Practical Preimplantation Genetic Testing

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Third Edition

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Preface

Before introducing the present edition of the book, we have to mention the changes of standardized terminology*, according to which the term "preimplantation genetic testing" (PGT) replaces the previous term "preimplantation genetic diagnosis" (PGD) and a quite inadequate "jargon" "preimplantation genetic screening" (PGS) used as an alternative to PGD for aneuploidy. Accordingly, "PGT-M" will be used as an abbreviation for preimplantation genetic testing for monogenic disorders, "PGT-A" for preimplantation genetic testing for aneuploidy, "PGT-SR" for preimplantation genetic testing for structural rearrangements, and "PGT-HLA" for preimplantation genetic testing for human leukocyte antigens (HLA). So, the title of the third edition is changed from "Practical Preimplantation Genetic Diagnosis" to "Practical Preimplantation Genetic Testing" to comply with the standardized international terminology for assisted reproductive technology (ART) and preimplantation genetics.

It should be mentioned that although PGT has become an established procedure for genetics and ART practices already for the last decade, its wider application has been obvious only after the introduction of the next-generation technologies in the last few years. At the present time, more than one third of ART centers and the majority of genetic practices in the United States have already been utilizing PGT services to allow at-risk couples to reproduce normally, without fear of having an affected offspring. The practical PGT experience at present may be estimated in hundreds of thousands, what makes the updating of this experience of practical utility to both medical profession and patients. This will include, first of all, an update of PGT accuracy, reliability, and safety to ensure an improved access to PGT of those who may benefit greatly from this technology. It is of note that PGT has now been applied in as many as 581 different conditions, with the accuracy in the leading PGT centers, such as ours, approaching almost 100%. In fact, PGT may now be performed for any genetic condition, even if it was identified in one of the parents or in the affected child *de novo*, with also a possibility of concomitant testing of a number of disorders in one test.

Updating will include also the progress in the primary prevention of genetic disorders described in the introductory section, which will now

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include the approaches for prospective identification of at-risk PGT couples, through the application of the expanded carrier screening using an increasing number of gene in the panels, as a means for an improved prospective access to PGT. In fact, because of dramatic technological improvements in all aspects of PGT, most of the sections will be updated, with addition of also new sections on the next-generation technologies and universal PGT with combined testing for single gene and chromosomal disorders, which has previously presented a real challenge. As we have presently accumulated the world's largest experience in this area, the guiding PGT strategies for different genetic disorders, will be presented, with emphasis on the most complicated cases that might be of special utility in the wider application of PGT technologies worldwide.

PGT indications continue to expand for those that have never been even predicted, so the new section will be devoted to borderline indications, which will include common adult-onset conditions with genetic predisposition and nongenetic indications. This section will include the expanding PGT application to heart disease and cancer, for which the number of requests has been increasing gradually, such as for breast cancer, with more than a hundred predisposing gene mutations tested by the present time.

A unique experience on PGT-HLA for stem cell transplantation treatment of congenital and acquired disorders will be addressed in a separate section, for which the increasing outcome data have become available. Started with our pioneering experience, still representing one of the largest in the world, PGT for HLA typing has become a method of choice in considering the treatment regiments for bone marrow failures, requiring HLA-compatible stem cell transplantation treatment. As the majority of couples requesting PGT-HLA are of advance reproductive age, our experience in overcoming this problem will be presented, which will help to avoid the potential problems in older PGT-HLA patients.

As there is still some controversy in the utility of PGT for aneuploidy, mainly due to the procedure accuracy in the past based on the use of FISH technique, a special section will be devoted to the outcome data that have been obtained since the shift of embryo biopsy to the blastocyst stage and the introduction of NGS-based 24-chromosome aneuploidy testing. This will also include the available RCT data, showing the obvious clinical impact of preselection of aneuploidy-free embryos. However, it is also obvious that not all the preselected euploid embryos have a potential to implant, so additional contributing factors will be addressed, such as cytoplasmic DNA contents, time-lapse parameters, genetic expression profile related to the euploid embryo competence, endometrial receptivity, and possible epigenetic influences of IVF-related procedures. The special emphasis will be on subchromosomal variations, with additional sections on segmental aneuploidies and mosaicism, which were not detected previously with the previously available inferior technologies but were shown to affect implantation and pregnancy outcome.

Significant improvement has been also achieved in PGT for chromosomal rearrangements, involving the application of next-generation technologies, which requires a significant updating of the corresponding section as well. In

addition, special emerging technologies for distinguishing normal from balanced rearrangements will be described, such as mate-pair sequencing, with presentation of the available practical experience. As the application of NGS allows PGT for both chromosomal rearrangements and aneuploidy, the reproductive impact of such a combined PGT will be evaluated.

It should be, however, noted that PGT is still an invasive procedure, and despite the lack of any detectable damage and considerable improvement of biopsy techniques with the shift from cleavage to blastocyst stage, the potential negative effect of embryo biopsy-based PGT cannot be totally excluded. So the current experience for attempting a noninvasive PGT will be described, including approaches with the use of blastocoel fluid and spent culture medium, as well as a possibility of PGT without IVF, with their limitations and possible practical applications in the future. Also, a recent progress in the development of noninvasive prenatal tests (NIPT) for the application to PGT will be demonstrated, as a realistic follow-up procedure after PGT.

Thus, a dramatic progress above in PGT and related areas requires an update in all sections, with substantial revision in PGT for both single-gene and chromosomal disorders. These updates are illustrated by our 30 years pioneering experience, including 6,204 PGT-M cycles for as many as 581 different monogenic disorders, currently performed together with PGT-A in the majority of cases. This and other large experiences in PGT-A, PGT-SR, and PGT-HLA, based on the use of the next-generation technologies, demonstrated an extremely high accuracy, safety, and reliability of PGT technology, which represents a practical PGT for improving the standard of ART and genetic practices.

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1 Place of Preimplantation Genetic Testing (PGT) Among Available Options for Prevention of Genetic Disorders

Preventing genetic disorders and birth defects is a universal goal. Dramatic progress has been made in corrective gene therapy in recent years using CRISPR, but prevention of genetic disorders remains the main approach. Primary preventive measures are ideally applied at the community level. Examples include suitable dietary intake or avoiding toxicants that can result in new mutations. Prevention is provided also by preconception and prenatal predictive testing for genetic and complex disorders and by prospective screening for genetic disorders, including that for common conditions specific for each ethnic group or expanding carrier screening [1–2]. The ideal time for offering many preventive measures is, in fact, the preconception or preimplantation stage because detection thereafter will involve the decision either to keep the pregnancy and confront long-term social, familial, and financial consequences that arise with a seriously affected child or to terminate a planned and wanted pregnancy.

The most relevant approaches for primary prevention of congenital disorders include (1) avoidance of new mutations through environmental programs, (2) reduction of maternal age related conditions through community education and family planning, (3) reduction of neural tube defects and a few other congenital malformations by periconceptional folic acid supplementation or multivitamin fortification of basic foodstuffs, (4) avoidance of alcohol and smoking during pregnancy, and (5) rubella vaccination. These actions can reduce congenital disorders of environmental origin through public health measures and those of biological origin through sophisticated approaches for molecularly detecting and managing individuals at genetic risk. In fact, most congenital and other complex conditions (e.g., NTD) have both genetic and environmental components; thus, the actions are first addressed to environmental causes, through the finding of the key components to modify the occurrence of congenital disorders. The decision to adopt any of the available preventive programs depends on health services development, ethnic distribution of certain congenital diseases, and local attitudes to genetic testing and termination of pregnancy. For example, induced abortions are still not permissible in many countries. The number of countries permitting prenatal genetic diagnosis and termination of pregnancies for medical indications is steadily increasing, but there is still restriction on the stage of pregnancy when termination for medical reasons can be performed. The impact of community-based preventive approaches is obvious from Down syndrome preventative programs in industrialized countries of Europe, South America, Asia, Oceania, and the United States. Pregnancies at increased risk are offered invasive testing and, if appropriate, selective pregnancy termination. Prenatal diagnosis offered to all women of advanced maternal age can result in the reduction of the birth

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prevalence of Down syndrome by more than 50% [3]. These programs were initially based solely on testing by invasive prenatal procedures (amniocentesis; chorionic villus sampling), but later women of all ages were screened based on maternal serum analysis and ultrasound criteria. Currently screening increasingly involves cell-free DNA in maternal plasma (NIPT). All these actions decrease number of invasive procedures required. However, this reduction is positively correlated with the number of pregnancy terminations. In some countries the effect of such program is still growing, while in others it seems to be reaching a plateau, reflecting differences in the development of the services as well as social and religious differences. That this reduction is achieved only through pregnancy terminations is, however, a cause for serious concern [3–4]. This is particularly relevant for high-income countries in which women use family planning to postpone childbearing, leading to a rebound in a higher proportion of older mothers.

The paradigmatic example of a highly effective population-based preventive measure at the primary prevention preconception stage is, as noted, prevention of neural tube defects (NTD) by folic acid or folic acid-containing multivitamins. Preimplantation genetic testing has not typically been considered analogous to traditional preventive strategies like NTD prevention by folic acid supplementation, of course, the reason being the necessity for in vitro fertilization. However, PGT is genuinely an established and realistic option for preconception prevention of genetic disorders. The role PGT plays in primary prevention for genetic disorders will be described in detail in this book.

1.1 Preconception Prevention of Congenital Anomalies by Folic Acid Containing Multivitamin Fortification Programs

Despite the need for integrating programs and combining all feasible approaches maximizing the benefits and minimizing the negative aspects of preventive programs for congenital malformations, the ideal is a primary preventive measure. The paradigmatic example is represented by vitamin/folic acid supplementation to prevent NTD and congenital disorders [5–14]. The success of this effect deserves attention. The strategy is folic acid dietary supplementation and folic acid food fortification (FFI (flour fortification initiative), www.Sph.emory.edu/wheatflour). Application of these approaches has resulted in the overall reduction of NTD by as much as by half (from 40.6 per 1000 to 20.6 per 1000).

Supplementation or fortification with folic acid or folic acid-containing multivitamins not only reduces the frequency of NTD by 75% but may reduce the population prevalence of other congenital disorders: cardiovascular, urinary tract, and limb deficiencies. More data are needed to determine efficacy of reducing birth defects other than NTD, such as pyloric stenosis. Still, the positive impact of folic acid in reducing congenital anomalies is in agreement with the following:

1. Mothers who give birth to a child with neural tube defects have mildly elevated blood and amniotic fluid levels of homocysteine.
2. Hyperhomocysteinemia and/or lack of methionine can induce neural tube defects in animal experiments.
3. Low maternal folate status is associated with increased risk for neural tube defects.
4. Vitamins of B group, including folate/folic acid, are important in homocysteine metabolism.
5. Vitamins B_6, B_{11}, and B_{12} are also able to reduce hyperhomocysteinemia.
6. Homocysteine accumulates if its conversion to methionine is slowed with a shortage of folate or vitamin B12 or both.

Elevated plasma homocysteine indicates suboptimal nucleic acid and amino acid metabolism and results in increased risk of cardiovascular disease through thickening the lining of blood vessels [15–18].

Folate deficiency is usually related to genetic factors. In many populations, approximately 20% are homozygous for a common polymor-

phism of the enzyme methyl tetrahydrofolate reductase (MTHFR). Valine replaces alanine at codon 677, reducing the enzyme activity in homozygotes by 50–70% and increasing risk of neural tube defects. Folic acid supplementation increases the supply of tetrahydrofolate, accelerates most folate-dependent metabolic reactions, and reduces plasma homocysteine levels [19–22].

In over 90% of pregnancies in which the fetus has a neural tube defect, there is no previous indication of increased risk. The only identifiable risk group consists of (a) women with a prior affected pregnancy, who have a 3–4% recurrence risk, and (b) women who are heterozygous for the MTHFR mutation. However, these groups account for only a small proportion of affected pregnancies. Trials of the effect of folic acid supplementation on the prevalence of neural tube defects were needed to generate conclusive scientific evidence for its preventive effect [5–14]. Recommendation evolved to recommend that (1) dietary supplementation with folic acid or with multivitamin preparations containing folic acid, before and during early pregnancy (periconceptional supplementation), markedly reduces both the first occurrence of neural tube defects and recurrence among women having had a previously affected pregnancy (3–4% risk); (2) benefit is greatest in regions in which a high baseline prevalence of neural tube defects is present, but efficacy exists also in lower prevalence areas; and (3) no harmful effects have been observed, based on levels of supplementation ranging from 360 μg to 5 mg of folic acid daily. Most recent data show that no harmful effects have been seen even with the extreme doses of folic acid [23].

The recommended current dietary folate intake for adults in the United States is 400 μg, representing a daily intake of 200 μg folic acid equivalents. The additional intake needed for pregnant women is recommended to be 600 μg [24]. The UK Committee on Medical Aspects of Food and Nutrition Policy recommendation of 240 μg of folic acid per 100 g flour [25] is approximately equivalent to an additional folic acid intake of 200 μg a day. To obtain adequate protection against risk of neural tube defects, the mean plasma folate should approximate 10 ng/ml. The mean plasma folate level in most populations is only 5 ng/ml [24–27]. There is considerable variation within and between populations, but a few individuals have a mean plasma folate in the recommended (IU ng/ml) range. Thus, food fortification alone needs to be provided as well as folic acid supplements. Groups of greatest likelihood for folate intakes and plasma folate being at the lower end of the range are those residing in regions lacking food fortification.

Folic acid fortification of all cereal grain products at a level of 140 μg/100 g flour has been mandatory in the United States and Canada since 1998 (Table 1.1) [28–31]. Since introduction, birth prevalence of neural tube defects has fallen by about 19–32%, with no adverse effects reported [28–33]. In Hungary, folic acid, vitamin B12, and vitamin B6 were added to bread in 1993, with average daily intake of folic acid, vitamin B12, and B6 from this source approximately 200 μg, 1 μg, and 1080 μg [7]. The prevalence of neural tube defects in Hungary has fallen by 41%.

Mandatory fortification of wheat flour with folic acid are in place in 53 countries, with maximum and minimum level of folic acid set according to the World Health Organization guidelines [28–29]. The potential global estimate of reduction of congenital disorders is presented

Table 1.1 Global estimate of reduction of neural tube defects (NTD), congenital heart disease (CHD), and limb reduction defects (LRD) by folic acid (FA) food fortification

	All three conditions	NTDs	CHD /LRD	NTDs %
Potential annual affected births	1,035,604	388,442	647,162	37.5
Est'd annual affected births with FA	659,090	137,402	521,689	20.8
Born malformation-free with FA	376,513	251,040	125,473	66.7

Based on Crider et al. [31]

Table 1.2 Reported fall in prevalence of neural tube defects following folic acid food fortification

Country/region	Year fortification started	% fall in NTD
The United States and Canada	1998	19–32
Hungary	1998	41
Costa Rica	1998	19–35
Chile	2000	19–35
South Africa (urban)	2003	19–35
Oman	1996	44

Based on Crider et al. [31]

in Table 1.2. The available actual figures support these estimates (see Table 1.1), although there were also conflicting data from updated US data, which may be due to potential misclassification and bias that can attenuate the measured association of folic acid supplementation with neural tube defects [30–32]. Overall, the data suggest at least 5–6 per thousand of NTDs and other malformations mentioned are the lowest achievable in these primary preventive measures, with conservative estimate of up to 300,000 NTDs preventable worldwide [31].

In addition, folic acid fortification is highly cost-effective [28, 33]. The cost is only about $1 per metric ton of flour, so low that the extra cost is insufficient to change the price of a loaf of bread. Again, however, the multivitamin food fortification does not substitute completely for periconception supplementation nor for multivitamin supplementation during pregnancy. Taking into consideration the estimated lifetime cost for a single patient with spina bifida ($250,000), complementary periconception supplementation program is highly cost-effective, the major benefit being avoidance of an affected child. Available experience from those countries which have implemented a national food staff fortification program is presented in the abovementioned Table 1.1.

In summary, food fortification and preconception vitamin supplementation programs are a paradigmatic prevention that has maximized numbers of healthy babies.

1.2 Genetic History and Avoidance of Congenital Disorders by Prenatal and Preimplantation Genetic Testing

Prevention requires one to inquire into the health status of first-degree relatives (siblings, parents, offspring), second-degree relatives (nephews, nieces, aunts, uncles, grandparents, or grandchildren), and third-degree relatives (maternal and paternal first cousins). Similar family history taking is required for sperm or oocyte donors and their family members. A positive family history for a genetic disorder may indicate need for prenatal or preimplantation testing. Most couples undergoing PGT for a monogenic disorder (PGT-M) will have been ascertained in this fashion. On the other hand, the couples undergoing PGT because of a prior affected child is low compared to the general population unaware of their 25% risk. Few will have undergone genetic screening. This gap is a major deficiency, to be discussed in this book.

Family history may reveal at-risk individuals in other ways. For example, a second-degree relative (e.g., grandparent) may have an autosomal dominant disorder. This should warrant physical examination of the potential parents (grandchildren) presenting for prenatal or preimplantation testing. Subtle and previously unappreciated clinical features in the couple could indicate presence of a transmitted mutant gene. For example, multiple café au lait spots connote neurofibromatosis, an autosomal dominant disorder.

A history of adverse reproductive outcomes should be sought, namely, queries for miscarriages, stillbirths, or as already noted liveborn infants with anomalies. This may require genetic tests. A woman having recurrent miscarriages carries an increased likelihood for a balanced chromosomal translocation if she is <25 years and has a sibling who also had miscarriages.

Advancing *maternal* age confers the well-known increased risk for aneuploid offspring. Increased *paternal* age confers only an arguable increased risk for aneuploid offspring, but risk is increased for sperm having a de novo single-gene mutation that could result in autosomal dominant disorder.

Exposure to teratogenic factors (prescription drugs, chemotherapeutic agents, radiation) should be sought. Standard tests enumerate drugs of potential teratogenic potential. Exposure to environmental toxicants (mercury, heavy metals, pesticides, plastics) should also be sought. Exposure to levels encountered by the general population usually poses little risk. However, this would not be so for continuous occupational exposures.

Carrier screening has been traditionally ethnicity-based but has now expanded to be panethnic. Ethnicity should be recorded, even though when self-reported often incorrect. Panethnic expanded carrier screening for disorders of Ashkenazi Jewish ancestry reveals that only one-half of carriers self-report themselves as Jewish. Still, autosomal recessive diseases sufficiently common in selected ethnic groups justify targeted screening in asymptomatic individuals.

In ongoing pregnancies one of the straightforward approaches for avoiding liveborns with congenital disorders is fetal ultrasound detection. Virtually all pregnant women receive an ultrasound, which, at 16–18 weeks, is informative and safe for detecting pregnancies with anomalies. This enables the choice of terminating an affected pregnancy or planning for postnatal treatment for the affected child. Effects may be evaluated by the community-based birth defect monitoring systems available in increasing number of countries. Because termination is usually requested only for the most severe disorders, fetal anomaly scanning selectively reduces the proportion of chromosomal abnormalities. The downside of ultrasound screening to detect anomalies is the need to terminate pregnancies that were desired. The benefit is that terminations are replaced in subsequent pregnancies and birth of wanted unaffected children.

Prenatal diagnosis of inherited (single-gene) disorders is another strategy for prevention of affected live births. Impact has not been high in population terms because ascertainment is usually retrospective, i.e., following birth of an affected child. The approach requires specialized diagnostic facilities and genetic counseling skills. Most parents having a child with a severe single-gene disorder utilize prenatal diagnosis in subsequent pregnancies or restrict further reproduction. This has a greater contemporary impact compared to earlier eras when a large final family size was the population norm. Prevention by prenatal management reduces birth prevalence by less than 10% when family size is small.

The high (25%–50%) recurrent risk of many carrier couples makes PGT-M highly relevant: (1) low treatment efficiency at present for therapeutic interventions; (2) high risks recurrence in each pregnancy; (3) relatively ineffective methods for reducing affected birth prevalence in the absence of high uptake of screening pregnancies; (4) requirements for innovative methods to detect risk, including not only population screening but extended family studies in populations where consanguineous marriage is common; (5) continual refinements of requisite DNA-based diagnosis; and (6) wide ranges of severity and age at onset, making very difficult the decision-making for people at risk for late-onset or ostensibly less severe disorders (e.g., cancer susceptibility genes). Many find termination of pregnancy difficult to accept and prefer PGT if available and reasonably reliable. Thus, PGT is providing important functions providing options for utilizing genetic knowledge to preserve the health of their families.

1.3 Prospective Carrier Screening as a Means for Improving PGT Uptake

If an asymptomatic individual is a heterozygote for a mutant allele and his or her partner likewise, a 25% risk exists for an autosomal recessive disorder. This couple would ordinarily not know their at-risk statuses until they had an affected child. For 50 years, screening programs have been offered to detect asymptomatic individuals who are clinically normal but heterozygote carriers. The detection rate for heterozygotes in autosomal recessive carrier screening programs is expected to be high (>95%) but not necessarily 100%. Still, a negative screening test reduces considerably the likelihood that an individual is a carrier and, hence, lowers risk for having an affected offspring.

The American College of Obstetricians and Gynecologists (ACOG) has long recommended a selected number of disorders for ethnicity-based carrier screening [34]. American College of Medical Genetics and Genomics (ACMG) offers recommendations that are similar, but not identical recommendations. Disorders recommended for screening before conception or early in pregnancy were traditionally restricted to specific ethnic groups and based on gene products (protein), as DNA methodology did not exist at that time. At present, panethnic carrier screening intermingles protein- and DNA-based methods for greatest efficiency in carrier detection. Updated recommendations have been summarized in joint statements by multiple organizations: ACOG, National Society of Genetic Counselors (NSGC), ACMG, and Perinatal Quality Foundation [35–36].

Carrier screening may concurrently involve both potential parents. However, cost-effective approach is to test the partner of greater risk (e.g., based on family history of the disease of interest). If one partner has a mutation that could result in an autosomal recessive disorder, the next step is to test the other partner. It is perhaps preferable to test both partners concurrently if they are of equal risk. Any couple being screened should be informed of their options: invasive prenatal diagnosis (chorionic villus sampling (CVS) or amniocentesis), PGT-M, donor gametes (eggs or sperm), or adoption. All would avoid offspring with the at-risk disorder.

Among the original carrier screening programs were those for Tay-Sachs disease in individuals of Ashkenazi Jewish ancestry. Screening for Tay-Sachs disease initially was based on ratios of the gene products hexosaminidase A and B, the former deficient in carriers and almost absent in affected individuals. Protein-based functional assays are still applicable and sometimes essential if one partner is not Jewish. However, DNA-based screening in the Jewish populations is highly efficient because only a few mutant alleles account for most heterozygotes. Over 95% of heterozygotes in the Jewish population are accounted for by: ΔF508, W1282X, G542X, N1303K, and 3849 + 10kbC → T. In non-Jewish individuals, the same mutations are not far less predictable. ACOG later recommended carrier screening in Jewish populations for Canavan disease and familial dysautonomia (IKBKAP). “Consideration” was recommended for other conditions prevalent in this population: mucolipidosis IV; Niemann-Pick disease type A; Fanconi anemia types A, B, C; Bloom syndrome; Gaucher disease (GBA); Jourbert syndrome; familial hyperinsulinemia (ABCC8); maple syrup urine integrated (BCKDHA, BCKDHB, DBT) disease; and Usher syndrome. By contrast with ACOG, ACMGG explicitly recommends offering Niemann-Pick (type A), Bloom syndrome, Fanconi anemia type C, mucolipidosis IV, and Gaucher disease. DNA panels are utilized for all these conditions, with carrier detection rates of 95–99% in the Jewish population. Carrier frequency and detection for non-Jewish partners are less established in these Ashkenazi disorders. Unlike Tay-Sachs disease, there are no suitable protein-based methods, for which reason screening is less efficient in couples of mixed ethnicities. ACOG Committee Opinions 691 and 690 detail recommendations [35–36].

Carrier screening programs for the hemoglobinopathies were developed in the 1970s [37]. Sickle cell disease is prevalent among individuals of African or African-American ori-

gin, 1 in 12 being carrier for sickle cell anemia. β-Thalassemias are prevalent among Greeks, Italians (Sicilians), Turks, Arabs, Southern Iranians, Azerbaijanis, and Asian Indians. β-Thalassemia carrier detection is based on anemia that is not due to iron deficiency origin: mean corpuscular volume (MCV) is less than 80 fL, and iron saturation levels are normal. Diagnosis is confirmed by hemoglobin electrophoresis. Hemoglobin electrophoresis will reveal diminished hemoglobin B and increased hemoglobin F. Detection of α-thalassemia requires DNA testing.

Cystic fibrosis was the first disorder screened solely by DNA testing. In 2001 American College of Medical Genetics (ACMG) and ACOG proposed a DNA panel consisting of 23 mutations [38]. Screening was "offered" only to Caucasians and Ashkenazi Jews on grounds of prevalence being higher than in Hispanic, African-American, and Asian populations. For these ethnicities, screening was "made available." The rationale was that carrier frequencies and, hence, detection rates were much lower in the latter ethnic groups. Lower heterozygote rates and lower detection rates meant that likelihood of heterozygosity not only was lower, but likelihood of being a heterozygote despite a negative screen was also lower. In other words, screening was less efficient. Recommendations were later updated later by ACOG [39], ACMG, and NSGC [40]. At present all these organizations now recommend carrier screening for all ethnicities [35, 36].

Spinal muscular atrophy (SMA) has long been under discussion. Carrier frequencies for autosomal recessive SMA are 1 in 40 to 1 in 60 in most populations [41], albeit lower in Hispanics [42]. The causative gene undergoing mutations causing SMA is survival motor neuron 1 (SMN1). Based on carrier rates being similar to cystic fibrosis, ACMG has long recommended population screening for SMA. Initially ACOG did not recommend population screening, but the 2017 Committee Opinion does so [35].

Fragile X syndrome (FMR1) is an X-linked disorder that is the most common inherited form of intellectual disability in males (1 in 3600). In the United States, the carrier frequency in women with no known risk factors is approximately 1 in 250. The molecular basis is expansion of trinucleotide CGG repeats in the FMR1 gene. Affected individuals have >200 GGT repeats. In females with 55–200 repeats, gametes may expand during meiosis to result in >200 and thus offspring with FMR1. Such individuals are said to have a "premutation." Population-based carrier screening for fragile X is recommended by neither ACOG, ACMG, nor NSGC. Screening in Europe and North America is recommended for women having a family history of fragile X, but not in the general population [43]. In Israel screening is more common.

How effective is genetic screening in preventing birth of offspring with genetic disorders? Prenatal genetic diagnosis and PGT-M as traditionally practiced have marginally reduced the number of births with these monogenic disorders in the population. However, many couples are at risk but do not realize their status. Approximately half of all individuals heterozygous for an Ashkenazic specific mutant had not self-reported themselves as Jewish [1]. In 2017 ACOG explicitly codified that "ethnic-specific, panethnic, and expanded carrier screening are (all) acceptable strategies for pre-pregnancy and prenatal carrier screening" [35]. Expanded carrier screening means all ethnicities, more genes, and increased information on genes screened. In 2015 joint counseling recommendations were published by ACOG, ACMG, NGSC, SMFM, and Perinatal Quality Foundation.

Such a strategy has become feasible by availability of the broad spectrum of sequencing methodology. This approach is panethnic. *Panethnic* screening entails *Expanded* carrier screening. ACOG explicitly states that "ethnic-specific, panethnic, and expanded carrier screening are acceptable strategies for prepregnancy and prenatal carrier screening" [35, 36].

Many more conditions are screened and the depth of screening is greater. In expanded carrier screening, several hundred additional genes are considered plausible candidates [44, 45]. Costs are no longer a major impediment. Copy number variants (CNV) are also

sought in certain genes traditionally difficult to sequence: FMR1, SMN1, congenital adrenal hyperplasia, and α-thalassemia. Detection rates are thus increased. Interrogating for CNV in cystic fibrosis and Duchenne/Becker muscular dystrophy has increased heterozygote detection to almost 100%. In the 2018 reported results of the Counsyl, using expanded (Foresight™) panel consisting of 254 genes, 1 in 22 couples was at risk for a detectable disorder; 1 in 300 fetuses was affected [45].

These advances have impacted PGT-M, increasingly following expanded carrier screening programs, as was demonstrated by our ongoing PGT-M experience, which represents the world's largest series in one center. In 2016, 38% of PGT-M cases at our center were ascertained by carrier screening. That is, the majority were ascertained following an affected proband. Two years later in 2018, the majority (63%) of PGT-M cases were ascertained by carrier screening (Fig. 1.1) [46]. The dynamics of increase of at-risk couples presenting for PGT-M through expanded carrier screening was observed for each genetic condition tested, compared to the baseline referrals through the traditional approach. Among the most frequently referred conditions were cystic fibrosis (CFTR), the uptake of which increased from 50% in 2016 to 82.7% in 2018 (Fig. 1.2); deafness (GLB2) with the uptake growing from 31.2% in 2015 to 86.4% in 2018 fragile X (FMR1), the uptake growing from 73% in 2016 to 84% in 2018; and thalassemia and sickle cell disease (HBB), increasing from 45.8% in 2016 to 60.9% in 2018. Thus, the overall number of prospective PGT-M cases for the last 3 years more than doubled after referral through expanded screening, with a similar dynamics for each condition tested. This may become the major source for performing PGT-M in the near future, allowing to offer PGT-M prospectively before the birth of an affected child.

The data show significant increase of the PGT-M uptake following expanded carrier screening, demonstrating the utility for offering PGT-M prospectively to the couples at risk. Gene panels for various adult-onset heritable disorders are also becoming common. This has led to at-risk couples wishing gamete and embryo to be tested, resulting in increased PGT-M.

Greatest applicability of PGT-M for adult-onset heritable disorders lies in heritable cancers.

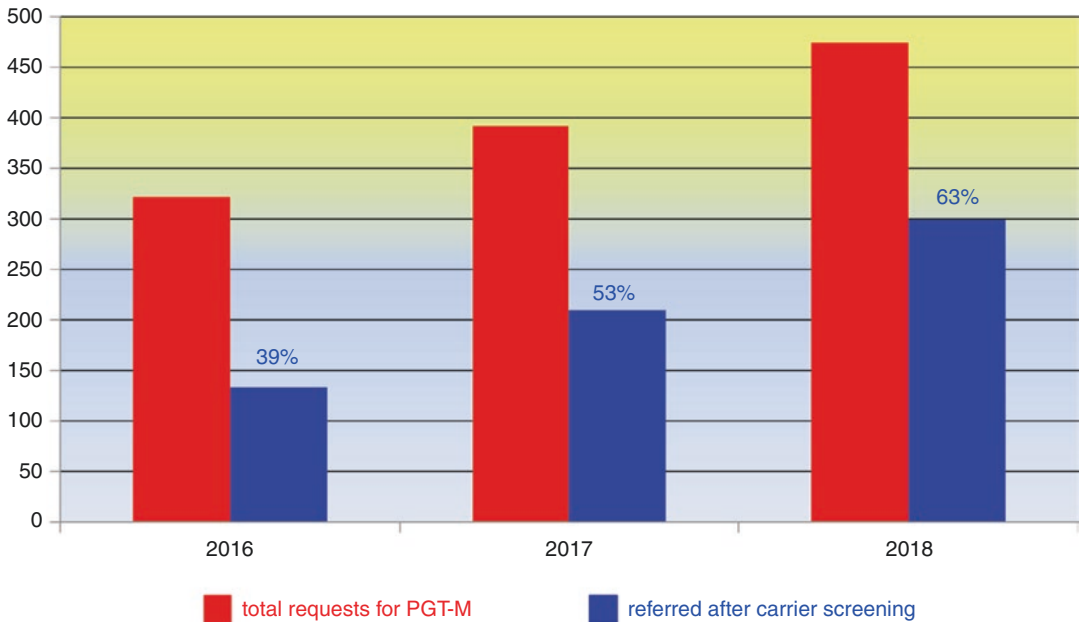

Fig. 1.1 Impact of expanded carrier screening on PGT-M uptake

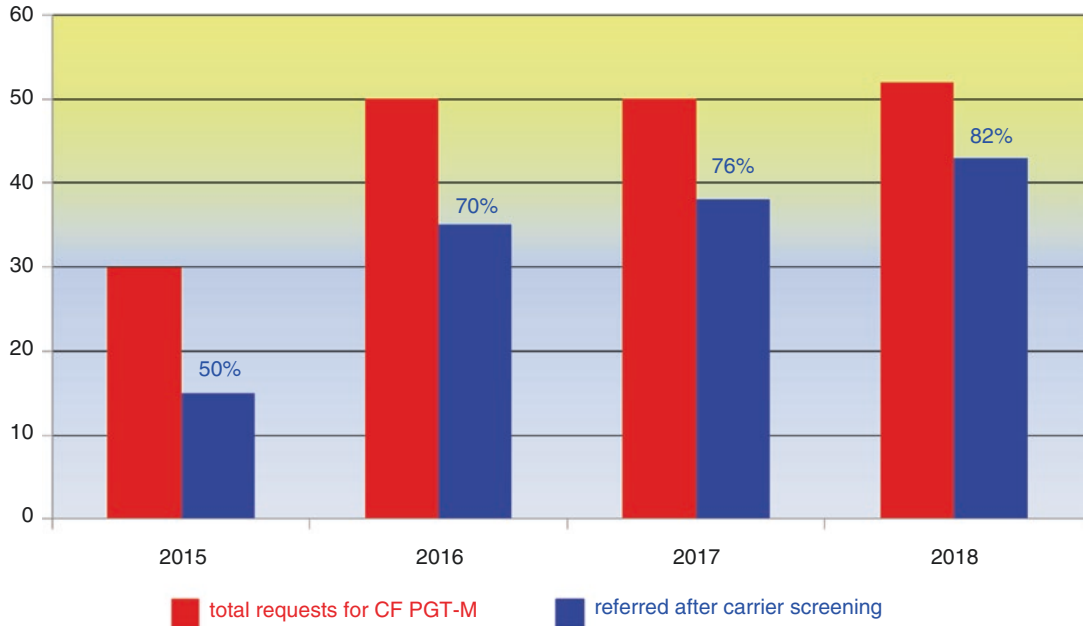

Fig. 1.2 Increase in PGT-M requests for cystic fibrosis (*CFTR* gene) after expanded carrier screening

The most recently reported population genomic screening for multiple conditions involved 2,688,192 young adults aged 18–25 years with the purpose of disease prevention [47]. This appeared to be highly cost-effective in significantly reducing the incidence and mortality of hereditary cancers and the burden of severe childhood-onset genetic diseases, compared with targeted testing. The NIH National Cancer Institute lists of Familial Cancer Susceptibility Syndromes numbers 39. Many are of adult-onset, autosomal dominant in inheritance. Interest is often initiated when a family member is found to have a heritable cancer and the mutation is identified. Unaffected relatives of reproductive age naturally seek to learn if they themselves have the same cancer-susceptible mutation. If this proves to be the case, avoiding transmission of their mutant alleles to offspring is sought through PGT-M. PGT-M is preferable to invasive prenatal genetic diagnosis because an ongoing pregnancy monitored by CVS or amniocentesis carries a 50% likelihood for an affected fetus; repeated clinical pregnancy terminations may be needed to achieve a normal offspring. By contrast, PGT-M allows selection of an unaffected embryo to transfer without fear of an affected clinical pregnancy. Despite obvious benefit, PGT-M for adult-onset cancers is diagnostically and emotionally complex. Chapter 4 will provide this strategy. Heritable cancers most often subjected to PGT include breast cancer, familial adenomatous polyposis 1 (FAP1), Fanconi anemia, neurofibromatosis, tuberous sclerosis, and hereditary nonpolyposis colon cancer (HNPCC) (see Chap. 4).

Adult-onset PGT-M is also increasingly utilized for adult-onset autosomal dominant cardiac disorders. Most common are Long QT syndrome (LGT1, LGT2, LGT8), hypertrophic cardiomyopathy (CMH1, CMH4, CMH8), and dilated cardiomyopathy (Type 1A, 1DD, 1E, 1G). Monogenic cardiac disorders also commonly subjected to PGT-M include Holt-Oram syndrome and Noonan syndrome, to mention only a few, described in Chap. 4).

In conclusion, the main objective of screening or prenatal genetic diagnosis is to assist couples to have an unaffected child of their own. PGT does this by embryo selection. We have noted that prenatal genetic diagnosis during an ongoing pregnancy is efficacious but requires termination of affected pregnancies. That is, prevention is

secondary. Pregnancy termination is not tolerated in many communities nor ethnic groups and is undesirable by all. Providing an option for couples at risk assuring that nonclinical pregnancy is affected is the goal. For this reason PGT has already become an integral part of preventive services for congenital disorders, providing a choice for those couples who are unable to accept prenatal screening and termination of pregnancy. PGT as the preferred form of prevention is performed under this assumption.

Less appreciated is that PGT can accomplish primary prevention in certain circumstances, i.e., preconception PGT for *primary* prevention; PGT represents an important component of ART and genetic practices. PGT management usually includes embryo selection with discarding affected embryos. However, in exceptional cases PGT can identify a transferable embryo without even having to discard or permanently cryopreserve an affected embryo. Examples will be provided in Chap. 2.

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2 Major Components of Preimplantation Genetic Testing

2.1 Introduction

Preimplantation genetic testing (PGT) is now an established clinical option in reproductive medicine [1–3]. Tens of thousands of PGT cases have been performed in hundreds of centers around the world, allowing at-risk couples to avoid producing offspring with genetic disorders. More importantly, children have been healthy, validating no ostensible evidence incurred by embryo biopsy or embryo culture (see Chap. 7).

Applied first in 1990 for preexisting Mendelian diseases [4, 5], namely, cystic fibrosis (CF) and X-linked disorders, PGT initially did not seem to be practical. Only a few babies were born during the first 3 years, and several misdiagnoses were reported [6, 7]. After the introduction of fluorescent in situ hybridization (FISH) analysis in 1993–1994 for PGT of chromosomal disorders [8–13] (Chap. 6), the number of PGT cycles began to double annually, yielding more than 100 unaffected children by the year 1996 [14, 15].

Application of PGT increased further when the ability to detect chromosomal rearrangements became possible in 1996, first using locus-specific FISH probes, then more widely available sub-telomeric probes [16, 17] (Chap. 6), haplotyping and microarray technology (array CGH) [18–29], and presently next-generation sequencing (NGS) (Chap. 3). Because many carriers of balanced translocations have a low likelihood of having an unaffected pregnancy, PGT for structural rearrangements (PGT-SR) has a clear advantage over the traditional prenatal diagnosis in assisting these couples to establish an unaffected pregnancy and deliver a child free from unbalanced translocation [1, 30–33]. Reproductive outcomes depend in turn governing efficiency of achieving an unaffected pregnancy on the origin and type of translocation. The majority result in early fetal loss and rarely in an affected birth; thus, it may take years until the translocation carriers are fortunate enough to have an unaffected offspring; thus, current recommendations of PGDIS, ESHRE and ASRM Practice Committee include chromosomal rearrangements as one of the main indications for PGT [34]. The experience of thousands of PGT-SR cycles accumulated to date demonstrates a fourfold reduction of spontaneous abortions in these couples, compared to their experience before PGT [32–37]**.** In addition, carrier couples can avoid transfer of translocation carrier embryos; approaches developed to distinguish carrier embryos from the normal ones may currently be offered to carriers of balanced translocations (Chap. 6).

The natural extension of PGT's ability to allow transfer of euploid embryos (PGT-A) would be expected to have positive impact on the liveborn pregnancy outcome, especially in poor prognosis IVF patients (prior IVF failures, maternal age over 37, repeated miscarriages). Introduction of commercially available FISH probes in 1998–1999, followed a decade later by

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current 24-chromosome testing by microarray analysis and NGS, have led to the accumulated experience of tens of thousands of PGT-A cycles worldwide [35, 37–41] (Chaps. 6 and 7), demonstrating the usefulness of PGT-A in assisted reproduction practices. According to the experience of centers, the overall pregnancy rate per transfer is higher than that in non-PGT IVF patients of comparable age groups, although the details of its application to different patient groups are still debated (see Chap. 7). The current IVF practice of transferring embryos based solely on morphological criteria is inefficient, given that half of these embryos are chromosomally abnormal and would compromise the reproductive outcome (Chap. 7). Introduction of 24-chromosome testing combined with blastocyst biopsy and current strategy of single embryo transfer further improves reproductive outcome in poor prognosis IVF patients, confirming the need for preselection of euploid embryos for transfer [18–29].

The application of PGT has further expanded with its introduction to late-onset diseases with genetic predisposition [42] (Chap. 4), an indication that had never been considered for the traditional prenatal diagnosis. For patients with inherited pathological adult-onset predisposition, PGT provides a realistic reason for undertaking pregnancy. Despite 50% risk, offspring without genetic predisposition to the disease can be obtained. Prospective parents at such risk and their physicians should be aware of this option, especially when there is no opportunity to diagnose the disease until it is fully manifested (Chap. 4).

Another unique option of PGT is HLA typing as a component of PGT (PGT-HLA) [43] (Chap. 5). In this application PGT offers not only preventative technology to avoid an affected offspring but also an approach for treating (older) siblings with congenital or acquired bone marrow diseases for which there is still no other therapy. This may in future be applied to any condition that can be treated by embryonic stem cell transplantation.

PGT- HLA was first applied to couples desiring of having an unaffected (younger) child free from the genetic disorder in the older sibling. In addition to diagnosis to assure a genetically normal embryo, HLA-matched, unaffected embryos were chosen. At delivery cord blood (otherwise to be discarded) was gathered for stem cell transplantation. As will be described, this approach has been also used without testing of the causative gene, with the sole purpose of finding a matching HLA progeny for a source of stem cell transplantation for affected siblings with congenital or acquired bone marrow disease or cancer [44] (see Chap. 5).

As will be described in this book, 30 years of PGT experience has demonstrated considerable progress. Hundreds of thousands of PGT attempts worldwide have resulted in birth of a large cohort of apparently unaffected children, with no detrimental effect on embryo development. There are no significant differences in the overall congenital malformation rate after PGT compared to population prevalence [45–47] (Chap. 7). With the highly improved accuracy of genetic analysis and indications expanding well beyond those for prenatal diagnosis, up to hundred thousand PGT cycles are now performed annually. This reflects PGT offering a special attraction not possible with traditional prenatal diagnosis, namely, avoiding clinical pregnancy termination. As mentioned, this is extremely useful for translocation carriers, couples at risk for producing offspring with common diseases of autosomal dominant or recessive etiology, and couples wishing to have not only an unaffected child but also an HLA-compatible stem cell donor for treatment of an older moribund sib with a congenital disorder. Yet the greatest numerical impact of PGT involves assisted reproduction practices to increase pregnancy rates (Chaps. 6 and 7). The estimated number of ART centers using PGT solely for this purpose is well over one-third of cycles in the United States. Thus, improved IVF efficiency through aneuploidy testing has become standard, despite the technology being quite sophisticated.

Special expertise and training in the main components of PGT is required.

Pivotal to PGT is obtaining biopsy material from oocytes and embryos. Biopsy material for performing PGT may be obtained from three major sources:

1. Matured and fertilized oocytes from which the first and second polar body (PB1 and PB2) are removed
2. Eight-cell cleavage-stage embryo, from which a single blastomere is removed
3. Blastocyst-stage embryo, from which not less than 5, but not more than 10, cells are removed

Material obtained is tested for single-gene disorders using PCR analysis, or used for chromosomal abnormalities, previously done by fluorescent in situ hybridization (FISH) and now by microarray (array CGH) or next-generation sequencing (NGS) (Chaps. 3 and 6). Each of these PGT methods has its advantages and disadvantages, with method selected depending on circumstances; in some cases combination of these methods may be required. Despite reduction in embryo cell number after biopsy, having a potential deleterious influence on embryo viability, blastomere or blastocyst biopsy allows detection of paternally derived abnormalities. On the other hand, removal of PB1 and PB2 should not have much effect on the embryo viability as polar bodies are naturally extruded from oocytes as result of maturation and fertilization. Polar bodies provide no information on the paternally derived anomalies, even if this constitutes less than 5% of chromosomal errors in preimplantation embryos.

2.2 Polar Body Sampling

Introduced almost 30 years ago [5], PB biopsy is still one of the alternative approaches in PGT, although its application has been limited to ethnic and social groups that cannot accept embryo biopsy or wish to avoid technical problems in certain PGT indications. The rationale for performing PGT by the use of PB is based on the fact that PBs are the by-products of female meiosis and allow predicting by deduction of the resulting genotype of the maternal contribution to the embryo. Neither PB1, extruded as a result of the first meiotic division, nor PB2, extruded following the second meiotic division, has any known biological value for pre- and postimplantation development of the embryo. Initially, only PB1 was tested, based on the fact that in the absence of crossing over, PB1 will be homozygous for the allele not contained in the oocyte and PB2 [48, 49]. However, the PB1 approach was not applicable for predicting the eventual genotype of the oocytes if crossing over had occurred, because the primary oocyte in this case would be heterozygous for the mutant gene. Frequency of crossing over varies with the distance between the locus and the centromere, approaching as much as 50% for telomeric genes. Thus, PB1 approach is of limited value, unless the status of the oocyte can be deduced by PB2, which allows detecting hemizygous normal oocytes resulting after the second meiotic division. As will be described below, this PGT technique involves a two-step oocyte analysis, with a sequential testing of PB1 and PB2 (see details of micromanipulation setup and procedure steps elsewhere [48]).

In brief, PB1 and PB2 are removed following stimulation and oocyte retrieval using a standard IVF protocol. Following extrusion of PB1, the zona pellucida (ZP) is opened mechanically using a microneedle, or laser, and PB1 aspirated into a blunt micropipette. Oocytes are then inseminated with motile sperm, or using intracytoplasmic sperm injection (ICSI), and examined for the presence of pronuclei and extrusion of PB2, which is removed in the same manner as PB1. To avoid an additional invasive procedure, both PB1 and PB2 may be removed simultaneously, fixed, and analyzed on the same slide (acceptable only for FISH analysis). However, for PGT-M, PGT-SR, and PGT-A by microarray and NGS analysis, PB1 and PB2 are removed sequentially as mentioned above. The biopsied oocytes are then fertilized, returned to culture,

checked for cleavage, and transferred, depending on the genotype of the corresponding PB1 and PB2 [48].

As mentioned, PB1 and PB2 have no any known biological significance in pre- and post-implantation development to affect embryo viability, as also shown by the follow-up study. In this study, it was demonstrated that following the procedure, zygotes with two pronuclei were observed in 1192 (81.8%) of 1458 oocytes, compared to 30,972 (77.3%) of 40,092 in a routine non-PGT cycles, suggesting no difference in fertilization rate observed after PB1 removal in comparison with non-biopsied oocytes. There was also no difference in blastocyst formation of the embryos resulting from the biopsied oocytes. Blastocyst formation of embryos resulting from biopsied oocytes was observed in 1653 (50.2%) of 3293 embryos, not different from 49.8% (9726 of 19,529) non-biopsied embryos observed in routine IVF. Similarly, no detrimental effect was noted after PB2 removal, which was evident from cleavage rate, blastocyst formation, and the number of cells in the respective blastocysts [50]. As will be seen below, there was no difference after a sequential PB1–PB2 and embryo biopsy.

2.2.1 Polar Body Testing as a Preconception Testing Strategy

Although not used on a large scale, PB as a preconception strategy may have selected utility. In certain venues – Austria, Germany, Switzerland (until recently), and Malta – PGT has been restricted to micromanipulations only prior to fertilization. This strategy is also applicable to certain religious groups. While laws are being evolved in some of these communities, PB1-based testing still remains an option for haplotype analysis in PGT for de novo mutations of maternal origin (Chap. 4). PB1 testing is not sufficient to predict embryo genotype, unless PB2 is tested before pronuclear fusion. This may be combined with freezing of the oocytes at the pronuclear stage. After analysis, the oocytes predicted of having the normal maternal allele may be thawed and cultured to allow the pronuclear fusion, embryo development, and transfer in a subsequent menstrual cycle.

In fact, it is possible to complete the testing of PB2 in approximately 9 hours after removal. This avoids the need for freezing of the mutation or aneuploidy-free oocytes, allowing continued culture as usual and replacement on day 3 or day 5; abnormal oocytes are frozen at the pronuclear stage or discarded [51]. Because zygotes are not considered to be embryo until pronuclear fusion, and no abnormal oocytes are thawed and cultured, the establishment of the affected embryos is obviated; thus, this technique may be ethically more acceptable to many couples. This technique creates a new class of genetic testing, which may be called pre-embryonic genetic testing (PEGT), pushing the frontier of genotyping to an even earlier stage. The first attempt of PEGT in testing the feasibility of the approach included sickle cell anemia and Sandhoff disease (SHD).

PEGT was performed for a 33-year-old woman and her spouse at risk for producing a child with sickle cell disease. The couple could not accept neither a possible termination of a pregnancy following prenatal diagnosis nor any manipulation of the embryo. A standard IVF protocol was used, but the patient suffered hyperstimulation syndrome, which precluded transfer of embryos from that cycle. Twenty-eight mature oocytes were aspirated and placed in culture medium. Of the 28 aspirated oocytes, 14 extruded PB1s, which were removed. The oocytes were then fertilized by intracytoplasmic sperm injection. As soon as the PB2s were removed and prior to the fusion of the male and female pronuclei, all the oocytes were frozen. PB1 and PB2 were analyzed by multiplex nested PCR to ensure detecting a potential allele dropout (ADO), which occurs in approximately 5–10% of cases in the PB analyses (see Chap. 3). This involved a nested, multiplex PCR with primer sets for the sickle cell mutation and two linked short tandem repeat

(STR) markers, one located at the 5′ end of the beta-globin gene (5′ STR) and the other in the human tyrosine hydroxylase gene (THO-STR), for both of which the mother was heterozygous. To detect potential contamination with extraneous DNA and identify the embryo that implanted and established a pregnancy, additional non-linked STRs were amplified (the details of this first preconception PGT case were described elsewhere [48, 52]).

The pronuclear-stage oocytes predicted to be normal were thawed, cultured to develop into the cleaving embryos, and transferred back to the patient in the two subsequent clinical cycles. The oocytes predicted to contain the mutant maternal gene were not thawed, but analyzed directly at the pronuclear stage for the confirmation of PB diagnosis. Following intracytoplasmic sperm injection of 14 oocytes with extruded PB1, PB2s were extruded from 13 of them, with the results of both PB1 and PB2 available in 12 of these 13 oocytes. Overall, six oocytes were predicted to contain a normal allele, based on heterozygous status of PB1 and hemizygous mutant status of PB2.The frozen cycle with the transfer of two unaffected embryos resulted in a singleton pregnancy and birth of unaffected child, following confirmation of PB diagnosis by chorionic villus sampling (CVS).

The second case of PEGT was done without pronuclear stage freezing, based on the technological possibilities to complete testing before pronuclear fusion. It was offered to a 32-year-old woman and her spouse, who were at risk for producing a child with Sandhoff disease (SHD) and specifically requested PGT to be performed without any possible discard of embryos even if affected [51]. As seen from the pedigree shown in Fig. 2.1, the couple had one affected son with classical features of SHD, who died at the age of 1 year and 3 months despite bone marrow transplantation.

SHD results from the defect in the beta chain of hexosaminidase B gene (HEXB) on chromosome 5, which consists of 14 exons distributed over 40 Kb of DNA (MIM 268800; 606,873). Mutation in this gene causes beta-hexosaminidase deficiency, resulting in the lysosomal storage disease GM2-gangliosidosis. The same condition is caused by Tay–Sachs disease resulting from the defect of hexosaminidase A gene (HEXA).

The child inherited two different mutations from his parents: the paternally derived I 270 V mutation in exon 5 of HEXB gene, resulting from ATT to GTT substitution, and a large maternal 16Kb deletion (16Kb Del), involving as many as 5 exons, from exon 1 to exon 5. The paternal mutation was identified by the *Hinf* I restriction digestion, which cuts the normal allele into two fragments of 32 and 25 bp, leaving the mutant allele uncut, and the maternal 16Kb Del detected by a fragment size analysis. Five closely linked polymorphic markers, D5S1982, D5S1988, D5S2003, D5S349, and D5S1404, were tested simultaneously with the HEXB gene in a multiplex heminested PCR system.

A single PGT cycle was initiated, which was performed according to the modified timetable of the applied procedures of sequential PB1 and PB2 analysis. PB1 was removed as usual 3.5 hrs after aspiration, followed by ICSI. PB2 was removed soon after it was extruded, approximately within 6.5 hrs after ICSI, to allow sufficient time for the completion of the DNA analysis before pronuclear fusion (see Fig. 2.2). DNA analysis was done in less than 9 hrs overall, making it realistic to freeze the oocytes predicted to contain the deleted HEXB allele before syngamy (within 24 hrs after aspiration or 12 hrs after PB2 removal), and culture the HEXB deletion-free oocytes to blastocyst and transfer at day 5, following confirmation of the maternal mutation-free status of the embryos by the embryo biopsy.

Of 18 oocytes available for testing in a single PEGT cycle, 16 showed conclusive PB1 and PB2 results, of which 8 contained the maternal 16Kb deletion and frozen at the pronuclear stage (Fig. 2.1). Four of these oocytes contained heterozygous PB1 and normal PB2 (oocytes #3, # 9, #11, and #14) and four homozygous normal PB1 and mutant PB2 (Fig. 2.1b). The remaining eight

oocytes were free of the deletion, two originating from the oocytes with heterozygous PB1 and mutant PB2 (oocytes # 1 and # 5) and the others from the oocytes with homozygous mutant PB1 and normal PB2. As the predicted genotypes in these oocytes may erroneously appear opposite, due to a possible undetected ADO of one of the alleles in the actually heterozygous PB1, similar to the four mutant oocytes predicted on the basis of homozygous normal PB1 and mutant PB2, the testing for five closely linked polymorphic markers was essential, confirming all the predicted oocyte genotypes mentioned.

A follow-up blastomere analysis of the embryos deriving from the oocytes predicted to be free of maternal deletion showed complete correspondence to the PB testing. Six of these embryos appeared to contain also a normal paternal allele (embryos #1, 4, 5, 6, 8, and 10), while only two (embryos #16 and 18) inherited the paternally derived mutant allele, confirmed by all five linked polymorphic markers tested (Fig. 2.1).

Results showed that PEGT is a realistic option for couples who cannot accept traditional PGT, because of their objection to micromanipulation

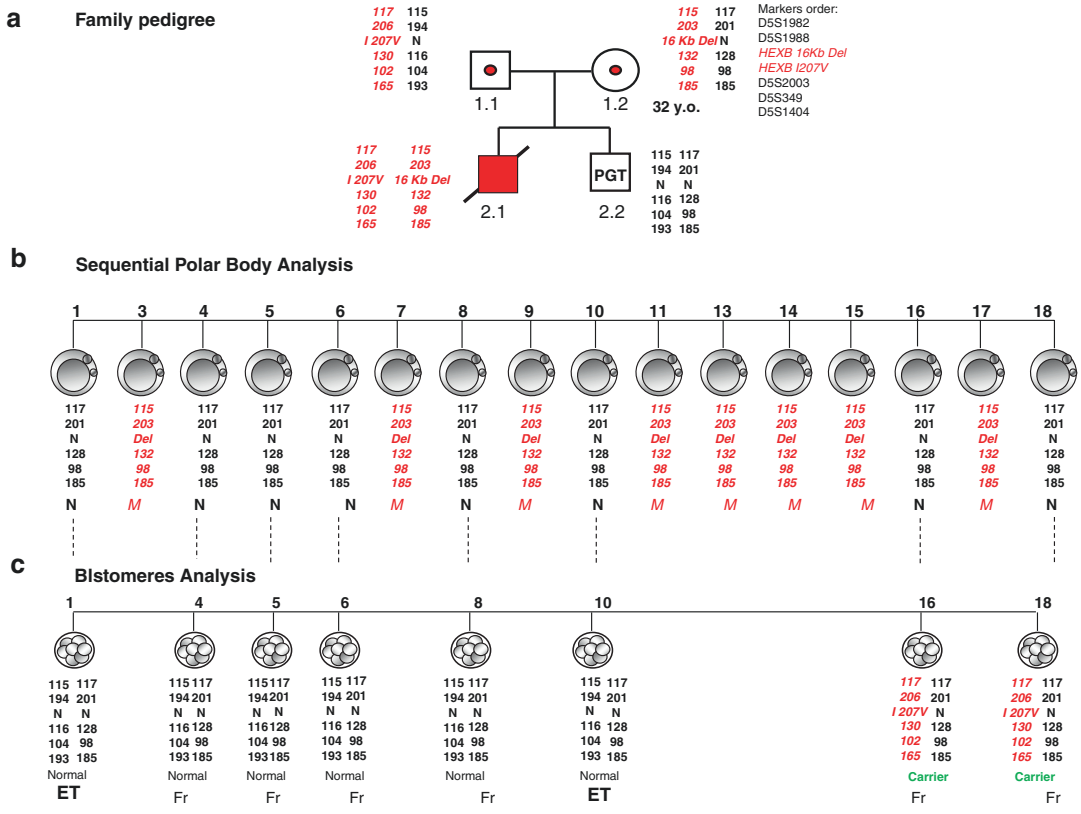

Fig. 2.1 Pre-embryonic testing for Sandhoff disease. Upper portion, showing pedigree (**a**) results of polar bodies (**b**) and blastomeres (**c**); and Figure 2.1. Bottom portion, showing the actual DNA analysis used: schematic presentation of the mutation and linked polymorphic markers (**A**); polar body analysis of the maternal 16 Kb deletion (N – normal; D – deletion) (**B**); Restriction map: HhaI enzyme created 2 fragments in normal gene, leaving the paternal mutation I 207V uncut (**C**); and Blastomere analysis for maternal deletion and paternal mutation, confirming the PB diagnosis (**D**)

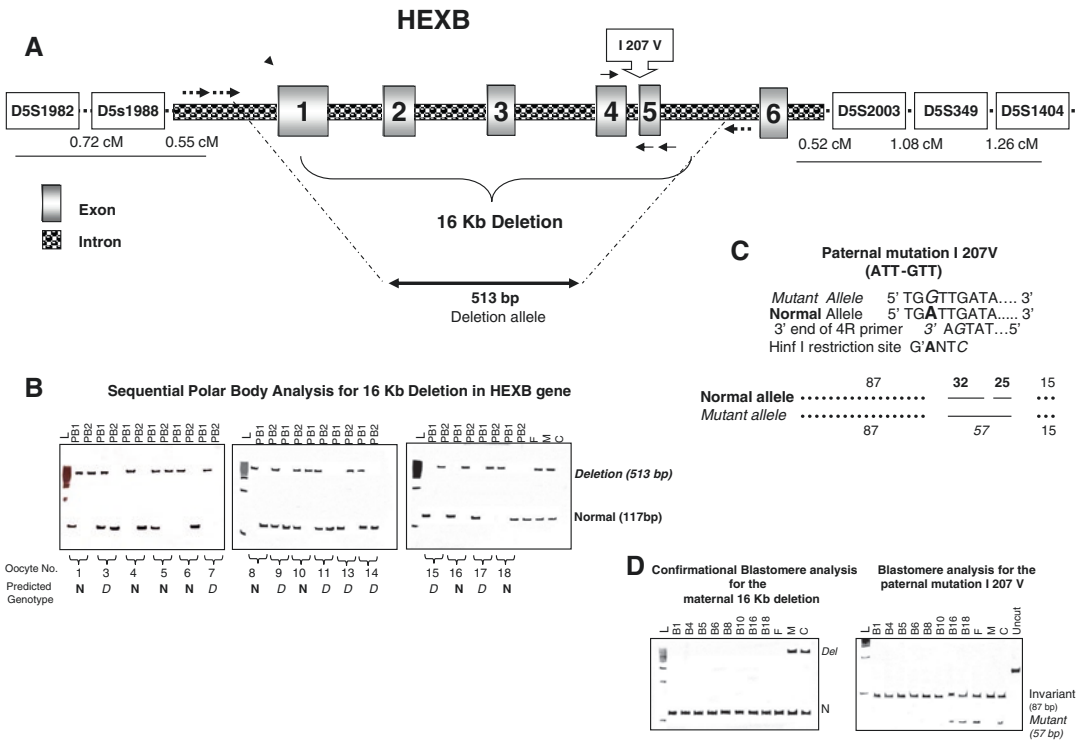

Fig. 2.1 (continued)

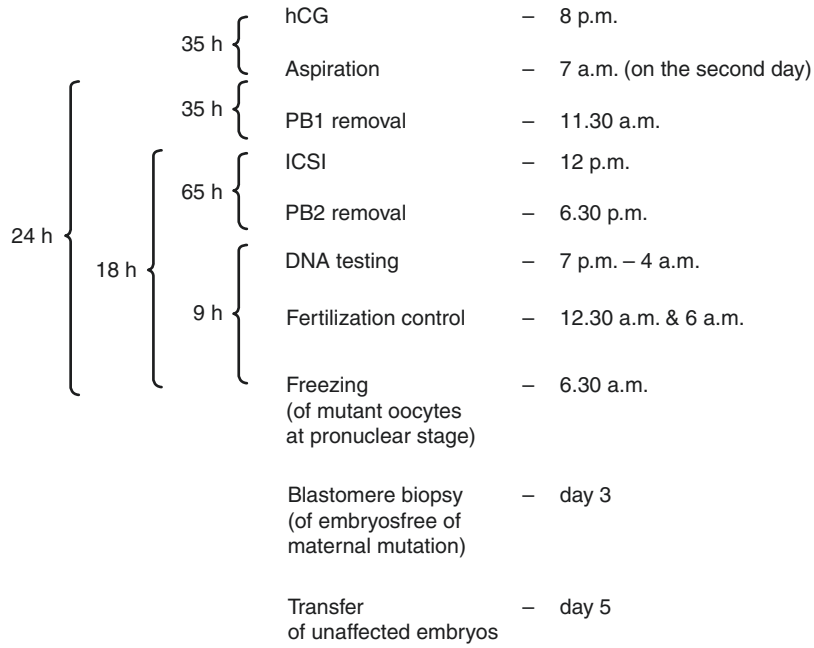

Fig. 2.2 Timeframe for pre-embryonic diagnosis of Sandhoff disease (see explanation in the figure)

and potential discard of the tested embryos. The previous PEGT case described involved freezing of all the tested oocytes at the pronuclear stage immediately after ICSI and extrusion of PB2. The present case is proceeded without freezing of the mutation-free oocytes, which were detected well before the pronuclear fusion and prior to when a decision to discard could not be avoided. Although all oocytes could have been frozen irrespective of DNA diagnosis (as in the previous case), recovering all frozen pronuclear stage oocytes might not be possible. Those not recovered may have included preselected unaffected embryos that if not transferred could have negatively affected the PEGT outcome. PEGT in the same clinical cycle is clearly an important practical step, which has become realistic because of DNA analysis being completed within less than 9 hrs.

PEGT may be also applied for aneuploidy, as given the great majority of chromosomal disorders deriving from the female meiosis and testable by PB analysis. Available experience is presently limited to translocation or aneuploidy testing by PB1 analysis, which, as mentioned, leaves meiosis II errors undetected. As seen from the present results, detection of the second meiosis errors is currently feasible within the timeframe available prior to pronuclear fusion; thus, PEGT for chromosomal disorders may in future be also applied in those countries where PGT is still not acceptable because of the potential discard of the affected embryos with the currently used methods.

Presented data demonstrate feasibility of performing PEGT for single-gene disorders, which resulted in obtaining unaffected pregnancies and birth of healthy children. Of course, PGT-M may be performed by the use of PB1 analysis alone, as described in the first case of PGT by PB1 [5]. Although this allowed preselection of a few mutation-free oocytes inferred from the homozygous abnormal status of PB1, the majority of oocytes were heterozygous after the first meiotic division, so the genotype of the resulting embryos could not be predicted, thus limiting the number of normal embryos for transfer.

Data also show that to avoid discard of preimplantation embryos reaching the cleavage stage by the time the PB genotyping results were obtained, freezing of oocytes may be applied immediately after ICSI and extrusion of PB2, as well as prior to fusion of the male and female pronuclei (the actual point considered to be the beginning of the embryonic period of development [53]). In fact, freezing may be omitted entirely, as developments in PCR analysis allow completing the genetic diagnosis before pronuclei fusion. This opens a possibility for application of PGT for couples who are unable to accept any intervention and discard of the human embryos.

2.2.2 Analysis of Sperm

No method has yet become available for testing the outcome of male meiosis because genetic analysis destroys the sperm, rendering it useless for fertilization. To overcome this problem, an original technique has been introduced, allowing duplicating a sperm before genetic analysis. One of the duplicated sperms can be used for testing whereas the other for fertilization and consequent transfer of the resulting embryos, i.e., provided genetic analysis of the corresponding duplicate shows normal genotype [54, 55]. In this way the establishment and discard of any embryo containing paternal mutation may be avoided. However, more data are necessary to define special conditions required for the faithful replication of human sperm genome, i.e., assuring that the haploid cell pairs obtained from sperm duplication are identical. Still lacking is validation for preconception testing and application to exclude paternally derived mutations.

The genotype of sperm may be also tested following the testicular biopsy culture and tracking the developmental progression of spermatocytes through meiosis in vitro. This provides the possibility of meiosis outcome analysis to infer the genotype of the resulting sperm to be used for fertilization. However, this is still not practical. There have also been attempts to approach preconception testing through development of artificial gametes, using techniques of somatic cell haploidization