical compounds known as endocrine-disrupting chemicals (EDC) influence the endocrine systems of animals and humans. There is a growing concern about the effects of these EDCs in reproductive health. A link between EDC exposure and spontaneous miscarriage is suspected, but the results we have are limited [28]. Environmental exposure to metals has been associated with pregnancy loss. Impaired fetal growth, fetal loss, and neonatal deaths were significantly associated with heavy metals exposure during pregnancy. Even the existing controversial scientific literature acknowledges this issue [29–31].

One of the most common EDCs is bisphenol A (BPA). It is used to manufacture a wide range of products in common use, for example, products in direct contact with food (plastic bottles, food containers, etc.), medical equipment, and paints. Although little is known about the effects of EDCs on miscarriage, a number of studies suggest the relationship between exposure and miscarriage or other adverse prenatal outcome [28,32,33]. Hunt et al. demonstrated that daily oral dosing exposure (BPA) causes meiotic aneuploidy in the female mouse [34]. Therefore, in couples with known exposure to endocrine disruptors, the chromosomal analysis of POC could be indicated.

Limitations

Isolation of Fetal Tissue in the Sample Collection

Tissue collection is one of the important limitations to avoiding or ruling out MCC and providing accurate results in most of the samples. General recommendations for proper fetal tissue collection are as follows:

- Take a tissue sample with a minimum size of 3 × 3 mm (preferably a small but clean sample without blood rather than a larger size sample).
- Remove the largest blood clots from tissue sample.
- Use sterile saline solution to wash the samples.
- Place the tissue on the sterile cup with enough saline solution to cover the sample.
- Draw blood from the mother or gestational carrier before or after a D&C procedure to rule out MCC or detect polyploidy in any further analysis. Extract the blood into the EDTA tube to prevent clotting.

Figure 11.6 shows the appearance of the villi of the decidua in a clean tissue (Figure 11.6a) and the appearance of a hematic sample (Figure 11.6b). In a tissue sample collected under good conditions, the decidua appears pink, dense, and leaf-like. Once villi are well rinsed they tend to float and have a paler appearance and feathers compared with the decidua.

Mosaicism

Every technology has its own limitations. Recent data from Shah et al. [35] have shown there is a 33% discordant rate between results due to MCC, balanced chromosome rearrangements, polyploidy, and placental mosaicism, but regardless of the platform used, mosaicism was detected in 18% of all the samples.

More specifically, confined placental mosaicism (CPM) is a condition characterized by the discrepancy between the chromosomal/genetic makeup of the baby and the placenta. Approximately 2% of viable pregnancies studied using chorionic villus sampling (CVS) at 9–11 weeks gestation is confined to the placenta [35].

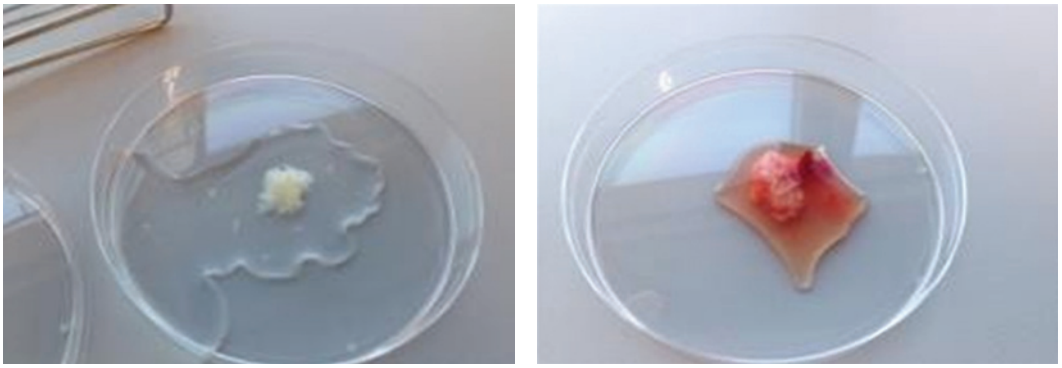
Detection of Del/Dup below the Resolution of the Platform

NGS technology improves the detection of Del/Dup, offering better resolution than conventional karyotyping. Nevertheless, some Del/Dup below the resolution of the platforms (6 Mb) could not be detected and resulted in a misdiagnosis [36]. Such de novo microdeletions represent less than 1% of the cases.

POC Decision-Making Workflow

Following a miscarriage, all women (or couples) should have access to support, follow-up, and formal counseling when necessary. For this purpose, in our lab, a standard operating procedure has been established for decision making in couples who suffer a spontaneous miscarriage (Figure 11.7). After a first trimester miscarriage, the POC and maternal or gestational carrier blood are collected. The POC is evaluated for 24-chromosome screening by NGS.

- If a normal result is found, we must discard the MCC by performing STR analysis. If the analysis shows no MCC, then we recommend the physician consider other potential causes of miscarriage.
- If the result is abnormal, it could be a whole chromosome aneuploidy or a small deletion (loss) or duplication (gain) of a part of the chromosome (Del/Dup). If an abnormal Del/Dup is found, we recommend checking if the abnormality is de novo or inherited. In some cases, the result could



(a) (b)

FIGURE 11.6 Tissue sample collection. (a) Villi of the decidua in clean tissue. (b) Villi of the decidua of a hematic sample.

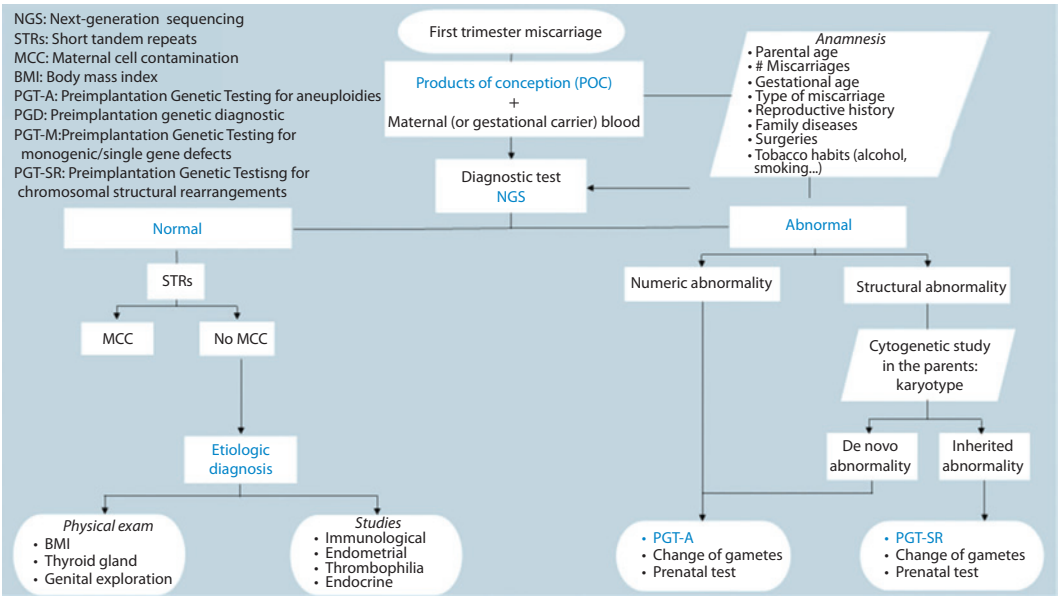


FIGURE 11.7 POC decision-making workflow.

suggest the presence of a balanced chromosome reorganization in one of the members of the couple. In this case we would recommend performing a karyotype in both members to establish the risk of miscarriage in further gestations. Either in de novo or inherited abnormal results, we would recommend Preimplantation genetic testing for monogenic/single gene defects (PGT-M) for aneuploidy or for structural rearrangements. Other options could be change of gametes or prenatal test.

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12

Non-Invasive Prenatal Testing for Aneuploidy and Beyond

Miguel Milán Sánchez, Emilia Mateu, Pere Mir Pardo, and David Blesa Jarque

CONTENTS

Introduction

Non-invasive prenatal testing (NIPT) was first made possible in the 1970s with the introduction of two-dimensional fetal imaging. Tests for indirect markers associated with, but not 100% predictive of, fetal chromosomal anomalies, such as alpha-fetoprotein, pregnancy-associated plasma protein A, and the free beta subunit of human chorionic gonadotropin, also became available. The current first trimester screen (FTS) calculates the risk of fetal aneuploidy based on indirect biochemical markers, ultrasound measurements at different weeks of fetal gestation, and maternal age. FTS has an aneuploidy detection rate of 85%–95% with a 5% false-positive rate [1]. Invasive prenatal testing is recommended to more accurately determine the chromosomal status of the fetus if the calculated risk value of aneuploidy is greater than 1/100 to 1/270, depending on the institutional policy and the combination of markers studied. The new generation of NIPT utilizes fetal DNA found in the maternal bloodstream to check for aneuploidy instead of relying on a proxy marker associated with aneuploidies [2]. Since the first description of circulating free placental DNA (cfpDNA) within the maternal bloodstream, there have been many improvements in its detection [3–7]. As a consequence, NIPT has a higher specificity and sensitivity than the FTS [8,9].

NIPT has been used primarily in high-risk populations. However, recent studies demonstrate that NIPT is superior to conventional screening methods in low-risk populations. NIPT has provided significantly lower false-positive rates and higher positive predictive values for the detection of trisomy 18 and trisomy 21 compared to standard screenings in general obstetrical populations [8,10,11]. Therefore, the American College of Obstetricians and Gynecologists and the Society for Maternal Fetal Medicine have updated their position statement on the use and regulation of NIPT [12]. They now include NIPT in conjunction with counseling on its limitations and benefits as a prenatal screening tool for any pregnant woman, regardless of her risk of fetal aneuploidy.

The higher specificity and sensitivity of the tests and the ability to perform the tests as early as the tenth week of gestation make NIPT highly attractive for practitioners and patients. There is an increasing demand for NIPT in the general obstetric population as an alternative or complement to FTS to significantly decrease the use of invasive prenatal procedures [13]. NIPT is not without limitations, however; there is much to be gained from studying its use in a prenatal clinic. A clinical decision algorithm for NIPT is proposed in Figure 12.1.

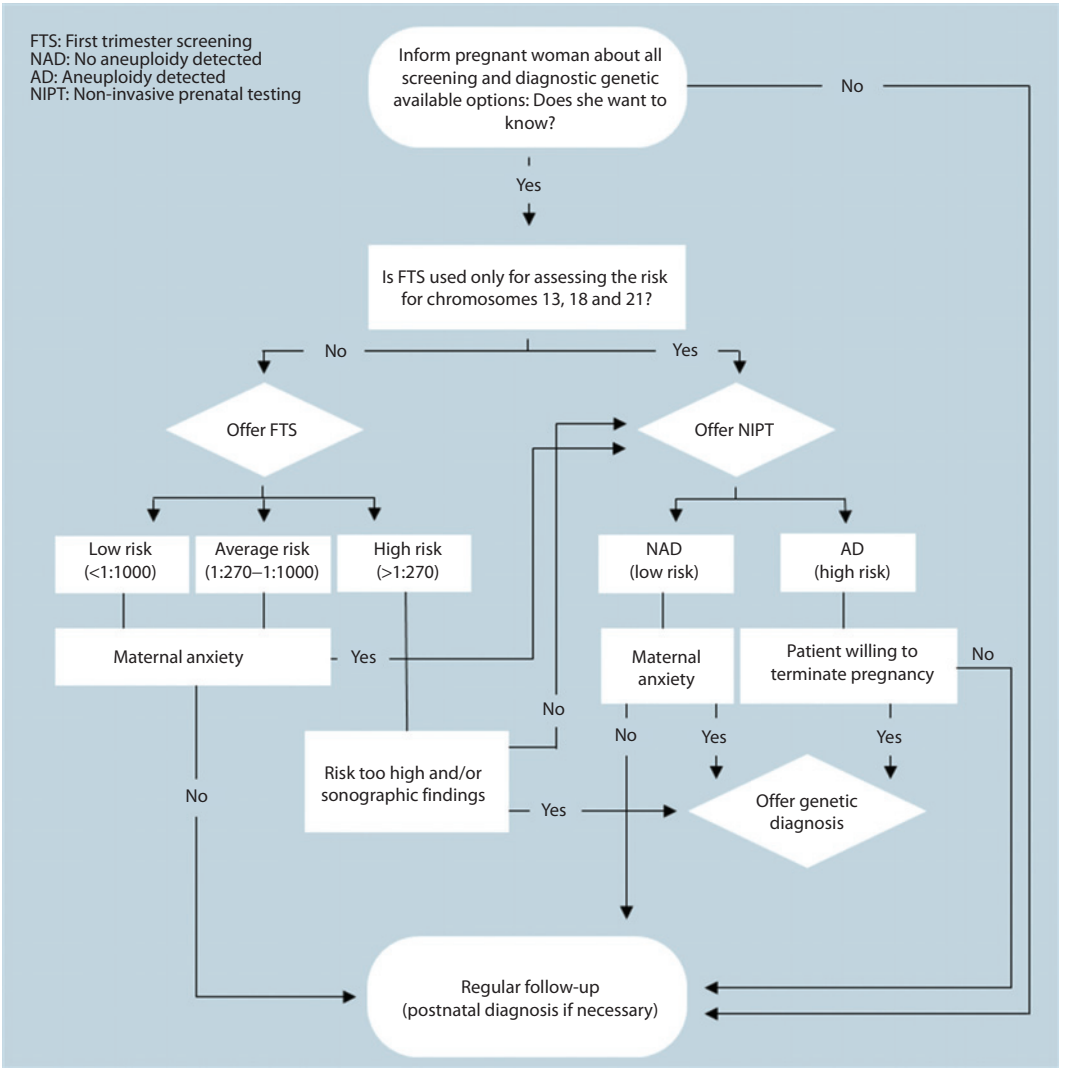

FIGURE 12.1 Clinical decision algorithm for NIPT.

Cell-Free Placental DNA Overview

Cell-free DNA (cfDNA) present in the bloodstream during pregnancy is a mix of maternal and placental DNA. The cfpDNA is present as small fragments produced by natural apoptosis events. Less than 1% of cfpDNA fragments are bigger than 313 base pairs [14]. Maternal and placental DNAs are differentially packaged by nucleosomes, resulting in different fragmentation patterns and, consequently, size differences between cell free circulating maternal DNA and cfpDNA [15].

The placenta is the main source of non-maternal cfDNA. During anembryonic pregnancies, in which the fetus fails to develop, the unique fetal tissue is the placenta. Similar cfDNA levels have been found between anembryonic and embryonic pregnancies, demonstrating the placenta as the main source of fetal cfDNA. In addition, DNA methylation profiles found in non-maternal cfDNA coincide with those found in placental cfDNA. In cases of fetoplacental discrepancies, genetic information from non-maternal cfDNA found in the bloodstream coincides with the placental genetic information [16]. The best accepted mechanism of DNA release from the placenta into the bloodstream is through apoptosis of trophoblastic cells [17,18].

One of the most important cfpDNA features is that all cfpDNA fragments disappear from the maternal bloodstream within 1–2 days postpartum [19]. Therefore, a previous pregnancy should not interfere with the NIPT results of a future pregnancy.

Benefits and Limitations of Cell-Free Placental DNA Testing

Current non-invasive prenatal screening technologies are based on sequencing cfpDNA present in maternal plasma by next-generation sequencing (NGS), also called massively parallel sequencing (MPS). In a single sequencing run, this technology allows the rapid sequencing of millions of DNA fragments simultaneously [20]. There are two primary approaches.

Random Sequencing

cfDNA is randomly amplified by polymerase chain reaction (PCR). All the products resulting from amplification are sequenced without differentiation between maternal and placental DNA. The obtained sequences are aligned to a reference genome to map their chromosomal origin. Finally, the number of sequences corresponding to each chromosome of interest is compared with a euploid reference sequencing run, and the fetal chromosomal copy number is determined [21,22]. The number of reads obtained with random sequencing range from 10 to 30 million per sample [21,23,24]. Although only some chromosomes may be analyzed for aneuploidy, all the sequencing information is used to normalize the data and make the samples comparable. In addition to aneuploidies, this methodology allows the detection of the most prevalent microdeletions [25]. This method is advantageous because it is independent of measuring and reporting the fetal fraction (FF). FF refers to the percentage of cfpDNA with respect to the total amount of cfDNA in the sample. The higher the amount of cfpDNA sequenced and analyzed, the higher the statistical power to correctly identify aneuploidies in low FF scenarios. On the other hand, one disadvantage to using this approach is the interference of maternal karyotype abnormalities, especially with those technologies that use normalized chromosome values (NCV) for the analysis. When an alteration is detected in the chromosomal information needed to calculate the NCV, a result is not reported for the test chromosomes. In addition, this methodology is the most expensive due to the comprehensive sequencing.

The second major approach is targeted sequencing. cfDNA is selectively amplified to enrich only those sequences from chromosomes of interest, the test chromosomes. There are two methods used with this approach.

SNP-Based Sequencing

This method is based on specific amplification and sequencing of single-nucleotide polymorphisms (SNPs). SNPs are the most common type of genetic variation in the human genome, and each SNP represents a change in one single nucleotide [26]. For analysis purposes, informative SNPs are those in which the mother is homozygous and the fetus is heterozygous. Parental genomic DNA is needed for this method. The required maternal genomic DNA is obtained from the buffy coat. Paternal genomic DNA is optional and can be obtained from a blood or a buccal swab sample. All the samples undergo PCR amplification specific to selected SNPs, and PCR products are then sequenced. A specific algorithm subtracts parental genomic information to deduce placental DNA data and determine the copy number of each interrogated chromosome [27,28]. The number of sequencing reads obtained in this method is on average 6 million reads per sample [27,29]. It is also possible to report deletions and microdeletions using this approach [30].

The SNP-based approach has several advantages. This method discriminates between placental and maternal DNA, makes it possible to ignore the maternal karyotype, and leads to better gender classification. In addition, SNP sequencing is unique in that, by detecting the presence of additional placental haplotypes [31], it is able to detect triploidy and the presence of a vanishing twin not previously identified by sonography. Finally, SNP technology is less costly than shotgun sequencing–based technologies. A major disadvantage is the constraint of the target obstetric population for testing: it is not possible to screen in cases of oocyte donation, surrogate pregnancies, multiple gestations, a vanishing twin (a vanishing twin is detected, but not analyzed), or parents related by blood. In addition, this approach cannot be performed if the FF of DNA is lower than 4%.

Targeted Massively Parallel Sequencing

This method, originally called digital analysis of selected regions (DANSR), implies targeted PCR amplification, sequencing, counting, and sequence comparison with a reference genome to determine fetal copy number. To assess important factors related to aneuploidy risk in the screening, the fetal-fraction optimized risk of trisomy evaluation (FORTE) algorithm was designed. It considers FF and maternal age, and adds this information to the direct study of cfDNA to achieve a more complete analysis. This methodology uses non-polymorphic loci to determine chromosome copy number and polymorphic loci to identify the FF [24,32]. This test is the least costly and produces the lowest number of sequencing reads among the three methodologies described here (the number of reads per sample ranges from 420,000 to 1 million). It shares one drawback with each of the other methods. As with any shotgun-based approach, an abnormal maternal karyotype can interfere with the analysis. In addition, as with other SNP-based methodologies, at least 4% of the FF is necessary to obtain accurate results.

Regardless of the technology used for the NIPT, limitations are common. It is important to bear in mind the following considerations for proper pre-test and post-test counseling. First, NIPT is a screening method, not a diagnostic. None of the described methods has a 100% detection rate; further, false-positive and false-negative results do occur. Second, the source of cfDNA is the placenta. If feto-placental discrepancies or confined placental mosaicism is present, a false-negative or false-positive could result [33]. In cases of fetal mosaicism, the detection depends on the degree of mosaicism. The larger the aneuploid cell population is, the higher the likelihood of detection; however, false-negative results are possible [34].

Fetal Fraction and Result Interpretation: Does the Percentage Matter?

As explained above, FF refers to the percentage of cfpDNA with respect to the total amount of cfDNA in the bloodstream. cfpDNA becomes detectable a few days post-conception [35]. The FF continually increases with gestational age about 0.1% per week until 20 weeks gestation, then increases 0.6% per

week from week 21 to birth [36]. At the clinical level, NIPT has been validated for detecting aneuploidies from 10 weeks gestation onward when the average FF is 10%, ranging from 0.6% to 50% [5].

Several factors and conditions can affect FF in the maternal bloodstream. FF can be reduced due to increased maternal weight [36,37] or by drug treatments for certain clinical conditions, such as integrase inhibitor treatment in HIV patients [38]. Maternal blood volume increases and maternal cell apoptosis have been proposed as mechanisms for cfpDNA dilution and consequently FF reduction. It is controversial, but, FF may also be reduced by some aneuploidies and digynic triploidy [39,40], probably as a reflection of a decreased placental volume. A correlation with small placental volume and low FF for fetal trisomy 18, trisomy 13, or digynic triploidy has been described [39,41].

Since some conditions may decrease the expected FF based on gestational age, it is important to know the minimum amount of cfpDNA needed to accurately detect aneuploidies. In general, and for almost all the NIPT providers, an arbitrary lower limit is set at 4% [34]. Other laboratories, however, do not estimate the amount of cfpDNA and report test performances as good as those companies considering the FF in the analyses. The American College of Obstetricians and Gynecologists and the Society for Maternal Fetal Medicine agree that the percentage of cfpDNA is important, but have not set a standard [42]. As shown in Figure 12.2, the higher the FF, the easier an aneuploidy is separated from the euploid status. Empirical evidence does not exist regarding the FF threshold needed for detecting chromosomal alterations for individual NIPT platforms, and all providers have published extensive clinical validation trials with comparable results. There are no statistical differences in false-negative rates between the different NIPT methodologies when providers use their optimal obstetrical population [5,11,33,43,44]. However, although similar test performances are reported for all companies, companies have not provided detailed information regarding test performance in samples within specific FF ranges along the broad FF spectrum. These data are of paramount importance for clinicians. Providing test performance for specific FF ranges may allow clinicians to calculate more accurate positive and negative predictive values, enabling more informed patient decisions regarding their future child.

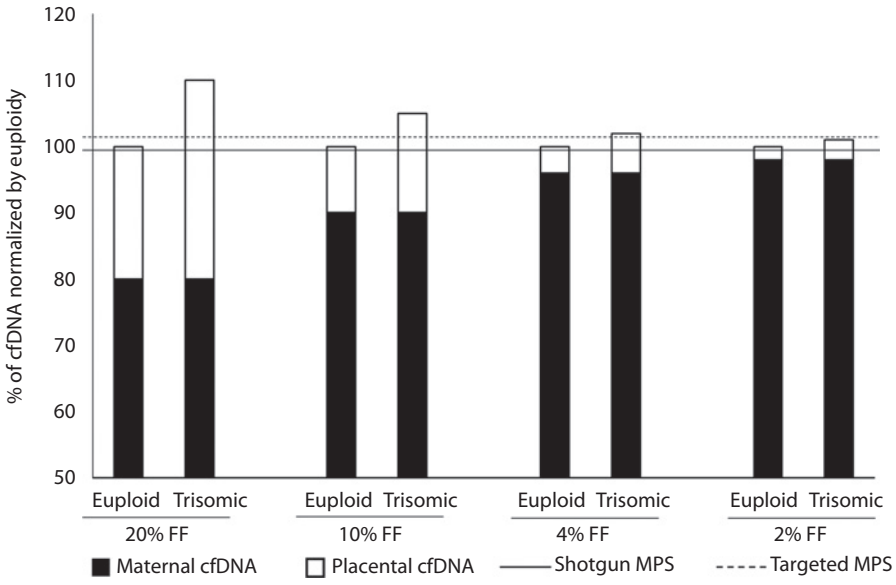

FIGURE 12.2 Importance of fetal fraction (FF) in the level of aneuploidy detection using different NIPT approaches. As shown, the higher the FF, the better the trisomy classification. Solid and dashed lines represent the sensitivity of each sequencing approach to detect a trisomy in the corresponding FF (as stated by each NIPT company; targeted MPS can correctly classify aneuploidies over 4% of FF, whereas shotgun MPS can correctly classify aneuploidies under this limit). cfDNA, circulating free DNA; MPS, massively parallel sequencing.

How to Prescribe cfpDNA Assessment of Aneuploidies

In brief, three main strategies should be considered by obstetricians and gynecologists when considering NIPT [13,45,46].

NIPT as a Secondary Screening

FTS is to be used as the primary screening method with NIPT only offered to women with a high aneuploidy risk.

- *Advantages:* Good detection rate of aneuploidy and cost-neutral or cost savings in prenatal care.
- *Disadvantage:* No improved aneuploidy detection in average and low-risk populations.

NIPT as a Replacement for Combined First Trimester Screening

FTS is not performed and NIPT is offered to all pregnant women.

- *Advantages:* Better detection rate of aneuploidies in all pregnant women and a lower percentage of women with a false-positive result than occurs with FTS alone.
- *Disadvantages:* Elevated cost in prenatal care and loss of information unrelated to aneuploidies for the tested chromosomes [47,48].

Contingent NIPT

New cutoff levels are adapted to FTS. NIPT is offered for average- and high-risk patients typically with risk values for aneuploidy between 1/50 and 1/1,000. Those individuals with a risk level of 1/50 or greater are offered invasive genetic diagnostic procedures [47,48].

- *Advantages:* No loss of extra information unrelated to aneuploidies for tested chromosomes and a high detection rate of aneuploidies.
- *Disadvantage:* No improved aneuploidy detection in low-risk populations.

NIPT is changing the way in which prenatal screening is offered to pregnant women. The superior positive and negative predictive values of NIPT compared to FTS [45] make NIPT the first-line screening method for many clinicians. However, cost-effectiveness evaluations should be considered when the testing costs are defrayed by public health systems or by insurance companies. In addition, patient desires and obstetrician information requirements should be considered when deciding what screening methodology is the best for prenatal care. At the end of this chapter, a general clinical decision algorithm for NIPT is proposed.

Beyond Chromosomal Aneuploidies

Several molecular approaches regarding fetal aneuploidy detection, fetal development monitoring, etc. have been developed for different purposes using maternal blood. Some of the most important research fronts are briefly reviewed in this section.

Monogenic Diseases

Although not extensively used at the clinical level, non-invasive diagnosis for single-gene disorders could be performed for inherited autosomal dominant, inherited autosomal recessive, and sex-linked diseases. Using cfpDNA, a quantitative comparison between the wildtype allele and a disease-causing allele is necessary to identify if the fetus has inherited a disease [49].

In 2008 investigators developed a quantitative approach named relative mutation dosage (RMD) analysis based on a digital PCR method [49,50]. Digital PCR analysis allows the measurement of wild-type and disease-causing alleles in maternal plasma so small differences in allele concentrations can be calculated [51]. This technique depends on the quality of the DNA sample and the total amount of cfpDNA [52]. Recent studies have shown that this method is clinically useful for the detection of thalassemia [51,53], hemophilia [54], and methylmalonic acidemia [55].

In 2010 a new platform using MPS of maternal plasma DNA was developed under the name of relative haplotype dosage (RHDO) analysis [16]. It is based on the analysis of haplotype blocks defined by heterozygous SNPs that flank a disease-causing mutation. The number of molecules that appear in two homologous chromosomes having the mutation and the normal allele are compared. The fetal genotype is deduced from specific ratios of mutant haplotypes versus wildtype haplotypes [50,52,56]. This diagnostic analysis has a high sensitivity and specificity [52], and allows the screening of pathogenic mutations in multiple gene regions and for multiple diseases. However, it still requires parental genomic DNA to determine the parental haplotypes [49,57]. RHDO has been used for a number of monogenic diseases including thalassemia [58], congenital adrenal hyperplasia [59], and Duchenne muscular dystrophy [60].

RNA Sequencing

Fetal RNA is also present in maternal plasma [61]. The detection of fetal RNA in maternal plasma allows a rapid screening of new markers, including those with intracellular localization and noncoding mRNA [62]. In addition, this approach can provide valuable information regarding the gene expression patterns of fetal tissues. Overall, with the discovery of new RNA markers, fetal RNA analysis, and thus gene expression, in maternal plasma may allow the non-invasive monitoring of pregnancy health in a multitude of clinical conditions [61]. Transcriptome analysis can be also informative for placental function and dysfunction and allow the early diagnosis of diseases with placental origin, like HELLP syndrome and preeclampsia [63,64].

However, detection of circulating RNA in the plasma has been challenging for several reasons: (1) There is more ribosomal RNA present than messenger RNA. (2) RNA is easily degraded and less stable than DNA, affecting the quality of the data. (3) The concentration of fetus-derived RNA transcripts in plasma depends on the expression of each gene and can vary greatly between genes and individuals [65]. (4) Finally, in terms of quantity, fetal RNA behaves as cfpDNA in maternal plasma. The ratio of fetal to maternal RNA increases with gestational age. RNA concentrations have been measured in plasma samples from pregnant women in their first, second, and third trimesters of pregnancy [66]. In the early pregnancy group, the mean fetal contribution was 3.70%. This proportion increased to 11.28% on average in the late pregnancy group.

Epigenetics in cfpDNA Sequencing

Epigenetic modifications play crucial roles in prenatal development, but monitoring them during pregnancy has been difficult since fetal tissues are not readily accessible. Since the discovery of cfpDNA in maternal plasma, it has been possible to assess that there is a difference in the methylation status of genes between the fetus and the mother in an allele-specific manner. For example, differential DNA methylation within the imprinted region between the insulin-like growth factor 2 and H19 genes has been detected between placental DNA and maternal DNA in maternal plasma [67]. In addition, differential methylation of the specific genomic region *maspin* (*SERPINB5*) was found between the placental and the maternal cells [68]. This was detectable in maternal plasma only during pregnancy, making it the first universal epigenetic fetal marker. Many different hypermethylated or hypomethylated placental-derived sequences have been described in relation to chromosomal aneuploidies [26,69].

Two main methods are used for the detection of fetal epigenetic markers in maternal plasma: bisulfite modification of the template DNA or methylation-specific restriction enzyme analysis [70]. The former is based on the change of unmethylated cytosine residues into uracil, leaving methylated cytosine unchanged. Methylation-specific PCR and DNA sequencing can be used to distinguish DNA sequences according to individual epigenetic profiles [71]. Using this approach, it was shown that cfpDNA in maternal plasma was hypomethylated and its concentration was 33.9% in pregnant women and only 4.5% after delivery. In addition, it was demonstrated that hypermethylated DNA molecules are larger than

hypomethylated molecules. This finding suggests that hypomethylated sequences are more prone to enzymatic degradation because they are less densely packed with histones.

The methylation specific restriction enzyme analysis is based on the use of methylation-sensitive restriction enzymes. These enzymes cannot cut a sequence if it contains a methylated base. Therefore, the maternal plasma can be treated with these enzymes and due to methylation differences, the unmethylated maternal DNA can be removed and the remaining methylated fetal DNA can be measured.

In the next few years, new technical approaches may offer non-invasive ways to clinically monitor important pregnancy-associated conditions based on the isolation and analysis of molecules from fetal origin: a fetal dialogue of genetic and non-genetic conditions that we are just starting to understand.

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13

Obstacles to Implementing New Technology

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CONTENTS

Introduction

In previous chapters we reviewed how genetics contributes to the likelihood of conceiving a healthy baby in the shortest period of time for a couple undergoing in vitro fertilization (IVF) [1]. In this chapter, we discuss in detail preimplantation genetic testing (PGT-A), a genetic test that has received increasing attention in recent years. Both new clinics and clinics with established programs can face operational issues that affect the implementation of PGT-A technology. We have observed operational barriers in mature markets in experienced clinics in the United States, Spain, and Brazil. It is not unusual to find clinics in these countries with long waiting lists for patients who want to use PGT-A. The backlog is usually attributable to lack of space or lack of trained personnel in the IVF lab. A cycle that includes PGT-A requires additional time from the embryologist. Physicians who decide to increase the percentage of their cycles with PGT-A might not be able to do so due to operational constraints at their IVF labs. We recommend that clinics who want to increase their PGT-A cases plan their resources and equipment carefully in advance.

Aspects of PGT-A

Although PGT-A provides advantages in assisted reproduction, it also has several drawbacks. These include:

1. Operational aspects, such as equipment and training
2. Cost and affordability
3. Non-transfer “drama”

This chapter does not discuss the ethical or religious aspects of the screening, which could also present obstacles for clinicians and patients.

Operational Aspects: Equipment and Training

PGT-A requires a biopsy of the embryo, which means that clinics must have certain equipment and proper expertise [2–3]. Biopsy imposes stress on the embryo, and clinics must have a properly trained embryologist since a deficient biopsy could result in failure of the program [4–5]. One of the worst situations for patients, clinicians, and reference labs is when a clinic that is not properly prepared to implement PGT-A decides to launch a PGT-A program. In such cases, the number of developed embryos will be low, the survival rate after biopsy will be limited, and the transfer of normal embryos will be compromised [6–7]. When this happens, the clinical team becomes frustrated and may have doubts about the program. We recommend that clinics planning to start a PGT-A program contact a recognized reference lab that can help them establish the program, train the embryologist, and validate the protocols before handling actual cases. International genetics labs offer the services of a senior embryologist consultant who travels to the clinic and spends several days with the IVF team preparing them to start a PGT-A program.

Both new clinics and clinics with established programs can face operational issues that affect the implementation of PGT-A technology. We have observed operational barriers in mature markets in experienced clinics in the United States, Spain, and Brazil. It is not unusual to find clinics in these countries with long waiting lists for patients who want to use PGT-A. The backlog is usually attributable to lack of space or lack of trained personnel in the IVF lab. A cycle that includes PGT-A requires additional time from the embryologist. Physicians who decide to increase the percentage of their cycles with PGT-A might not be able to do so due to operational constraints at their IVF labs. We recommend that clinics who want to increase their PGT-A cases plan their resources and equipment carefully in advance.

Cost and Affordability

Cost is the overwhelming reason why clinicians choose not to recommend PGT-A. In general, we have observed a decrease in the cost of genetic tools in IVF in the last 5 years through new technologies and increased competition; this is also the case for PGT-A. The introduction of next-generation sequencing (NGS) has enabled reference labs to significantly reduce the cost of their diagnostic services [8–10]. This cost reduction ranges from 25% to 50%,[1] depending on the market. The trend in some countries, such as the United States, to move a high percentage of cycles to frozen cycles also helps reference labs optimize their operations and increase efficiency while reducing costs [11–13].

In previous chapters, we discussed the current status of PGT-A and the latest results of randomized clinical trials demonstrating its contribution to improved success rates per transfer in any age range [14–16]. PGT-A allows for single embryo transfer, reducing the number of multiple pregnancies [17–20]. In general, clinicians worldwide agree that PGT-A helps them select the best embryo, increasing the success rate per transfer and reducing the risk of miscarriage. However, they are not usually ready to include PGT-A as a routine practice for all their patients. Although we have observed an increase in clinics that have decided to use PGT-A for all their patients, they are still a minority.

Several factors affect the cost of PGT-A: the cost of the biopsy, the biopsy strategy (day 3 vs. day 5), the type of cycle (fresh vs. frozen), the margin that the clinic applies to the biopsy, the type of technology used (FISH, aCGH, or NGS), the cost of the genetics lab, the genetics lab pricing models, and the transportation of samples.

Next we describe the different cost components of PGT-A:

1. *Intracytoplasmic sperm injection (ICSI):* ICSI is recommended as part of performing PGT-A to prevent sample contamination that could affect the result of the test and lead to a false negative or positive result [21]. This cost is not necessarily linked to PGT-A since most IVF cycles today are performed with ICSI.
2. *Biopsy fee:* Several costs are related to the embryo biopsy to obtain the sample that will be diagnosed by PGT-A. This cost differs, depending on the type of biopsy.
    a. *Day 3:* All embryos are biopsied at the same time with the same set of pipettes. This procedure is faster and more efficient in terms of equipment consumption and the

embryologist's use of time. This type of biopsy is more commonly used outside the United States. Although there might be some savings at the IVF lab, they are offset by the fact that more embryos will be tested by PGT-A versus the number of embryos if biopsy occurs on day 5. In general, we see a certain number of embryos that do not survive to day 5 of development and therefore the number of embryos requiring PGT-A testing is lower [22]. The pricing model of the reference lab is also highly relevant to the cost of PGT-A. A day 3 biopsy will allow for a fresh cycle when clinics do not have a reference genetics lab close by because it allows more time for the logistics of sending the sample from the clinic to the lab. In countries with no access to a genetics lab, where clinics need to send samples abroad, a day 5 biopsy forces the clinic to vitrify embryos. Instead, due to the fact that with day 3 biopsies there are 48 hours from biopsy to embryo transfer, fresh cycles are possible.

b. *Day 5:* This biopsy strategy is gradually growing in popularity among clinics. Most cycles in the United States are already day 5. There are a few pros and cons to using day 5 embryos:
   i. Pro:
      – Fewer embryos to biopsy than day 3.
   ii. Cons:
      – Generally, on day 5, not all embryos will be ready for biopsy at the same time and, therefore [23], the embryologist will have to perform biopsies for the same patient more than once. This has cost implications due to (1) additional complexity in the IVF lab and more time required of the embryologist and (2) additional consumables.

3. *Fresh versus frozen:* The preference of the doctor regarding the use of fresh versus frozen cycles also impacts costs [11–13]. Some publications [24] indicate that deferring the transfer to a non-stimulated endometrium can improve the pregnancy rates. This strategy will also impact cost.
   a. Vitrification typically involves two components: cost of embryologist's labor and consumables to vitrify embryos.
   b. Transportation: Frozen cycles do not require express delivery, while fresh cycles do. This allows for the use of more economical fares or carriers. The cost of transportation for fresh cycles can be as much as six times more expensive than frozen. In addition, frozen cases allow clinics to group several cases in the shipment, which also reduces the cost per patient.
   c. IVF lab costs and reference lab costs: Frozen cycles increase the efficiencies of both the IVF and PGT-A labs [11–13].
      i. In the IVF clinic, planning, personnel, and equipment related to the transfer of the embryos can be optimized, eliminating transfers on weekends and holidays, avoiding peaks of workload, and in general contributing to streamlining the overall costs of the clinic.
      ii. In the PGT-A reference labs the same logic applies. Fresh cases, especially from the day 5 biopsy, force labs to have resources working overnight and always add a higher level of stress in the process. This is also relevant to testing, especially for arrays and NGS.

4. *PGT-A technologies:* We have already learned that pregnancy results can differ, depending on the technology. FISH technology (PGT-A 1.0) cannot be compared to arrays or NGS (PGT-A 2.0) and their costs are also different. NGS already has an impact on the cost of PGT-A since it helps reduce the cost per sample, especially for reference labs with high volume and samples related to frozen cases. NGS becomes a more efficient technology than arrays or PCR when a high volume of samples is processed together in the same run since the cost of reagents can be divided among a larger number of samples. NGS allows labs to process runs of 15, 24, or even 96 samples at the same time.

5. *The genetics lab pricing models:* Here, we describe different pricing models that have been observed in markets around the world. Market competition has increased the creativity of labs, and labs are launching new formulas to help patients afford PGT-A.
   a. Up to 8 embryos with extra cost for additional embryos (traditional pricing model). This model benefits patients with a high ovarian reserve and high stimulation protocols that generate large numbers of embryos. It is also beneficial for patients using PGT-A for family balancing programs in markets where this practice is allowed.
   b. Packs of embryos (1–4, 5–8, and so on): This pricing model helps patients with a low number of embryos. It adapts better to a larger patient base.
   c. Flat fee program: This is a novelty in some markets. Reference labs agree to set a fixed price for PGT-A independent of the number of embryos. The price is set based on the average number of embryos in each clinic. A clinic specializing in Family Planning patients will have a larger average number of embryos versus a clinic specializing in complicated cases (this clinic will serve patients with advanced maternal age and poor respondents). This program is very useful in reducing the uncertainty of the final cost to patients. Most patients highly value a fixed cost of IVF treatment.
   d. Batching programs: This type of pricing model has increased in popularity recently. Some labs offer up to 8 embryos over the course of 9 months with the possibility of sending several sets of embryos (up to 8) during a period of time. Other labs offer the same proposal with the limitation that once a normal embryo is diagnosed, the program ends. In any case, all these programs benefit poor respondents. With previous pricing models, when treating poor respondent patients, several stimulations would take place until a certain number of embryos were available (i.e., 6 embryos) so that PGT-A usage would be maximized. The disadvantage of this strategy is that unnecessary stimulations are applied to patients when a normal embryo is obtained at the first or second stimulation. Therefore, drug cost is wasted and patients undergo unnecessary discomfort.

These are the major cost factors related to PGT-A and the overall view of the total cost. If PGT-A can benefit all patients and reduce the number of unsuccessful transfers, could we define a financial model that could help patients and physicians understand the real cost of a baby taken home for each strategy? Could we use the known pregnancy rates for the different groups of patients to calculate the expected average final cost with or without PGT-A?

When talking with clinics, we often hear that many patients cannot afford PGT-A and that they prefer not to include it unless it is requested by the patient or when they think a patient can afford it. But have any studies made a financial comparison of the cost of the baby taken home when not using PGT-A versus when it is used? What is the basis of the decision when the costs are not clear? We know that a patient will need fewer transfers when using PGT-A, and we also know that PGT-A will reduce the number of multiple pregnancies. PGT-A will also significantly reduce the number of miscarriages. These three aspects will have an impact on the cost to the patient from both economical and emotional points of view.

In the following section we describe a financial model in an attempt to answer the above questions. Most of the variables included in the model are averages for different clinics and countries; to use this information in a clinic, the variables and costs need to be updated. However, the logic of the model will apply to most markets and clinics throughout the world.

Financial Model PGT-A versus Non-PGT-A

Here we weigh the option of including the PGT-A in a cycle. We are not going to include the variable of fresh versus frozen but only the variables that will affect both strategies. Let us start defining them.

1. *Patient age:* The age of the patient is a very relevant variable in defining the cost efficiency of PGT-A. Advanced maternal age patients will have a higher percentage of abnormal embryos, so a tool that helps identify the normal embryos will help reduce the number of unsuccessful transfers.

2. *Male factor:* Embryos created with sperm from males with severe male factor (<2 million sperm/ml) will have a higher number of chromosomally abnormal embryos, increasing the number of unnecessary transfers.
3. *Number of previous miscarriages:* Patients with previous miscarriages will have a greater chance of having Asherman syndrome [25–27].
4. *Patient response profile:* Patients could be classified as Low, Normal, or High depending of the number of expected oocytes.
5. *Biopsy day:* This must be taken into account to estimate the number of embryos needed to be tested. As explained previously, at day 3 of embryonic development we will find more embryos and also a higher percentage of abnormality than at day 5.
6. *Number of embryos to be transferred:* This is a critical variable in the model since transferring more than one embryo will increase the chances of finding a normal one, although it will also increase the likelihood of multiple pregnancies.
7. *Probability of miscarriage:* This variable needs to be taken into account when comparing both strategies since it will have an impact both financially and emotionally for the couple. We have built a table relating the age of the patient and the probability of ending up with a miscarriage from randomized and retrospective data.
8. *Probability of a D&C procedure:* As with miscarriage, we need to consider the cost associated with a D&C procedure, which does not include the cost of lost days of work after the procedure and a delay in pregnancy. According to the American Pregnancy Association, around 50% of miscarriages require a D&C procedure [28].
9. *Probability of Asherman syndrome:* It is important that patients understand not only the associated cost of both strategies but also the additional risks of transferring abnormal embryos. The risk of Asherman syndrome increases with the number of D&Cs performed. After a single termination the risk is 16%; however, after three or more D&Cs, the risk increases to 32% [29].
10. *Expected days to achieve a normal pregnancy:* Patients must understand the average expected time required to achieve a normal pregnancy [30]. A couple using PGT-A will require 100 days less to achieve a pregnancy than a couple not using PGT-A.

Using data from randomized clinical trials and complementing it with retrospective data from several clinics, we have assessed a percentage of abnormal embryos by age along with the impact of male factor.

Based on these data and the variables described above, we can assess the number of expected embryos, the number of expected normal embryos, the expected average number of transfers to achieve a normal pregnancy, the probability of a miscarriage, the probability of a D&C, the probability of Asherman syndrome, and the average expected number of days to obtain a normal embryo and pregnancy.

Once the above variables are calculated or estimated, the costs are also defined. The most important variable in the model is the number of expected transfers. This is the variable that has the highest impact on the cost. Typically, an IVF cycle includes one transfer. Every additional transfer will have a cost that can range from 20% to 30% of the initial cost and that is similar to the cost of PGT-A.

Here, we illustrate how the model will help patients and doctors make the comparison. We will describe two examples:

1. Female, 38 years old, with good ovarian reserve (high responder), no previous miscarriage, and normal male factor
2. Female, 41 years old, with low ovarian reserve (low responder), two previous miscarriages, and abnormal male factor

In both examples we have not included the cost of average days lost at work due to D&C procedure or the psychological cost related to miscarriage.

Example 1

Couple description (Table 13.1): Female, 38 years old, with good ovarian reserve (high responder), no previous miscarriage, and normal male factor.

Financial information about the clinic (example costs, Table 13.2):

1. *Cycle cost:* The cost that the patient would pay including ICSI and one transfer.
2. *Medication cost:* Patient cost of the stimulation drugs.
3. *Additional transfers:* Typically additional transfers are paid separately from the cost of the cycle.
4. *PGT-A cost:* Cost for the patient of including a PGT-A service in their IVF treatment.
5. *Cost of the biopsy:* Cost for the patient that the clinic will charge for performing the biopsy; not included in the cycle cost.
6. *Cost of amniocentesis or NIPT:* A patient with advanced maternal age who gets pregnant would be categorized as a high-risk patient and therefore a prenatal test would be recommended, either amniocentesis or a non-invasive prenatal test.

The intention of this model is to generate two types of information:

1. Clinical data
2. Financial data

Patients would have relevant information that would enable them to make an informed decision.

Table 13.3 provides the expected average information for the clinical aspect of the treatment. Let's describe the variables:

1. *Percentage of abnormal embryos:* Critical variable of the model that allows for the estimation of the abnormal and normal embryos expected for a certain age.
2. *Expected normal embryos:* After the assessment of the ovarian reserve, we can estimate the expected number of embryos that will arrive at the blastocyst stage, and using the percentage above, we can estimate the average number of normal embryos that a certain patient might obtain.
3. *Probability to obtain a normal embryo:* It is crucial for a couple to fully understand the chances of selecting a normal embryo from a group of embryos. It is generally accepted that the

TABLE 13.1

Clinical Data

Female age	38
Male factor	No
Day of biopsy	5
Number of previous miscarriages	1
Patient response profile	High
Expected number of embryos	4
Number of embryos to be transferred	1

TABLE 13.2

Cost Data (Euros)

Cycle cost	6,000
Medication cost	1.200
Additional transfer	1.950
PGT-A cost from lab	1,500
Cost of biopsy	700
Cost of amnio or NIPT	750

TABLE 13.3

Average Percentages for Clinical Results

Clinical Results	Non-PGT-A	With PGT-A
% of abnormal embryos	62%	62%
Expected normal embryos	1.52	1.49
Probability of choosing a normal embryo at first transfer	38%	99%
Probability of pregnancy at first transfer	23%	59%
Probability of miscarriage	36%	8%
Probability of multiple pregnancy	1.4%	1.4%
Probability of D&C	18%	4%
Asherman syndrome risk	3%	0.3%
Expected number of months to baby at home	19.5	16
Expected number of transfers to select a normal	2	1

TABLE 13.4

Financial Results

Financial Results	Non-PGT-A	With PGT-A
Cycle cost	6.000	6.000
Medication	1.200	1.200
Cost of additional transfers	1.995	0
Cost of PGT-A	0	2.200
Cost of D&C	292	65
NIPT	492	0
Expected cost of transferring a normal embryo	9.487	9.465

morphology of embryos will not allow an embryologist to select the euploid embryo and therefore the selection of a normal embryo from a group can be calculated with statistics. In the case of this patient, the couple has a 38% probability of selecting the right embryo without using PGT-A. In the case where PGT-A is applied, we consider that the probability increases to the limit of the reliability of the technique (around 99%).

4. *Expected number of transfers:* The most relevant variable of the model. Using this model, we can conclude that this variable is the one with the largest impact in the expected average cost of the process of finding a normal embryo and consequently in obtaining a healthy baby at home. Taking into consideration that each additional transfer has a cost from 20% to 30% of the cost of a cycle, in many cases adding PGT-A to the treatment will be less costly than not doing it in the first place. The calculation of the number of transfers is performed based on the statistical formula of hypergeometric distribution. Later in this chapter, we will also address the "non-transfer drama" that some clinicians have when, due to PGT-A, all embryos are identified as abnormal.
5. *Probability of multiple pregnancies:* It is widely practiced in the IVF community to aim for single embryo transfer to reduce the number of multiple pregnancies. There are two main reasons for this strategy: (1) health advantages to babies born singleton versus those born with multiple pregnancy and (2) financial disadvantages of multiple pregnancy. According to a study presented as a poster at the 61st Annual Clinical Meeting of the American Congress of Obstetricians and Gynecologists, New Orleans, LA, May 4–8, 2013, pregnancies with the delivery of twins cost approximately five times as much compared to singleton pregnancies, and pregnancies with the delivery of triplets or more cost nearly 20 times as much.

Once we have all the clinical data for our theoretical patient, we can analyze the expected average cost for both strategies, with and without PGT-A (Table 13.4).

For the couple described in this example, according to our calculations, PGT-A would not be more expensive than a non-PGT-A strategy.

Example 2

Female, 41 years old, with low ovarian reserve (low responder), two previous miscarriages, and abnormal male factor (summarized in Table 13.5 with average percentages in Table 13.6 and financial results in Table 13.7).

TABLE 13.5

Clinical Data

Female age	41
Male factor	Yes
Day of biopsy	5
Number of previous miscarriages	2
Patient response profile	Low
Expected number of embryos	2
Number of embryos to be transferred	1

TABLE 13.6

Average Percentages

Clinical Results	**Non-PGT-A**	**With PGT-A**
% of abnormal embryos	79%	79%
Expected normal embryos	0.42	0.41
Probability to choose a normal embryo first transfer	21%	99%
Probability of pregnancy at first transfer	13%	59%
Probability of miscarriage	44%	8%
Probability of multiple pregnancy	1.4%	1.4%
Probability of D&C	22%	4%
Asherman syndrome risk	7%	0.7%
Expected number of months to baby at home	19.5	16
Expected number of transfers to select a normal	2	1

TABLE 13.7

Financial Results

Financial Results	**Non-PGT-A**	**With PGT-A**
Cycle cost	6,000	6,000
Medication	3,600	3,600
Cost of additional transfers	1,950	0
Cost of PGT-A	0	2,200
Cost of D&C	356	65
NIPT	555	0
Expected cost of transferring a normal embryo	11,906	11,865
Cost of multiple pregnancy		
Total expected cost	11,906	11,865

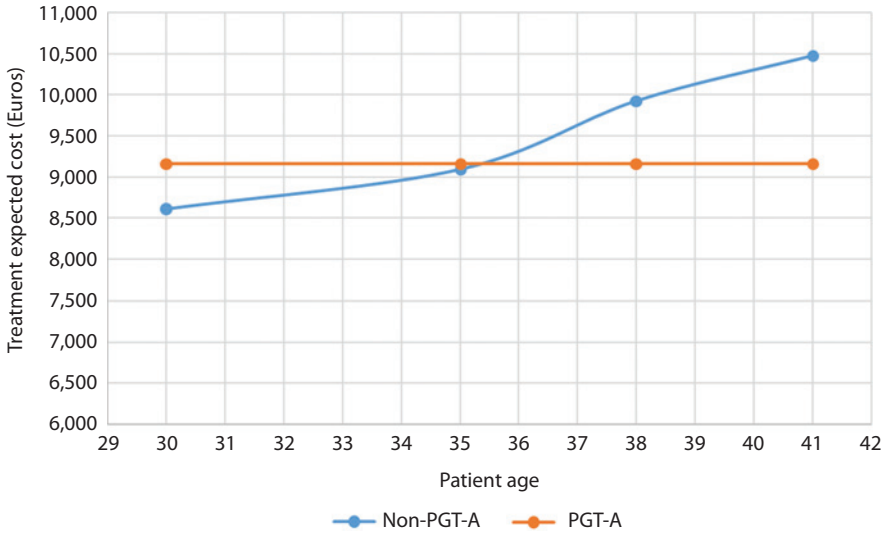

FIGURE 13.1 Cost comparison of treatment strategies. Non-PGT-A, non-preimplantation genetic screening; PGT-A, preimplantation genetic testing for aneuploidy.

An analysis of the cost comparison of both treatment strategies is represented in Figure 13.1. The PGT-A strategy might be more cost-efficient for patients older than 35 years old.

The Non-Transfer Drama

Another barrier that some doctors find to adopting PGT-A is what I call "the non-transfer drama." In this situation, PGT-A is performed and all embryos are diagnosed as abnormal. Some clinicians refer to this situation as a "drama" for them and their patients. We believe that being able to filter the abnormal embryos is a great advantage that will avoid unsuccessful transfers that result in repeated frustration and expensive processes. When all embryos are identified as abnormal, there is a reason that explains the infertility and clinicians can have an explanation that otherwise would be creating doubt in patients. The reason for infertility is the genetic profile of the embryos and not the skills of the clinic.

Conclusion

PGT-A requires a biopsy of the embryo, which means that clinics must have certain equipment and proper expertise [2–3]. Biopsy imposes stress on the embryos, and clinics must have a properly trained embryologist since a deficient biopsy could result in failure of the program [4–5].

Among the main barriers of adoption, cost is the overwhelming reason why clinicians choose not to recommend PGT-A. The introduction of new technologies like NGS has allowed reference labs to significantly reduce the cost of their diagnostic services [8–10]. In addition, reference labs have also launched new price proposals that cover not only patients with a high number of embryos but also the poor respondent patients. These two factors might reduce the cost barrier in the future and more patients might be able to afford the service.

From the cost point of view is also important to use a comparison financial model before assessing what option is more cost-effective for patients. We have designed a model taking into account the most relevant variables that could help assess the most attractive cost strategy for each couple depending on their own clinical profile. In general, we have observed that PGT-A is not necessarily

more expensive that a non-PGT-A treatment due mainly to the additional transfers that non-PGT-A treatment will require to find the normal embryo. Only patients under 35 years old might find a small cost disadvantage ranging from 6% more expensive for 30-year-old patients to 2% more expensive for 34-year-old patients.

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14

Dynamics and Ethics of Reproductive Genetics

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CONTENTS

Introduction

This chapter on ethical aspects of new genetic diagnostic technologies in reproductive medicine focuses on the use of these technologies in four interconnected contexts, moving from individual patient care to forms of reproductive population screening. In the first half of the chapter we discuss different applications of preimplantation genetic testing (PGT). This includes some new developments in preimplantation genetic diagnosis (PGD), the current debate about aneuploidy screening (PGS-A), and the future prospect of comprehensive genomic embryo screening. In the second half of the chapter we consider proposals for considerably widening the scope of genetic testing in the context of gamete donor screening, with an eye to improving the safety of third-party assisted reproduction. The idea to test all donors (and recipients) for carrier status for a wide array of autosomal recessive disorders directly connects with our next theme: the ethics of offering expanded preconception carrier testing as a form of population

screening. From there, we move on to discuss how new genomic technologies have already changed the face of prenatal screening and will continue to do so. In our concluding remarks we briefly reflect on the relation between the dynamics of the field and the ethical debate about new scenarios and challenges.

Preimplantation Genetic Diagnosis (PGD)

Since its introduction in 1989, PGD has become an established reproductive option in many countries for couples at high genetic risk of having an affected child. Its main advantage is that these couples can have a genetically linked child without confronting the traumatic decision of selectively terminating a wanted pregnancy after prenatal diagnosis (PD). Undoubtedly, PGD remains somewhat controversial [1]. Critics' objections include that embryo selection is at odds with the "sanctity of human life," that IVF/PGD carries disproportional burdens and risks for women, and the slippery slope argument, which points to possible future abuses of PGD. Such criticism, however, is not widely held to be convincing. The dominant view holds that the preimplantation embryo has a relatively low independent moral status, that balancing the burdens and risks of the different reproductive options, including the use of donor gametes, PD, and PGD, for women/couples at high risk of having an affected child is rather personal, and that, apart from the fact that the critics themselves widely disagree about what precisely constitutes "abuse," the slippery slope argument does not seem to be a strong argument to reject and prohibit PGD altogether. It simply underscores the need to engage in an ethical and societal debate about "where to draw the line."

That said, PGD raises different sorts of ethical issues. A first set of issues regards the indications, "the front door," so to say. What diseases are "serious enough" to qualify for PGD? Or, somewhat broader, what reasons are good enough to justify PGD? A second issue regards "the back door": what to think about the rule, "never transfer an affected embryo"?

Which Indications?

PGD within the "Medical Model"

There is a strong consensus that PGD is morally justified if it is linked to the "medical model," i.e., if it aims at avoiding the conception of a child affected with a serious disease or handicap. It is regularly proposed to make a detailed, restrictive list of acceptable indications. Such a list is, however, problematic. Many disorders have a variable expression; the list would require constant updating taking into account therapeutic developments; it could well entail a discriminatory message about the ("unworthy") lives of people affected with the conditions enlisted; and, last but not least, it does not take into account the family history and personal experiences and circumstances of the applicants (see below).

The aim of PGD to avoid children with serious congenital or childhood disorders is widely accepted, probably because it is assumed that such disorders will have most adverse consequences. More contested applications include PGD for late- or midlife-onset disorders, even if these are untreatable, such as Huntington's disease. Critics object that PGD for such disorders is unwarranted as the child will have (many) decades of good and unimpaired living. This criticism seems to disregard, however, that the prospect of HD imposes a severe burden on the members of affected families. Still, in more recent debates at least three ethical questions arise [2]. First, what about the future loss of parental competence in carriers of HD? Can reproductive physicians involved in IVF accept this risk, taking into account their professional responsibility to avoid high risks of serious suffering/harm for the possible future child? Although the development of HD in a parent is, no doubt, always burdensome for children, many children are able to cope. Further discussion is needed to see how relevant variables including the coping skills of the partner not affected with HD and the quality of the network of the family would allow decisions to be made on a case-by-case basis [3]. Second, what about exclusion-PGD? Though the procedure is "unnecessary" in 50% of the cases, the weighing of other relevant factors, including the wish not to know one's genetic status and the burdens involved in IVF/PGD, is rather personal [4]. And third, what about applicants who carry a reduced penetrance allele (RPA), entailing 35–39 CAG repeats, for HD? PGD may indicate that the embryo carries an RPA or (after expansion) a full penetrance allele (FPA). It is estimated that

some two-thirds of the future children carrying an RPA will have HD in (late) adulthood, before the age of 75. Furthermore, these children are at significant risk that their own children will carry an FPA (after expansion). PGD aimed at the non-transfer of embryos with an FPA or an RPA is morally justified in view of this risk combination.

An even more controversial indication regards preventable/treatable later-onset conditions, especially if caused by mutations with an incomplete penetrance, like hereditary breast and ovarian cancer (HBOC). But obviously, although the penetrance is incomplete, it is still very high; the cumulative risk for breast and ovarian cancer may be even higher than 90%. Preventive surgery mostly has major implications for women's welfare, whereas it is not 100% effective. PGD for these and other hereditary cancers and also for some of the cardiogenetic conditions may, we would argue, be morally justified. The view that the availability of treatment makes PGD obsolete is one-dimensional and too restrictive: if children's and families' quality of life is seriously (adversely) affected even though treatment is available, PGD may still be a justified option.

Contextualized Proportionality

It is often assumed that the moral acceptability of PGD depends on whether (1) the efforts, burdens and possible risks of IVF for women involved, (2) the possible risks of IVF and the (thus far, theoretical) risks of PGD for future children thus conceived, (3) the inherent embryo loss, and (4) the costs are in proportion to the benefit of avoiding the conception of an affected child, taking account of the seriousness and age of onset of the condition, the penetrance of the mutation, and possible preventive and/or therapeutic interventions for carriers and/or affected people. Although this way to make the proportionality criteria operational is already quite complex, it disregards three contextual elements that may directly influence the proportionality of IVF/PGD [5].

First, the family history, the obstetric history and personal circumstances of the applicants, may strongly color their perception of seriousness; a disorder that may not be evidently serious from a medical point of view may totally undermine the reproductive confidence of the applicants. It is remarkable and inconsistent to see that while "personal" elements are regularly taken into account when considering applicants' requests for traditional PD, they are often neglected in discussions and guidelines regarding PGD.

Second, the "fertility status" of the applicants is crucial. In the most common situation, fertile people undergo IVF/PGD simply in order to avoid the birth of an affected child. Alternatively, a couple opts for IVF/ICSI because of subfertility and wants to add PGD in order to avoid the transmission of a particular genetic disorder. The proportionality principle may, then, imply that in the latter situation the criteria for PGD may be somewhat more permissive; after all, because the couple will have IVF/ICSI *anyway* and "not selecting" between the embryos available is mostly not an option, the decision to engage in targeted PGD is relatively simple to justify. Consider, e.g., PGD to select female embryos in the case of ICSI for males with an Yq microdeletion.

And third, when people make use of IVF/PGD primarily for a disease that most, if not all, would qualify as serious, they may ask for "combination PGD," including a second, less serious, disease that runs in their family. Think, e.g., of a combination of cystic fibrosis (CF) (serious and qualifying for PGD) and phenylketonuria (PKU) (of debatable seriousness, according to many people, and therefore a debatable PGD indication in itself). Again, as the decision to engage in IVF/PGD has already been taken and considered to be proportional, a more permissive policy regarding the additional step seems to be justified.

Clearly, the justification and implications of such threefold contextualization of proportionality need further analysis and debate.

PGD for "Indirectly Medical" Cases

Some applications of PGD do not fit the medical model stricto sensu, as (at least part of) the testing is not linked with possible health problems of the future child, whereas there still is a link to the medical model in the wider sense, in that the testing may be relevant for the health of a "third party" [6]. These applications are, then, "indirectly medical." A first example is PGD/HLA typing. The main ethical condition is that the future child should be truly welcome; it should not be valued just as a cell bank. Obviously,

the low "take home baby rate" (THBR) should be clearly communicated to the applicants beforehand [7]. A second example is PGD/sex selection to avoid reproductive dilemmas in (healthy) future children. Consider the case of a male patient suffering from hemophilia. Some of these males prefer to conceive male progeny only, because sons will not carry the mutation, whereas all daughters would be obligate carriers. PGD aimed at avoiding this transgenerational risk may well be morally justified as long as flow cytometric sorting of sperm is experimental and not widely available.

PGD within the "Autonomy Model"

According to this view, prospective parents are free to use PGD in order to select embryos on the basis of any characteristic they prefer. While critics argue that selecting for non-medical characteristics violates the autonomy of the future child as the child is reduced to an object of parental ambitions and ideals, others contend that embryo selection on the basis of non-medical characteristics that do not limit the possible life plans of the future child or that are useful in carrying out almost any life plan ("general purpose means") need not be unjustified [8,9]. Regardless, the technical possibilities to use PGD for selecting "super-" or "designer babies," whatever that may be, are regularly widely exaggerated in the media.

A paradigm case for the autonomy model is PGD/sex selection for non-medical reasons. Non-medical sex selection is prohibited in many countries. From an ethical point of view, however, this is not evident [10,11]. The objection that such selection is inherently sexist is simply untenable. Think of "family balancing," where parents would ideally have children of both sexes. The fear that it will result in a distortion of the sex ratio does not seem convincing either, at least not in Western countries, where a preference for boys is weak or absent. When arguments against allowing sex selection for non-medical reasons, especially for family balancing, are weak, banning the practice may amount to an unjustified infringement of reproductive freedom.

The Back Door, or How Selective Should a Selective Transfer Be?

Sometimes the result of PGD is inconclusive and, therefore, the genetic status of the embryo unknown ("failed PGD"). In other cases, all embryos available in a given cycle, unfortunately, prove to be affected. Prospective parents may sometimes ask to transfer one of these. Taking account of the professional (co-)responsibility for the welfare of the child, most PGD centers accept the policy to never transfer an affected embryo and likewise abstain from embryo transfer in the case of PGD failure, even if there are no other embryos available and it is the applicants' last chance to have a genetically related child. After all, this policy meets the primary aim of PGD and seems to best fit the basic principle to avoid a high risk of serious suffering/harm.

Though this policy may be ethically sound, in principle, there may well be justified exceptions [5]. For example, when one, as we have suggested, would allow a more contextualized use of the proportionality criterion, PGD need not necessarily be restricted to clearly "serious" disorders. Consider, for example, PGD for a microdeletion on the Y-chromosome or additional testing for, say, PKU in the context of PGD for CF. In such cases, relaxing the policy to never transfer an affected embryo would not conflict with the responsibility to avoid a high risk of serious suffering. A more permissive policy may also be justified in the case of incidental findings causing milder syndromes, like an XXY-karyotype linked with Klinefelter syndrome. Needless to say, such situations may become more frequent in the context of genome-wide PGT (see below). It will be important to check whether another IVF/PGD cycle, aimed at transferring a non-affected embryo, would be possible and proportional from the applicants' perspective, but this requires case-by-case decisions, not dogmatic principles.

Preimplantation Genetic Screening (PGS)

PGS is inherent in regular IVF. Think of PGS to check the number of pronuclei (PGS-PN). This section focuses on two more controversial types of PGS.

PGS for Aneuploidy

There are no valid categorical moral objections to PGS for aneuploidy (PGS-A). After all, its primary aim, i.e., to increase the success rate of IVF and/or to decrease the time to pregnancy, is commendable, while the means, namely to exclude embryos affected with serious chromosomal aberrations, which often lack viability, from transfer is clearly morally acceptable. PGS-A is still controversial because of the lack of high-quality evidence of its efficacy. Proponents claim that at least three more recent studies about improved strategies for PGS-A—using either polar body biopsy or trofoblast biopsy instead of a day 3 biopsy, plus CGH and SNP arrays allowing the testing of all chromosomes—now clearly show PGS-A does the job [12–14]. The quality of the evidence, however, is still contested [15]. Furthermore, it is not always made clear what job precisely is "done"; while the original aim of PGS-A was to increase the THBR of IVF, the aim nowadays is formulated more often in terms of reducing the time to pregnancy, which is something different. Premature claims of the "proven efficacy" of PGS-A are at odds not only with the interests and the right to adequate information of IVF-patients but also with professionals' responsibility to avoid futile interventions and to enable society to distribute scarce resources available for publicly funded health care in a just and evidence-based way. Furthermore, this may undermine trust in reproductive medicine generally. The message that PGS-A should only be offered in the context of randomized controlled trials is still valid [15–17].

"Comprehensive" PGS

In theory, NGS technology allows PGS to test for chromosomal aberrations, all (more common) Mendelian disorders, many susceptibilities for complex disorders, and genetic co-determinants for non-medical traits, including personality traits, *simultaneously*. This approach may seem to be ideal, as one could at the same time select the most viable embryo for transfer, optimally reduce the risk of having an affected child, and select "the best embryo" for transfer, the embryo with the best prospects of a healthy and flourishing life. However, such "comprehensive" PGS raises a series of ethical questions and issues [1,18], including the following.

A rather practical concern regards the quality of the information generated by whole genome sequencing and analysis on a single-cell basis. The more false-positive results, the lower the number of embryos available for transfer, and the lower the take home baby rate. Furthermore, knowledge about genotype-phenotype correlations is not as solid as is often assumed [19]. Finally, much of the information generated will have a rather low predictive value, which will easily undermine the clinical utility of the screening.

Next, the complexity of comprehensive PGS would make traditional informed consent simply impossible. The conditions for acceptable alternatives, like "generic consent," need further scrutiny. Another autonomy-related concern is that patients would regularly be confronted with complex trade-offs; after all, all embryos will prove to carry lots of predispositions for a greater number of common disorders. And again, who has decision-making authority regarding (non-)transfers (see earlier)? Even if all parties agree that "the best embryo" should be transferred, doctors and applicants may well disagree about what this means if confronted with complex risk profiles of larger numbers of embryos.

Finally, comprehensive PGS may provide unsolicited, predictive genetic information about (one of) the prospective parents themselves, thereby undermining their right not to know. Likewise, it may violate the future child's right to informational self-determination, i.e., the right to later decide for itself whether or not to be predictively tested for future diseases. In theory, the latter could be prevented by not transferring embryos carrying such risk factors, but, again, as we are all "fellow mutants," there may well be no embryo suitable for transfer.

Our conclusion is that comprehensive PGS would be fully disproportional at the moment. It meets neither basic "technical" criteria related to analytic and clinical validity nor the proportionality criterion. Furthermore, some ethical issues need further proactive reflection.

This reflection should include possible alternative testing scenarios, particularly combinations of pre-conception screening (PCS) of IVF couples followed by more targeted PGT. This scenario could have various advantages [1,18]. It could facilitate prospective parents' reproductive autonomy, in that they would have both more time for reflection and more reproductive options if proven to be at high risk of

having an affected child, and avoid invasions of the right not to know of both prospective parents and future children. The question as to whether such "more targeted" PGT should include screening for de novo mutations—which play an important role in sporadic genetic disorders and handicaps—needs further scrutiny, as (and insofar as) these mutations may be difficult to interpret, undermining the proportionality of such inclusion [20].

Genetic Screening of Gamete Donors

According to current guidelines, genetic screening of candidate donors is mainly a matter of a thorough medical history both of the donor and his or her first-degree relatives, taken by a trained clinical geneticist. This should lead to excluding donors who either have or have had a serious hereditary disorder themselves, or with a positive family history for such disorders. Although in healthy candidate donors, further transmission risks may be detected through adding genetic tests, current guidelines are reticent about this. Some recommend no testing at all (except for carrier status for recessive disorders with a higher frequency in the donor's population of origin), whereas other guidelines recommend routine karyotyping in view of the small risk (<0.2%) that healthy young donors would carry a balanced chromosomal translocation. Commercially operating centers or sperm banks often perform genetic testing (e.g., testing for fragile-X [FXS] carrier status) beyond what national or professional guidelines for donor screening require [21].

Call for Wider Genetic Donor Testing

Cases in which a rare but serious disorder is found either in a donor whose gametes have been used for donation or in his or her (donor) offspring lead to much media attention and often a call is made for enlarging the scope of genetic testing. With the arrival of next-generation sequencing (NGS), it has been suggested that "donor screening should be orientated towards full genome testing as soon as such a possibility becomes economically feasible and our understanding of genetic mutations makes such an endeavour worthwhile" [22]. As in past years prices have already come down considerably, and as our knowledge of the genome increases, the scenario of comprehensive genetic donor screening may seem within reach. This might theoretically include testing donors for all kinds of genetic risks, including autosomal and X-linked recessive disorders, dominant disorders that because of a low penetrance or high variability may have remained under the radar of family history screening, as well as risk factors for multifactorial disorders.

Proportionality of Genetic Donor Testing

Against the idea of wider testing, it is sometimes argued that there is no need to make donor conception any safer than (natural or assisted) reproduction between partners. But this ignores a morally relevant difference: partners want to reproduce together, whereas people needing donor gametes do not (in most cases) want to have children only with this particular donor. Moreover, professionals providing medically assisted reproduction have a responsibility to reduce reproductive risks resulting from their services to the extent that doing so is reasonably possible [23]. The moral case for wider testing in donor conception therefore depends on whether this would be proportional [21]. This involves balancing the possible benefits and the inevitable drawbacks of such a policy, taking account of the interests of all stakeholders. Potential negative effects for recipients include higher costs affecting accessibility in countries where donor conception is only available commercially, and draining the donor pool as a result of excluding donors with relatively small risks. But consequences for the donor and his or her close relatives should also be taken into account. For them, findings of genetic testing may be beneficial, but may also turn out to be psychosocially harmful, especially if findings reveal a serious genetic risk that is not medically actionable.

An example of this is testing of oocyte donors for FXS carrier status (expanded *FMR1* alleles). Fragile X is the most important known cause of inherited intellectual disability. However, the proposition of a general population offer of FXS carrier screening is contested precisely because such screening is medically and psychologically complex and may act as "a double edged sword" [24]. On the one hand, it may enable carriers to make informed reproductive choices and alert them in time of the risk of fragile X-associated ovarian insufficiency. On the other hand, some findings and some choices are fraught with uncertainty. Moreover, carriers are at risk of developing fragile X-associated tremor/ataxia syndrome, a serious and untreatable disorder, later in life. As there is no good reason for maintaining that these concerns are of less weight in a donor conception context than in population screening, it is at least not obvious that routine testing of oocyte donors for FXS carrier status would be proportional.

Expanded Universal Carrier Testing of Gamete Donors

A conceivable development for the near future is the use of NGS for routinely testing all gamete donors (and recipients) for carrier status of a wide range of autosomal recessive disorders. To mark the difference with traditional testing for only one or a few of such conditions, this is known as "expanded" carrier testing. And because all donors would be tested, regardless of their ethnic background, this would also be an instance of "universal" carrier testing. The case for this approach is based on the fact that, although (in a general population) autosomal recessive disorders are individually rare, together they account for a considerable burden of disease [24].

Importantly, the concern that wider testing would drain the donor pool does not apply here, as carriers can still be used as donors by matching them with recipients who have tested negatively for the same disorder (recipients would have to be tested as well). Moreover, as carriers are healthy, negative psychosocial consequences for those tested (donors and recipients) can in principle be avoided. However, this requires proper pre- and post-test counseling, also to avoid false reassurance, and a careful selection of disorders and mutations in a test panel targeted to disorders with a well-understood genotype-phenotype relationship [25]. In this connection, it is important to note that present databases may overestimate penetrance, or list variants as pathogenic that are in fact benign [19].

Even if such expanded carrier testing in the context of donor conception may be justified in principle, there is a clear need to further spell out conditions for its responsible implementation. Recent publications reporting the first experiences of centers that have started using this testing technology in medically assisted reproduction (including both donor conception and assisted reproduction between partners) not only show that finding consensus on gene panel definition is still an important challenge but also that this is not merely a technical but also very much an ethical debate [26,27]. The temptation to follow the technological imperative and test for more genes "just because it is possible" should be resisted. And although proper pre- and post-test information and counseling is an absolute precondition, this should not be used as a justification for ever-wider testing without a separate consideration of whether this would indeed be proportional in the light of the interests of all stakeholders. Apart from concerns about the interests of donors who in this context may be the weaker party and who deserve to be treated as persons rather than being reduced to the sperm or oocytes that they contribute, a relevant concern is also that higher costs will have the adverse effect of undermining the accessibility of donor conception for recipients. Although testing costs as such are expected to come down, counseling costs will remain high, especially for testing beyond a limited set of well understood genes or disorders with similar implications.

Carrier Testing as Population Screening

Given that carriers of autosomal recessive disorders are healthy, that 1%–2% of couples are carrier couples, and that of these the great majority is not aware of their 1:4 risk of having an affected child, the logic of testing for carrier status is as relevant for reproduction between partners as it is in donor conception. By informing couples of their at risk status, carrier screening allows them to avoid the birth of a child with a serious disorder. With a pregnancy already under way, they can decide to have prenatal diagnosis and a possible termination should the fetus be affected. Ideally, they would receive this information prior

to pregnancy ("preconception carrier screening"), as this not only gives them more time to decide but also further reproductive options to choose from: refrain from having children, have children through donor conception, or have children through IVF and PGD. They might even decide to break up and find other partners.

From Targeted Ancestry-Based to Expanded Universal Carrier Screening

Carrier screening for autosomal recessive disorders is not new. It has been offered to individuals or couples in certain high-risk communities since the 1970s. The first initiatives addressed ethnic groups with a higher frequency of specific recessive disorders. Well-known examples are beta thalassemia carrier screening in several high-risk populations in the Mediterranean region [28] and carrier screening in Ashkenazi Jewish populations for Tay Sachs disease and other recessively inherited conditions more prevalent in those groups [29]. A more recent development is the step toward "universal" testing taken in guidelines of American professional societies. For example, the American College of Obstetricians and Gynecologists recommends offering carrier testing for CF to all persons of reproductive age [30]. In Europe, initiatives beyond specific higher-risk groups have been limited.

The emergence of new genomic technologies has now made it possible to think of expanded universal carrier screening: an offer to all couples or persons of reproductive age to have themselves tested for carrier status for up to several hundred recessive disorders. Although individually rare, together these amount to a reproductive risk comparable to that of a 36-year-old woman of having a child with Down syndrome, a risk for which screening programs have been available already for several decades [31]. Commercial laboratories in North America, Australia, and Europe have already started offering expanded universal testing to interested clients. However, it is still an open question whether a screening offer along these lines responds to a felt need among the general population. If it does, its implementation should be subject to conditions for responsible screening as discussed in a recent position paper of the European Society of Human Genetics [32].

Prevention or Autonomy

Two different accounts of the aim of carrier screening programs can be distinguished [10]. One is the classical prevention aim of public health programs, including most forms of population screening. With this understanding, carrier screening is offered in order to reduce the health impact of the targeted disorder (disorders) upon the community. This is how the aim of traditional ethnicity-based carrier screening programs is often understood. The alternative understanding derives from the tradition of reproductive genetic counseling and prenatal screening for fetal abnormalities such as Down syndrome and states that the aim of reproductive screening should be understood as providing women or couples with options for reproductive choice. Historically, this was fueled by a concern that the public health prevention paradigm, if applied in this context, might lead to instrumentalizing individual reproductive decisions in the interests of societal (cost reduction) or flatly "eugenic" goals. Although there may still be a justification for prevention-aimed carrier screening, especially in the context of community-supported ("bottom-up") programs targeted at high-impact disorders, the dominant view is that the autonomy paradigm (providing options for reproductive choice) is the morally preferable account of why carrier screening should be offered. This is not just a semantic discussion, as these different aims provide the moral context for determining how the program should be set up and evaluated. For instance, for a prevention-aimed program a *prenatal* (carrier) screening offer may be a preferred approach because the target population is more easily reached. By contrast, under the autonomy aim the offer is ideally made *preconceptionally*, as this would provide the couple with more reproductive options. And if the aim is autonomy rather than prevention, the program should be held to a higher standard of informed decision making and be evaluated in terms of whether these standards are met. Clearly, this may pose quite a challenge for the scenario of expanded universal carrier screening [10,32].

Parental Responsibility

Overlapping with the debate about autonomy and prevention is the question whether and to what extent prospective parents have a responsibility to use genetic technologies to avoid health risks to their offspring [33,34]. Whereas the principle of reproductive autonomy suggests that all that morally counts is whether prospective parents have been able to make their own decisions with regard to whether or not to test, or whether or not to know, or whether or not to select or terminate, it is clear that this cannot be the whole story. The responsibility that, according to a broad international consensus, professionals have to refrain from cooperating in the creation of a child if there is a high risk that its life will fall below a standard of reasonable welfare [35], can only be accounted for if this has its counterpart in a parental responsibility not to engage in reproduction in similar circumstances. Concretely, an infertile couple of which both partners are known carriers of a very serious disorder (e.g., Tay Sachs disease or another condition leading to a miserable life of much pain and suffering) should only be given IVF on the condition that they are also willing to have PGD and embryo selection (or PD and a possible abortion). Similarly, a fertile couple at the same risk of a child with a comparable disorder has a moral responsibility to refrain from having children through natural reproduction if they have no intention to avoid that outcome through PD and abortion.

But whereas this only sets a minimum requirement in order to avoid cases of serious suffering, some have argued that the morality of reproductive decisions in this context depends on whether they are made in accordance with a maximizing principle. This principle of "procreative beneficence" holds that "[i]f couples (or single reproducers) have decided to have a child, and selection is possible, then they have a significant moral reason to select the child, of the possible children they could have, whose life can be expected, in light of the relevant available information, to go best or at least not worse than any of the others" [34]. In a recent paper it has been explored what this might mean for the context of preconception choices including choices pertaining to carrier testing. Does it follow that couples wanting to have children have a moral obligation to make use of carrier screening options if these are available? The authors conclude that much depends on how precisely the condition that "selection is possible" should be understood. This, they say, requires further qualification in light of the principle of proportionality. The concern here is that the reasoning behind "procreative beneficence" may lead to imposing unreasonable burdens upon couples in terms of costs and other sacrifices [33]. However, apart from such considerations, a more fundamental issue is whether prospective parents do indeed have a responsibility to use medical technologies (not just carrier screening but also IVF and comprehensive PGS, and even further into the future perhaps embryo enhancement through crispr-cas9 or similar technologies) in order to create the best possible child that they together could have. To whom would they owe this duty, if it cannot be to the child, given that but for their choice a different child would have existed [34]? Should this then be construed as a responsibility toward society that as a whole will be better off if children have healthier and otherwise better lives? If so, can this be maintained without raising concerns that this amounts to a problematic form of (population) eugenics? How does this parental responsibility connect to the responsibility of professionals to as much as reasonable possible reduce reproductive risks? Clearly, what this shows is that the autonomy paradigm is under discussion, but that this has not yet led to a sustainable alternative framework, capable of guiding the field of repro-genomics into its new future.

Prenatal Screening

The area where the autonomy paradigm has always been strongest is prenatal diagnosis and screening. This is because reproductive decision making in a prenatal context is more directly related to debates about abortion. New developments in this area include the non-invasive prenatal test (NIPT) for fetal aneuploidy based on massive parallel sequencing of cell-free DNA in maternal plasma, the use of the same technology for prenatal diagnosis of monogenetic disorders (NIPD), and the introduction of new genomic tests (chromosomal microarrays, soon to be followed by NGS) at the stage of invasive diagnostic testing.

Ethics of NIPT

The great and also morally important benefit of NIPT is that because of its much better test characteristics than traditional first trimester screening, its introduction has led to a drastic reduction in the invasive testing rate, not only leading to earlier reassurance for many more women, but also reducing the number of iatrogenic miscarriages [36]. There is a concern that "paradoxically" this better test may make it more difficult to ensure that women make informed decisions. But this should be countered by appropriate information and counseling. More challenging is the question what the future scope of prenatal screening should be. Whereas initially debate about NIPT was about missing abnormalities that would be found with traditional approaches, current debate is about NIPT beyond common trisomies. Driven by the struggle for market share, several companies have started offering expanded NIPT panels that also test for selected microdeletion or -duplication syndromes with a phenotype including developmental delay, intellectual disability, dysmorphic features, and other malformations. There is no good reason why prenatal genetic screening should be limited to Down syndrome and a few other aneuploidies. Still this expansion is premature, as validation studies are lacking and clinical utility is challenged by lack of certainty about phenotypic implications [37]. An important concern is that due to the lower predictive value of NIPT for these conditions, the main benefit that NIPT has brought to prenatal screening, namely a lower invasive testing and miscarriage rate, may be lost, and that this will lead to serious challenges for decision making and counseling. Similar concerns apply to NIPT for sex chromosomal abnormalities[36].

Scope of Prenatal Screening

A further broadening of the scope of prenatal testing is already taking place at the follow-up stage, where it is rapidly becoming accepted practice to offer microarray testing to all women who come for amniocentesis or chorion villus sampling, also where a positive aneuploidy screen (without ultrasound abnormalities) is the only indication [38]. The reasoning is that where the risks of invasive testing have already been taken, it is better to look for more rather than less. But as this amounts to changing the game from diagnosis to opportunistic screening, it does require information and consent [39]. Among the challenges of this screening is the risk of having to deal with variants of unknown significance (VOUS) or other difficult-to-handle outcomes [40].

NGS-based technologies may in future scenarios be used as well. And given proof of principle regarding the analysis of the entire fetal genome in maternal plasma [41], it is to be expected that this will not remain confined to the invasive testing stage. If the dynamics of the field is not to be determined by the "technological imperative," a debate about the scope of prenatal screening is urgently needed [42]. The normative framework built around the autonomy aim does not seem to provide much guidance here. That is not surprising, as the framework was designed to answer different challenges. In this emerging new context, the autonomy paradigm needs qualification. What should be promoted is not "pure reproductive autonomy" but meaningful choices related to the possibility of having a child with a serious disorder. There are three reasons for this: (1) pure autonomy (choice for the sake of choice) cannot possibly be the aim of a publicly funded screening programme; (2) testing for everything will paradoxically undermine rather than serve well-informed decision making; and (3) the possibility of broad-scope prenatal screening brings a new stakeholder on the stage, the future person, whose interests in being protected against psychosocial and informational harm have not until now played a role in the ethics of prenatal screening. Based on these considerations, a recent joint position statement of the European and American Human Genetics Societies (ESHG, ASHG), has recommended that "pending further research and debate" expansion of the scope of screening should be limited to "serious congenital and childhood onset disorders" [36].

Concluding Remarks

There is a dialectical relation between the dynamics of new repro-genomic technologies leading to emerging opportunities for reproductive decision making and the normative framework in terms of which the ethical (and legal) aspects and implications of this development are being understood and discussed in

society. Whereas the framework determines under what conditions the application of new technologies may be regarded as acceptable, the adequacy of the framework and its ability to provide meaningful guidance may in turn need reconsideration in the light of the dynamics of the field. At several points in this chapter, it has become clear that a core principle of the current framework is that of "reproductive autonomy." However, it is also clear that this principle does not provide much guidance if it comes to choices positively determining the health and well-being of future children. Whether and how the principle of "procreative beneficence" can be part of a qualified and sustainable normative framework for this field is an urgent question for ethical debate and analysis. The need for this is obvious also in the light of potential reproductive applications of crispr-cas9 or similar new gene modification technologies [43]. The suggestion that given PGD there is no real need for such applications is not convincing [44]. In addition to rare situations where PGD cannot be of any help (e.g., if both partners are homozygous for a recessive disorder such as CF), a safe and effective embryo modification technology will be of use more generally in cases where PGD for multiple conditions leads to a low chance that any given cycle will yield transferrable embryos (see earlier). If safety concerns can be answered, such possible uses seem to provide a good reason for at least considering the step toward embryo modification [45]. This prospect requires a proactive ethical analysis that takes into account the highly different ways in which an autonomy or procreative beneficence framework would approach the challenges that arise here.

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