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# Practical Preimplantation Genetic Diagnosis

Second Edition

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# Foreword

The casual reader may not fully appreciate the significance of the well-chosen title for this unique and welcome second edition of *Practical Preimplantation Genetic Diagnosis*. The title is not only apropos but provided by the team that truly made it possible.

Prenatal genetic diagnosis began in the 1950s, with detection of X-chromatin in amniotic fluid cells. By the late 1960s amniocentesis allowed diagnosis of chromosomal abnormalities and selected inborn errors of metabolism. Prenatal genetic laboratories sprang up worldwide, and by the 1970s antenatal testing became standard for certain patients. The next decade (1980s) brought two key advances. First was noninvasive screening, an invasive procedure like amniocentesis sometimes performed only if risk was high enough to be justified. Concurrently, there was movement toward prenatal diagnosis earlier in pregnancy, as witnessed by first trimester chorionic villus sampling (CVS). Still, diagnosis was not possible before 10–12 weeks and, hence, clinical pregnancy termination necessary.

Although proof of principle for preimplantation genetic diagnosis (PGD) had been shown in 1967 by Prof. Robert Edwards through X-chromatin analysis in rabbit blastocysts, application in the human awaited advances in human assisted reproductive technologies (ART) and of molecular techniques. The first human PGD finally came only in 1990. The later 1980s by Yury Verlinsky and colleagues with near concurrent reports by Alan Handyside and colleagues. During the next few years, there was great interest in PGD among the genetic community; however, relatively few clinical cases were performed, mostly for Mendelian disorders. With introduction of fluorescent in situ hybridization (FISH), however, PGD was suddenly burgeoning. By 2000, novel indications were added, including aneuploidy testing to identify chromosomally normal embryos. Indeed, this is now the most common indication for PGD, despite recent controversy on the impact of aneuploidy testing on the reproductive outcome.

*Practical Preimplantation Genetic Diagnosis* systematically covers indications and technology. One is taken through PGD from start (embryo biopsy) to end (consequences). And, we learn from the group that has performed a considerable proportion of the world's cases. Techniques for obtaining embryonic DNA are first described (polar body biopsy or blastomere biopsy), followed by methods for single cell genetic analysis (FISH, PCR, or microarray-based approaches). The spectrum of detected Mendelian disorders and their detection follows, including PGD for de novo mutations; until

recently, the latter could not be accomplished. Once controversial topics like late-onset genetic disorders are covered, including PGD for genetic predisposition to different cancers and cardiovascular diseases. Especially lucid coverage concerns on PGD to obtain HLA compatible embryos. PGD not only avoids another genetically affected offspring, but provides umbilical cord-derived stem cells for transplantation into an older moribund sib.

Both numerical and structural chromosomal abnormalities are discussed in the light of the current application of microarray technology. We are provided novel information on the unexpectedly high *sequential* error rate in meiosis I followed by meiosis II. Clinical outcome, safety and extremely high accuracy of PGD are discussed. Throughout, discussion flows seamlessly between indication and counseling (reflecting the impeccable genetic credentials of the group) and laboratory performance (likewise). Illustrations are highly informative, and especially invaluable in understanding PGD for Mendelian disorders. The protocol must involve the linkage analysis, which is clearly explained.

Overall, one comes away with a keen appreciation of how PGD has moved from "boutique" medicine, practiced only rarely by the cognoscenti, to a technique rapidly becoming de rigueur in reference centers worldwide. Considerable information is new and not heretofore available even in peer review format. The result is a pleasing combination of reading a textbook peppered with peer review information. The sense of movement in PGD is thus palpable.

This second editorial *Practical Preimplantation Genetic Diagnosis* remains a gem. In this age of edited works, a text written by only one or two is rare. That our authors practically invented the field, and certainly have made it practical, makes this volume truly extraordinary. Our persisting pain over the tragic, premature, loss of Yury Verlinsky – the group's founder, pioneer in PGD, and author with Dr. Kuliev of the first edition – is mitigating by this stellar text. Dr. Verlinsky would be proud of his legacy as recounted and promulgated. This volume should thus be at the reach of every reproductive geneticist, ART lab, and reproductive biologist. The avid student will come away fully informed and up to date, ready for tomorrow's advances.

White Plains, NY Joe Leigh Simpson, M.D., FACOG, FACMG

# Preface

Although treatment is the major goal in the control of genetic disease, like in other fields of medicine, this is not yet a reality for most of inherited conditions. Even with a dramatic advancement in the field of gene therapy, there are still not, unfortunately, many successful stories, to allow predicting its sound impact in the near future. Therefore, in the absence of radical treatment, prevention of genetic disorders is still one of the available options for genetically disadvantaged people. Among the measures for prevention, the avoidance of the birth of an affected child in couples at genetic risk has become a quite acceptable option in many populations. This is based on a population screening and prenatal diagnosis, and has been quite successful, resulting in an almost eradication of new cases of some genetic diseases from several Mediterranean and some large Meddle Eastern populations. However, this has generated an increasing number of abortions following prenatal diagnosis, leading to a growing concern and negative reaction in society to the preventive genetics programs. For example, some ethnic groups cannot accept any control measures regarding congenital diseases, because of pregnancy termination not being allowed due to social or religion reasons. Even in those communities where abortion is allowed, some couples have to experience two or more terminations of pregnancies before they can have a normal child. No doubt these families might require an alternative option to achieve their desired family size without the need for termination of pregnancy.

The present book is devoted to a principally new approach in prevention of genetic disorders, which avoids the need for prenatal diagnosis and termination of pregnancy. Accordingly, a novel concept of pre-pregnancy diagnosis, which is called preimplantation genetic diagnosis (PGD), is introduced, which is based on the control of the processes of the oocyte maturation, fertilization, and implantation, so to select and transfer back to patients only normal embryos, and achieve an unaffected pregnancy resulting in the birth of a healthy child. In this way, the couples at high risk of having an offspring with genetic disease have an option to control the outcomes of their pregnancy from the onset. So the place of this approach in the context of other approaches for prevention of genetic disorders available is discussed, together with other primary preventive measures, which may allow presently avoiding up to a half of congenital malformations presented at birth (Chap. 1). Although the option of PGD involves an ovarian stimulation and in vitro fertilization (IVF), the description of the available experience demonstrates that this has appeared to be an acceptable procedure in many ethnic groups all over the world.

In fact, PGD is now entering its third decade as an established procedure for genetics and assisted reproduction practices, with exciting new developments that are changing the whole concept of prevention of congenital disorders, to allow the couples at risk to reproduce as normally as possible without much fear of having an affected offspring. The availability of the practical experience of tens of thousands of PGD cases makes it necessary to update the current information provided to medical profession and patients on its accuracy, reliability, and safety to ensure a wider clinical application, an improved access to PGD services of those at need who may benefit greatly from this technology. The dramatic developments in PGD technology are obvious from more than 250 different conditions for which PGD have been applied, with over 99.5% accuracy in the leading PGD centers. There is also not any restriction in provision of PGD, which may presently be performed for any genetic condition, even if no relevant haplotypes are available, such as in cases that the conditions were first identified in one of the parents or only in the affected child.

So the present edition of the book updates the progress in prevention of genetic disorders to demonstrate the important place of PGD in primary preventive measures and its increasing role in providing the whole range of reproduction options to couples at risk. Because of the above improvements of PGD methods, Chaps. 2 and 3 are considerably updated to provide the basis for improved accuracy to be achieved not only in leading PGD centers but also worldwide. This includes the methods for both direct and indirect testing for mutations, as a more universal approach for tracing their inheritance, with special emphasis on PGD for de novo mutations, which has previously presented a real challenge. As we have presently accumulated the world's largest experience in this area, we present PGD strategies for different genetic disorders, determined by de novo mutations of maternal or paternal origin, with dominant, recessive, and X-linked modes of inheritance (Chap. 3). Although the emphasis is mainly on the laboratory aspects, some of the ethical, social, and legal aspects will also be briefly explored (Chap. 8).

Indications for PGD were also expanded, with current wider application of PGD for diseases with genetic predisposition, such as different cancers and cardiovascular disorders. The number of requests for PGD of these common disorders is increasing gradually, with the progress of identification of the predisposing genes, with extremely high penetrance, such as in breast and colon cancer. So the description of our experience of a few hundreds of PGD cases for this group of conditions, which resulted in detection and transfer of embryos free of cancer predisposing genes in as high as 80% of cases, with one-third of them yielding the unaffected pregnancies and birth of healthy children, will help initiating similar services in other centers, facing the forthcoming increase of requests from this highly sensitive group of at-risk couples (Chap. 3). This section also includes the first cumulative experience of PGD for inherited cardiac disorders, allowing couples carrying cardiac disease predisposing genes to reproduce without much fear of having offspring with these genes at risk for premature or sudden death.

Because of tremendous progress in PGD for stem cell transplantation treatment of genetic and acquired disorders, the special section devoted to

preimplantation nongenetic testing involving HLA typing is substantially updated based on our pioneering experience still representing one of the largest in the world (Chap. 4). Since our first description of such a possibility more than 10 years ago, PGD for HLA matching has been performed in a few thousands of cases, resulting in a successful HLA-compatible stem cell transplantation in close to 100 siblings, with almost 100% success rate. The list of conditions for which this approach was applied is being gradually extended, so the description of this experience will help to avoid the potential problems of the observed recombination in the HLA gene cluster, affecting the selection of HLA matched embryos, and the clinical outcome of stem cell transplantation. This will promote a wider application of the stem cell therapy, which will be reality for increasing number genetic and acquired conditions, for which there is still no available treatment.

Despite recent controversy in PGD for chromosomal disorders, the present progress in improving the accuracy of the procedure through the adequate choice of biopsy material and microarray analysis for 24 chromosomes has demonstrated the clinical impact of avoiding aneuploid embryos from transfer. A highly improved detection of chromosomally abnormal oocytes and embryos by microarray technology is currently being validated for practical application due to the obvious need for detecting and avoiding the chromosomally abnormal embryos from transfer as a standard practice, so this is described in detail in a special section, with detailed discussion of the present controversy on PGD impact on pregnancy outcome (Chap. 6). The presented preliminary data on 24 chromosome testing confirm our extensive original experience of FISH analysis of over 20,000 oocytes and embryos, which is presented with special emphasis on chromosome-specific prevalence in relation to maternal age, their meiotic origin, and its possible impact on embryo viability. Because 96% of aneuploidies originate from female meiosis, the primary emphasis is still on testing for 24 chromosomes in the first and second polar body by array-CGH. On the other hand, to detect mitotic errors and paternally derived aneuploidies, the technique is being validated also for blastocyst biopsy, evidencing the accuracy for detecting post-zygotic errors, including mosaicism, which is still the major challenge of PGD for chromosomal disorders by embryo biopsy (Chap. 5).

It is further confirmed that PGD is the only hope for couples carrying balanced translocations (Chap. 5). In the light of these data, a pioneering work on different conversion methods to turn interphase nuclei of single biopsied blastomeres into metaphase chromosomes is described with presentation of the original experience of the application of these methods for PGD of translocations. On the other hand, further improvement in PGD for translocations is being achieved by application of microarray technology, although its utility is limited to the cost.

In Chap. 7, the original experience on the applications of PGD to the embryonic stem cells is described, providing the possibility of obtaining preimplantation embryos with known genotypes as a source for the establishment of custom-made embryonic stem cells. This section provides the data on the establishment of the world's largest collection of the genetic disease-specific embryonic stem cell lines, containing 87 lines with genetic and

chromosomal disorders and 12 lines with the allele conferring resistance to HIV. This is the unlimited source and unique in vitro model for analysing the primary mechanisms of congenital disorders, and development of the methods for cellular therapy.

So the second edition provides extensive review of the most recent developments of PGD, which includes PGD for expanding indications, such as de novo mutations, cancers, inherited cardiac diseases and combined PGD for single gene disorders, HLA typing and 24 chromosome testing in patients of advance reproductive age, in the light of the further prospects of the application of PGD to medical practice. This may be useful not only in planning and organization of such services but will also provide a working manual for the establishment and performance of PGD in the framework of IVF and genetic practices.

# Acknowledgments

Dedicated to the memory of my colleague and friend, co-author of the first edition of this book, Yury Verlinsky.

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# Contents

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# 1 Primary Prevention of Genetic Disorders and Place of Preimplantation Diagnosis

Gene therapy is still not a realistic option for genetic disorders, so prevention remains the main approach. Preventive measures may be applied at the community level by avoiding new mutations, protection from all possible environmental hazards, predictive testing for genetic and complex disorders, and prospective screening for common genetic disorders specific for each ethnic group. The optimal time for offering these preventive measures is the preconception or preimplantation stage (Fig. 1.1), as any detection afterward will involve the decision either to keep the pregnancy with the long-term social, familial, and financial consequences of a seriously affected child, or to terminate the planned and wanted pregnancy.

So the strategies for prevention range from preventing environmental hazards and vitamin supplementation programs to pre-pregnancy or prenatal diagnosis. The example of highly effective population-based preventive measures realized at the preconception stage has been prevention of neural-tube defects (NTD) and some other congenital abnormalities by folic acid or folic acid-containing multivitamins. On the other hand, preconception and preimplantation genetic diagnosis (PGD) has been established as a realistic option for primary prevention of genetic disorders as described in detail in this book.

The most relevant approaches for primary prevention of congenital disorders presently include (1) avoidance of new mutations through environmental programs, (2) reduction of pregnancy at advanced ages through community education and family planning, (3) periconceptional folic acid supplementation or multivitamin fortification of basic foodstuffs, (4) rubella vaccination, (5) avoidance of alcohol consumption and smoking during pregnancy, (6) prenatal diagnosis, and (7) pre-pregnancy (preimplantation) diagnosis. As seen in Fig. 1.1, the available actions could avoid congenital disorders of environmental origin, controllable by manipulating the environment, which can often be done by public health measures, and constitutional-remaining even when environmental causes are controlled, requiring more sophisticated approaches for detecting and managing risk. In addition, some conditions, such as NTD, have both

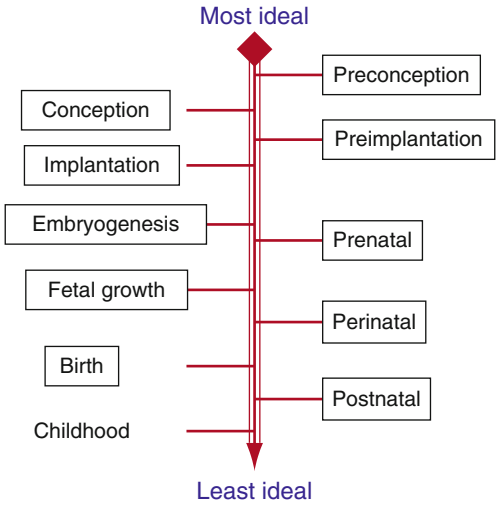


**Fig. 1.1** Options offered for couples at genetic risk for diagnosis and prevention. Stages of development are shown on the *left*, and the points for application of preventive measure on the *right*

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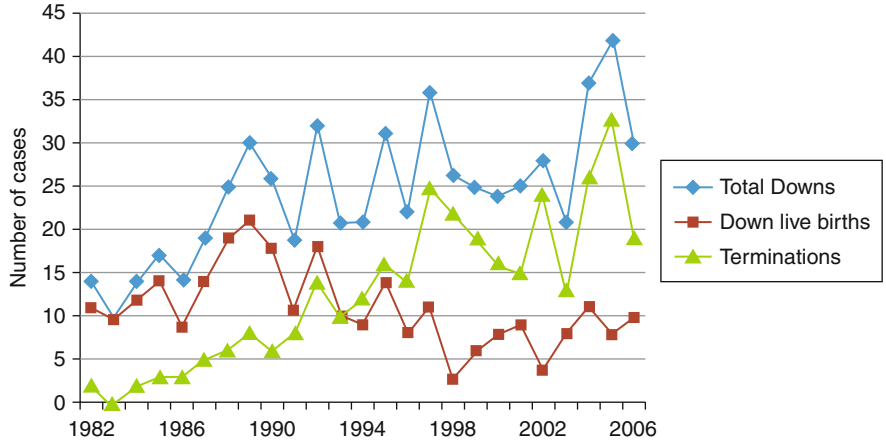


**Fig. 1.2** Prevention of Down syndrome, 1979–1999, in Strasbourg, France, by Prenatal Diagnosis and termination of pregnancy [1]. The data are representative for all industrialized countries where population screening and prenatal diagnosis are operational for more than two decades, resulting in tremendous reduction of Down syndrome prevalence (*orange curve*) compared to the expected prevalence in the absence of preventive measures (*blew curve*), which is achieved by the increase in the number of pregnancy terminations (*green curve*)

genetic and environmental components, so the actions are first addressed to environmental causes and finding the key components to modify the realization of the congenital disorder, as described below. It is understood that most populations only get round to addressing the constitutional disorders when they have largely controlled environmental causes. The decision to adopt any of the available preventive programs depends on differences in health services development, ethnic distribution of congenital disease, and the local attitudes to genetic screening and termination of pregnancy. For example, induced abortions are still not permissible in many countries on religious grounds. On the other hand, the number of countries permitting prenatal diagnosis and termination of pregnancies for medical indications is steadily increasing.

The impact of community-based preventive approaches is obvious from the Down syndrome prevention program in the majority of industrialized countries of Europe and the USA. The programs are based on prenatal maternal serum screening for all pregnant women to detect pregnancies at increased risk, followed by the offer of definitive diagnosis and selective pregnancy termination, plus prenatal diagnosis offered to all women of advanced maternal age, which has resulted in the reduction of the birth prevalence of Down syndrome by 50% or more [1] in some regions (Fig. 1.2). However, this reduction is proportional to the number of pregnancy terminations, which changed annually with the success of the prevention program, as seen from Fig. 1.3. In some countries the effect of such programs is still growing (www.eurocat-network.eu), while in others it seems to be reaching a plateau (Figs. 1.4 and 1.5), reflecting differences in the development of the service as well as social and religious differences. However, the fact that this reduction is achieved through pregnancy terminations proportional to the number of Down syndrome prevented (Fig. 1.3) is a cause for serious concern [1, 2]. This is particularly relevant for high-income countries where women use family planning to postpone childbearing, leading to a rebound in the proportion of older mothers.

At present the most powerful approach for avoiding congenital disorders at the community level involves the expanded use of fetal ultrasound, which has improved the detection rate of affected pregnancies, and enables the choice of whether to terminate the pregnancy or to plan early treatment for the affected child. The effects may be evaluated by the community-based birth defect-monitoring systems available in an increasing number of countries. As termination is usually

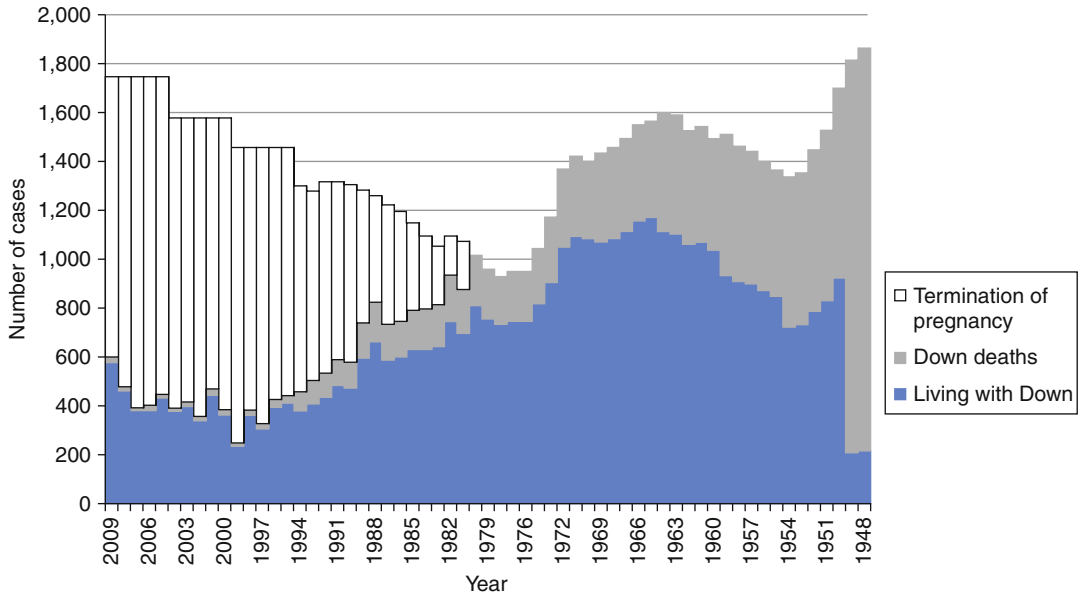


**Fig. 1.3** Outcomes of pregnancies with Down syndrome fetus in France since 1948

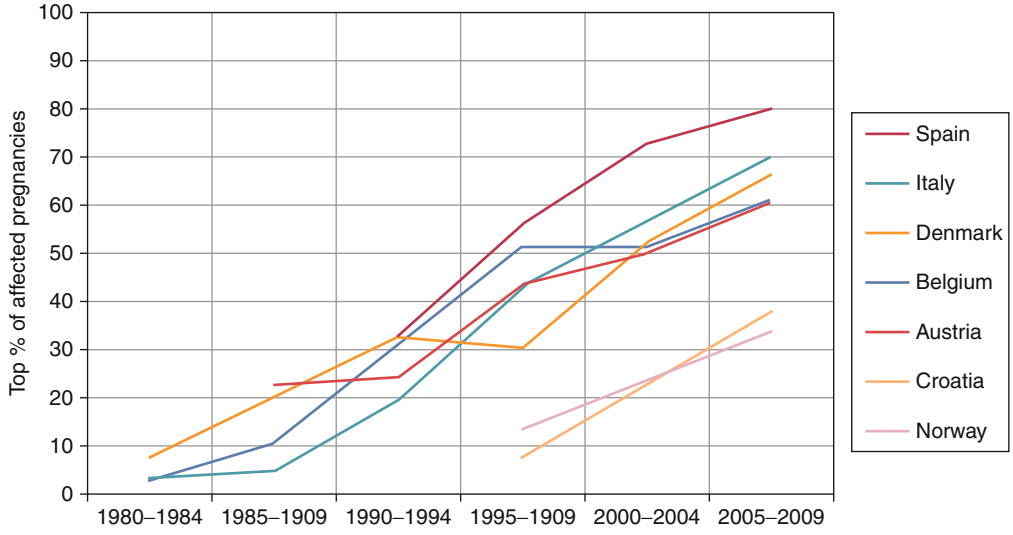


**Fig. 1.4** Proportion of Down syndrome pregnancy termination following Prenatal Diagnosis in European countries (EUROCAT)

requested only for the most severe disorders, fetal anomaly scanning selectively reduces the proportion of children born with lethal or incurable conditions, including NTD (Fig. 1.6).

Despite the need for integrating programs, to combine all feasible approaches, maximizing the benefits and minimizing the negative aspects of preventive programs for congenital malformations, the emphasis is on the primary preventive measures, such as the pre-pregnancy vitamin supplementation, which has been shown to be one of the most efficient approaches for primary prevention of congenital disorders [3–12]. The effectiveness of this approach has been amply demonstrated for

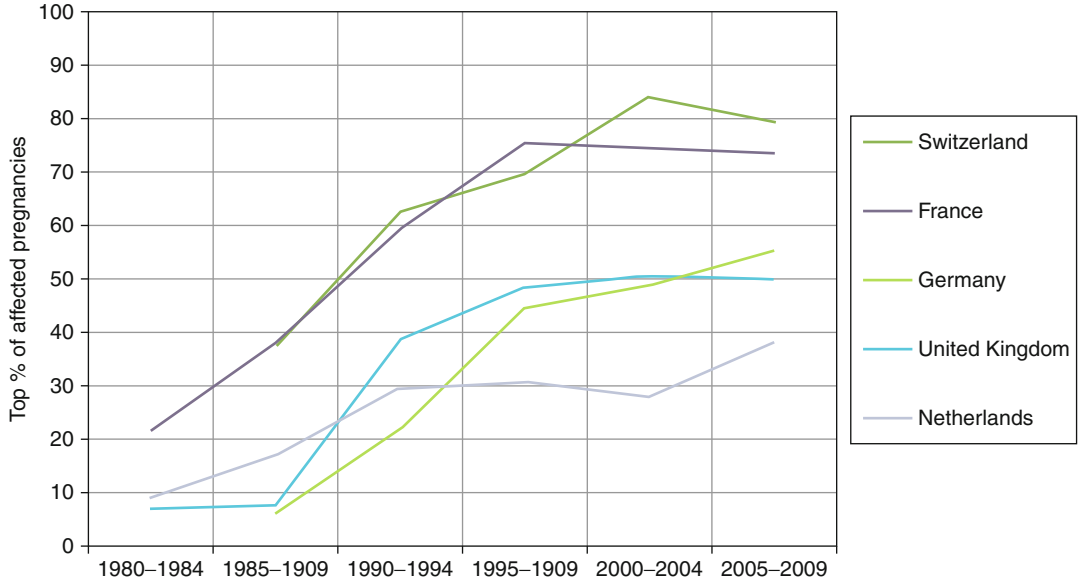


**Fig. 1.5** Proportion of Down syndrome pregnancy termination following Prenatal Diagnosis in European countries – different tendency (EUROCAT)

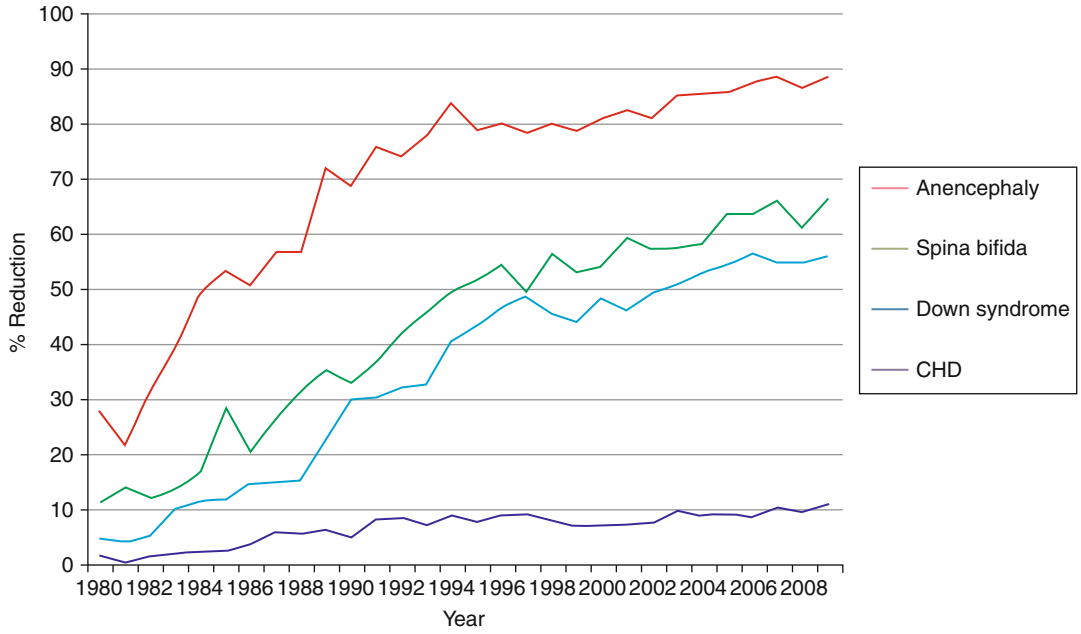


**Fig. 1.6** Percent reduction of birth prevalence of some major congenital malformations by routine fetal anomaly scanning and termination of pregnancy. *CHD* congenital heart disease

NTD, leading to adoption of folic acid food fortification in many parts of the world (flour fortification initiative – FFI, www.Sph.emory.edu/wheatflour). In some populations, the application of this approach resulted in the overall reduction of the prevalence of congenital disorders by as much as half (from 40.6 per 1,000 to 20.6 per 1,000). This included the reduction of the prevalence of NTD and some other congenital abnormalities (see below).

It is obvious at present that routine fetal anomaly scanning is a very powerful intervention for detecting and avoiding congenital malformations. The downside is the number of terminations of wanted pregnancies: this could and should be minimized by multivitamin supplementation and folic acid food fortification. The benefit here is the replacement of terminations, stillbirths, and affected live births by wanted unaffected children. PGD has no part to play in this area, but is particularly useful for inherited (single-gene) disorders, as this is the area where prevention has so far had the least impact. This is mainly because risk detection is retrospective – after the birth of an affected child – and requires specialized diagnostic facilities and genetic counseling skills. Many parents with a child having a severe single-gene disorder use prenatal diagnosis or restrict further reproduction: this can have a significant effect when a large final family size is the population norm, but it can reduce affected birth prevalence by less than 10% when family size is small – increasingly the global norm. The exceptions are the hemoglobin disorders and Tay Sachs disease, where carriers can be diagnosed prospectively by population screening and conventional laboratory tests. In both cases major reductions in affected birth prevalence have been recorded.

The particularly keen interest in PGD for single-gene disorders is not only because of the high and recurrent risk of carrier couples. PGD offers the most interesting challenge for single-gene disorders also because:

They are the most intractable group of congenital disorders – i.e., a relatively low proportion yield to available therapeutic interventions.

At-risk couples face exceptionally high risks in each pregnancy.

Present approaches have been relatively ineffective in reducing affected birth prevalences (except for thalassemia and Tay–Sachs, and possibly cystic fibrosis in some restricted areas).

They require innovative methods for risk detection (not only population screening but also, e.g., extended family studies in populations where consanguineous marriage is common).

They require continual refinements of DNA-based diagnosis.

They cover a wide range of severity and age at onset: this makes for very difficult decision-making for people at risk for later-onset or ostensibly less severe disorders (e.g., family cancer syndromes): many who would find termination of pregnancy hard to accept and would gladly go for PGD if available and reasonably reliable.

So, if one looks to the future, PGD could provide the most important contribution to enabling people to make use of increasing genetic knowledge to preserve the health of their families.

The fact that the diet supplementation with folic acid or folic acid-containing multivitamins may substantially reduce the population prevalence of four groups of congenital disorders, including neural tube defects, cardiovascular, urinary tract, and limb deficiencies, represents an important breakthrough in prevention of congenital disorders. Although more data are needed to further confirm this and investigate the possibility of reduction of other birth defects, such as pyloric stenosis, the impact of folic acid on the prevalence of congenital disorders is in agreement with the fact that (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 appears to be a risk factor for neural tube defects; (4) vitamins of B group including folate/folic acid are important in homocysteine metabolism; and (5) vitamins $B_6$, $B_{11}$, and $B_{12}$ are able to reduce hyperhomocysteinemia. It is known that homocysteine accumulates if conversion to methionine is slowed because of the shortage of folate or vitamin B12 or both, and a raised plasma homocysteine suggests suboptimal nucleic acid and amino acid metabolism. It also has direct harmful effects – e.g., it increases risk of cardiovascular disease through thickening the lining of blood vessels, and may also increase the risk of certain cancers and dementia [13–16].

It is also known that reactions catalyzed by tetrahydrofolate are crucial for cell growth and multiplication, making rapidly dividing cells particularly vulnerable to deficiency of either folate or vitamin B12. This may affect the embryo's morphogenetic movements, which

depend on focal rapid cell multiplication, increasing the risk of congenital malformation. Folate deficiency may be caused also by genetic factors, leading to rare variants of several enzymes involved in one-carbon transfer, which causes problems ranging from greatly increased plasma homocysteine levels, with very early onset cardiovascular disease, to developmental delay and neurological problems, with or without megaloblastic anemia. Variants of lesser effect in the same enzymes may contribute to genetic predisposition to cardiovascular disease and neural tube defects. For example, about 10% of many populations are homozygous for a common polymorphism of the enzyme methyl tetrahydrofolate reductase (MTHFR) (valine replaces alanine at codon 677). In homozygotes this reduces the enzyme activity by 50–70%, slows the regeneration of methionine leading to the raised plasma homocysteine, and increases risk of cardiovascular disease. So fetuses homozygous for the variant are at increased risk of neural tube defects. Variants of other enzymes, and other vitamins, may also influence homocysteine levels and the risk of neural tube defects. Accordingly, folic acid supplementation increases the supply of tetrahydrofolate, accelerates most folate-dependent metabolic reactions, and reduces plasma homocysteine levels [17–20].

The available data suggest that the cause of neural tube defects is not a primary lack of folate in the diet but an inborn error of vitamin $B_{11}$ and/or homocysteine metabolism. An interaction between genetic predisposition and nutrition, therefore, may have a causal role in the development of neural tube defects, i.e., a dietary deficiency may trigger the genetic predisposition. The genetic-nutrient interaction through genetic predisposition and low folate status is associated with a greater risk for neural tube defects than either variable alone.

There are different options for ensuring appropriate multivitamin/vitamin $B_{11}$ consumption in women of childbearing age, with each of them having their disadvantages and social feasibilities. The optimal daily intake of folate in the periconception period is 0.66 mg, whereas the usual intake per day is only about 0.18 mg. It seems also impossible to achieve a 3.7-fold increase in consumption through food intake alone, since this would require about 15 daily servings of broccoli or brussels sprouts. In addition, a large increase in the consumption of extra folate from natural foods is relatively ineffective at increasing folate levels. So the consumption of folate-rich foods may not be the most appropriate way to prevent the development of neural tube defects and other congenital abnormalities requiring the appropriate strategy for folic acid supplementation.

Over 90% of pregnancies where the fetus has a neural tube defect occur among women without any previous indication of increased risk. Identifiable risk groups include women with a prior affected pregnancy, who have a 3–4% recurrence risk, and 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 provide conclusive scientific evidence for its preventive effect [3–12], suggesting 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 with a previously affected pregnancy, who have an increased (3–4%) risk; (2) the effect is greatest in areas with a high baseline prevalence of neural tube defects, but also applies in lower prevalence areas; (3) no harmful effects have ever been observed, with levels of supplementation ranging from 360 μg to 5 mg of folic acid daily.

Because of the expected strong impact on prevalence of congenital malformations, the current dietary folate intake for adults in the USA is recommended at a dose 400 μg, representing a daily intake of 200 μg folic acid equivalents, the recommended intake for pregnant women being 400 μg dietary folate plus 400 μg folic acid, representing a daily intake of 600 μg folic acid equivalents [21]. The UK Committee on Medical Aspects of Food and Nutrition Policy recommendation of 240 μg of folic acid per 100 g flour [22] 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 be about 10 ng/ml, while the mean plasma folate level in most populations is around 5 ng/ml [21]. There is

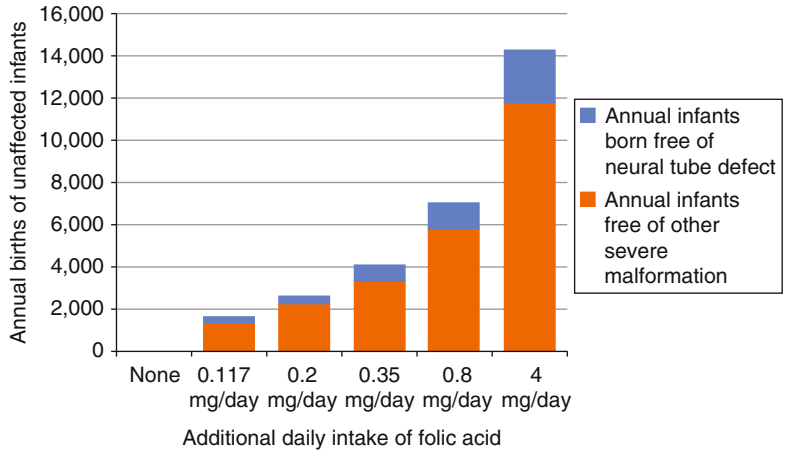


**Fig. 1.7** Projected effect of folic acid fortification on annual gain of infants free of congenital malformations in North America. The estimates were prepared by Bernadette Modell for WHO/EURO meetings on Prevention of Congenital Disorders, Copenhagen 2001 and Rome 2002 [25, 26], providing the expected reduction of congenital malformations overall depending on the additional folic acid intake (*orange portion of the bars*) and the proportion of avoided neural tube defects (NTD) (*blue portion of the bars*)

**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 |
| Estimated annual affected births w FA | 659,090 | 137,402 | 521,689 | 20.8 |
| Born malformation-free with FA | 376,513 | 251,040 | 125,473 | 66.7 |

Prepared by Bernadette Modell, personal communication

considerable variation within and between populations, but few have a mean plasma folate in the recommended range, and very few individuals could meet the above recommendations without food fortification or the use of folic acid supplements. The groups of greatest concern are those with folate intakes and plasma folate at the lower end of the range, for whom only food fortification is capable of bringing them into the recommended range.

Folic acid fortification of all cereal grain products at a level of 140 μg/100 g flour is mandatory in the USA and Canada, where the birth prevalence of neural tube defects has since fallen by about 20%, with no adverse effects reported [16, 23]. In Hungary, folic acid, vitamin B12, and vitamin B6 are being added to bread, with average daily intake of folic acid, vitamin B12, and B6 from this source at approximately 200, 1, and 1,080 μg, respectively [24]. The prevalence of neural tube defects has fallen by 41%. Based on this study and other relevant data on the potential reduction of congenital disorders through primary preventive measures, the expected overall reduction of the prevalence of congenital disorders in North America may be projected to be approximately 40,000 births of children with major congenital disorders [25] (Fig. 1.7). The potential global estimate of reduction of congenital disorders is presented in Table 1.1.

It is of importance to mention that folic acid fortification costs only about $1 per metric ton of flour. It is so minor that the extra cost is not sufficient even to change the price of a loaf of bread. However, the multivitamin food fortification does not substitute completely periconception supplementation, so the prospective pregnant

**Table 1.2** Reported fall in prevalence of neural tube defects (NTD) following folic acid food fortification

| Country/region | Year fortification started | NTD/1,000 pre-fortification | NTD/1,000 post fortification | % fall in NTD |
|---|---|---|---|---|
| Ontario Canada | 1998 | 1.13 | 0.58 | 49 |
| Nova Scotia Canada | 1998 | 2.58 | 1.17 | 55 |
| Chile | 2000 | 1.64 | 0.8 | 51 |
| South Africa (urban) | 2003 | 1.41 | 0.98 | 31 |
| Oman | 1996 | 5.5 | 3.1 | 44 |

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women should be informed about the need for folic acid and multivitamin supplementation, in addition to the intake of the multivitamin-fortified food. Taking into consideration the estimated lifetime cost for a single patient with spina bifida, which is approximately $250,000, a complementary periconception supplementation would be highly cost-effective, although the major benefit is the birth of an unaffected child. This approach may be best realized by advising oral contraceptive users to start taking folic acid-containing multivitamins as soon as they stop using contraception, and using a multivitamin supplementation containing a physiological dose (0.4–0.8 mg) of folic acid, which contributes to prevention in addition to more efficient reduction of neural tube defects (about 90% versus 70%), and prevention of some other congenital disorders of public health importance. The available experience from those countries which implemented a national foodstuff fortification program is presented in Table 1.2.

However, prevention of congenital malformations is not actually the major objective of the above programs, as replacing an affected live birth by abortion, as shown in Figs. 1.2, 1.3, 1.4, and 1.5, does not solve the problem, as the abortion of a wanted pregnancy is also an unfavorable pregnancy outcome. This makes primary prevention by food fortification, preconception vitamin supplementation, or PGD much more attractive interventions not associated with distress, because, on the contrary, they increase the number of healthy wanted babies born and it has a highly positive effect on the quality of life of parents and children. In evaluating the effects of folic acid fortification the appropriate evaluation of the program is therefore gain in the number of unaffected pregnancies (Fig. 1.7), rather than reduction in the number of affected live births [26]. The same consideration applies to PGD, the main objective of which is to assist couples to have an unaffected child of their own.

Thus, one of the most exciting possibilities to avoid the genetic disease before pregnancy is preconception and preimplantation genetic diagnosis (PGD), which has recently become available worldwide. Instead of prenatal screening and termination of affected pregnancies, which is not tolerated in many communities and ethnic groups, the pre-pregnancy diagnosis provides an option for couples at risk to plan unaffected pregnancies from the onset (Fig. 1.1). This is the reason the preconception and pre-pregnancy diagnosis or PGD 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. Together with other approaches for primary prevention, PGD may soon represent an important component of preconception clinics, which may soon shift their services from secondary preventive measures based on prospective carrier screening and prenatal diagnosis to preconception prevention and PGD, to ensure only unaffected pregnancy from the onset.

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# Approaches to Preimplantation Diagnosis

# 2

Introduced only in 1990 as an experimental procedure, preimplantation genetic diagnosis (PGD) is now becoming an established clinical option in reproductive medicine [1–3]. Thousands of apparently healthy children have been born after PGD, validating that there is no ostensible evidence of any incurred adverse effect. Over 100,000 PGD cases have presently been performed in more than 100 centers around the world, allowing at-risk couples not only to avoid producing offspring with genetic disorders but, more importantly, to have unaffected healthy babies of their own without facing the risk of pregnancy termination after traditional prenatal diagnosis. Without PGD it is likely that a few of these children would have been born.

Applied first for preexisting Mendelian diseases [4, 5], such as cystic fibrosis (CF) and X-linked disorders, PGD initially did not seem to be practical. Only a few babies were born during the first 3 years of work, and several misdiagnoses were reported [6, 7]. After the introduction of fluorescent in situ hybridization (FISH) analysis in 1993–1994 for PGD of chromosomal disorders [8–13] (Chap. 5); however, the number of PGD cycles began to double annually, yielding more than 100 unaffected children by the year 1996 [14, 15] (Fig. 2.1).

Application of PGD increased further when the ability to detect translocations became possible in 1996, first using locus-specific FISH probes, then more widely available subtelomeric probes [16, 17], (Chap. 5), and presently by haplotyping and also by microarray technology [18–29] (see below). Because many carriers of balanced translocations have a poor chance of having an unaffected pregnancy, PGD 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]. Of course, there are differences in the reproductive outcomes depending on the origin and type of translocation, with the majority resulting in early fetal loss and rarely in an affected birth. However, it may take years until the translocation carriers could be lucky enough to get at last an unaffected offspring, so the current recommendations of PGD International Society (PGDIS), European Society of Human Reproduction and Embryology (ESHRE), and American Society for Reproductive Medicine (ASRM) Practice Committee include chromosomal rearrangements as one of the main indications for PGD [34]. The experience of over 3,000 PGD cycles for translocations accumulated by the present time demonstrates at least a six fold reduction of spontaneous abortions in these couples, compared to their experience before PGD [32–37] (see Chap. 6).

The natural extension of PGD's ability to allow transfer of euploid embryos should have positive impact on the liveborn pregnancy outcome. This is especially applicable to poor prognosis in vitro fertilization (IVF) patients (prior IVF failures, maternal age over 37, repeated miscarriages). Introduction of commercially available 5-color probes in 1998–1999, and currently also 24 chromosome testing by microarray analysis, has led to the accumulated experience of more than

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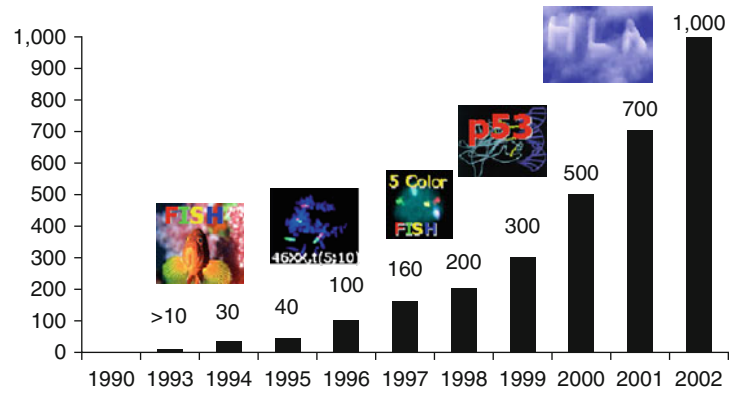


**Fig. 2.1** Babies born after (PGD): 1990–2002. *Bar graphs* showing the number of children born each year after application of PGD. Application of each novel technique for PGD is shown above the *bar graphs*, leading to the expanding indications, such as chromosomal aneuploidies in 1993–1994 (shown as FISH), translocations in 1996 (shown by example of translocation t (5; 10), five-color FISH in 1998, application to late-onset disease with genetic predisposition in 1999 (shown as an example of cancer predisposition caused by mutation in p53 gene mutations), and t application to non-disease testing in 2000 (shown as an example of preimplantation HLA typing)

50,000 clinical cycles worldwide for the aneuploidy testing [2, 35, 37–41] (Chap. 5). This has resulted in the birth of over 10,000 children, including a few with misdiagnosis, suggesting the continued need for the improvement of accuracy of aneuploidy testing. According to the experience of the majority of centers, the overall pregnancy rate per transfer is higher than that in non-PGD IVF patients of comparable age group (average age over 39 years), although this is still a controversial issue (see Chap. 6). Available data indicate that the current IVF practice of transferring embryos based solely on morphological criteria is inefficient and needs a revision, given that half of these embryos are chromosomally abnormal and would compromise the reproductive outcome (Sect. 6). The current introduction of 24 chromosome testing combined with polar body (PB) or blastocyst biopsy shows further improvement of reproductive outcomes in poor prognosis IVF patients, confirming the need for preselection of euploid embryos for transfer [18–29].

The application of PGD has further expanded with its introduction to late-onset diseases with genetic predisposition [24, 42] (Chap. 3), a novel indication never previously considered for the traditional prenatal diagnosis. For the patients with inherited pathological predisposition PGD provides a realistic reason for undertaking pregnancy, with a reasonable chance of having an unaffected offspring. Prospective parents at such risk should be aware of this emerging option, especially when there is no opportunity to diagnose the disease until it is fully realized, such as in cases of inherited cardiac diseases leading to premature or sudden death (Chap. 3).

Another unique option that can presently be considered, although involving ethical debate (Chap. 8), is HLA typing as part of PGD, which has never been considered in traditional prenatal diagnosis either [43] (Chap. 4). In this application PGD offers not only preventative technology to avoid affected offspring, but also a new method for treating (older) siblings with congenital or acquired bone marrow diseases, for which there is still no available therapy. This may in future be applied for any condition that can be treated by embryonic stem cell transplantation.

Preimplantation HLA typing was first applied to couples desiring 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 replaced. 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. 4).

As will be described in this book, the over 20 years PGD experience demonstrates considerable progress. As mentioned, over 100,000 PGD attempts worldwide have resulted in the birth of dozens of thousands of apparently unaffected children, with no detrimental effect on embryo development, as demonstrated by no significant differences in the overall congenital malformation rate after PGD from the population prevalence [45–47]. With the highly improved accuracy of genetic analysis and indications expanding well beyond those for prenatal diagnosis, thousands of PGD cycles are now performed annually. As seen in Fig. 2.1, already in the year 2006, only the annual experience has resulted in the birth of nearly the same number of children as during the entire preceding decade since the introduction of PGD. This is clearly because PGD offers a special attraction not possible with traditional prenatal diagnosis, such as avoiding clinical pregnancy termination. This is extremely attractive for translocation carriers, couples at risk for producing offspring with common diseases of autosomal-dominant or autosomal-recessive etiology, as well as for couples wishing to have not only an unaffected child but an HLA-compatible cord blood donor for treatment of an older moribund sibling with a congenital disorder. Yet the greatest numerical impact of PGD may be expected in standard assisted reproduction practices (Chap. 6), where improved IVF efficiency through aneuploidy testing will surely evolve to become standard, despite the recent controversy, which will be considered in detail throughout Chaps. 5 and 6.

## 2.1 Obtaining Biopsy Material

At present, biopsy material for performing preimplantation genetic diagnosis (PGD) may be obtained from three major sources:

1. Matured and fertilized oocytes, from which the first and second polar bodies (PB1 and PB2) are removed
2. Eight-cell cleavage-stage embryo, from which one or two blastomeres are biopsied
3. Blastocyst-stage embryos, from which up to a dozen cells may be removed

The biopsied material is tested for single-gene disorders using PCR analysis, or used for PGD for chromosomal abnormalities by fluorescent in-situ hybridization (FISH), or microarray analysis (see below).

Each of these PGD methods has advantages and disadvantages, and their choice depends on circumstances; however, in some cases, a combination of two or three methods might be required. Despite a possible embryo cell number reduction, which might have a potential influence on the embryo viability, blastomere biopsy allows detecting paternally derived abnormalities. Removal of PB1 and PB2, on the other hand, should not have any effect on the embryo viability as they are naturally extruded from oocytes as a result of maturation and fertilization, but they provide no information on the paternally derived anomalies, even if this constitutes less than 5% of chromosomal errors in preimplantation embryos. Approaches for preconception testing of the paternally derived mutations are being developed, although are still at a very initial stage with not yet considerable progress, as will be described in Sect. 2.1.3.

To perform PGD for chromosomal aneuploidies both PB1 and PB2 are removed simultaneously, next day after insemination of the matured oocytes or ICSI, and analyzed by FISH or microarray technology, as described in Sect. 2.2. PB1 is the by-product of the first meiotic division and normally contains a double signal for each chroSmosome, each representing a single chromatid (see below). Accordingly, in case of meiosis I error, instead of a double signal, four different patterns might be observed, ranging from no or one signal to three or four signals, suggesting either chromosomal nondisjunction, evidenced by no or four signals, or chromatid missegregation, represented by one or three signals [48]. The genotype of the oocytes will, accordingly, be opposite to PB1 genotype, that is, missing signals will suggest an extra chromosome material in the corresponding oocyte, while an extra signal (or signals) will indicate monosomy or nullisomy status of the

tested chromosome. In contrast to PB1, the normal FISH pattern of PB2 is represented by one signal for each chromosome (chromatid), so any deviation from this, such as no or two signals instead of one, will suggest a meiosis II error. Microarray technology also appeared to be extremely reliable in detecting the chromosomal status of polar bodies, as will be described in Sects. 2.2 and (Chap. 5), distinguishing also between chromosome and chromatid errors in PB1, not previously detectable by traditional CGH studies [24–27].

The method of blastomere biopsy was extensively used in PGD, despite its limitation due to the high mosaicism rate in cleaving embryos (see Chap. 5). The FISH pattern of blastomeres is represented by two signals for each chromosome tested, so any deviation from this number will suggest chromosomal abnormality. The same pattern applies to blastocyst analysis, which has an advantage of analyzing not one but a group of cells, obviating the problem of mosaicism at least to some extent. The aneuploidy testing in blastocyst biopsy was further improved by the application of microarray technology, which may pick up mosaicism of 10% and higher [23, 28, 29]. It is also expected that the microarray approach may improve the results in blastomere aneuploidy testing, possibly by avoiding the problems of artifactual loss of chromatid material during the slides preparation involved in the procedure of FISH analysis.

Although more data have to be collected to exclude completely the short-term and long-term side effects, the data available show no evidence for any detrimental effect of the PB, blastomere, or blastocyst biopsy. Overall, these methods were used now in a total of almost 100,000 clinical cycles, and resulted in the birth of dozens of thousands of unaffected children, showing a comparable prevalence of congenital abnormalities to that in the general population, which suggests no detrimental effect of any of the above biopsy procedures mentioned [41, 45–47].

### 2.1.1 Polar Body Diagnosis

Introduced 22 years ago [2, 5], PB biopsy has become one of the established approaches for PGD. The idea of performing PB PGD is based on the fact that polar bodies are the by-products of female meiosis, which allow predicting the resulting genotype of the maternal contribution to the embryos. Neither PB1, which is 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 [49]. However, the PB1 approach was not applicable for predicting the eventual genotype of the oocytes, if crossing over occurred, because the primary oocyte in this case will be heterozygous for the abnormal gene. As the frequency of crossing over varies with the distance between the locus and the centromere, approaching as much as 50% for telomeric genes, the PB1 approach appears to be of a limited value, unless the oocytes can be tested further on. So, the analysis of PB2 has been introduced to detect hemizygous normal oocytes resulting after the second meiotic division. As will be described below, this PGD technique presently involves a two-step oocyte analysis, which requires a sequential testing of PB1 and PB2 [50].

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, and PB1 aspirated into a blunt micropipette (see micromanipulation setup and procedure steps in Figs. 2.2, 2.3, and 2.4). The oocytes are then inseminated with motile sperm or by using introcytoplasmic sperm injection (ICSI), and examined for the presence of pronuclei and extrusion of PB2s, which are removed in the same manner as PB1 (Fig. 2.5). To avoid an additional invasive procedure, both PB1 and PB2 are removed simultaneously for FISH analysis (Fig. 2.6), and are fixed and analyzed on the same slide, while sequential PB1 and PB2 sampling procedure is used for microarray analysis and PGD for monogenic disorders. The biopsied oocytes are then returned to culture, checked for cleavage, and transferred, depending on the genotype of the corresponding PB1 and PB2 [48].

Although PB1 and PB2 have no any known biological significance in pre- and postimplantation

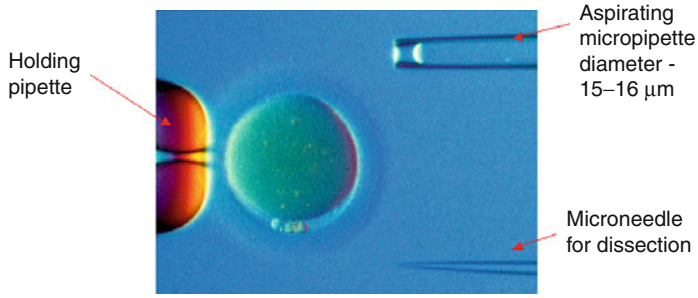


**Fig. 2.2** Required microtools for first polar body removal. Image of a metaphase II oocyte along with the three microtools that are required to perform first polar body (PB1) removal. A holding pipette is used (*left*) to hold the oocyte in position by gentle suction created by a hydraulic microsyringe system. On the right, a microneedle for partial zona dissection (*bottom*) and a micropipette (inner diameter 15 µm) for polar body removal are placed into a double tool holder. The micropipette is also attached to a hydraulic microsyringe (100 µl) system for fine control during the procedure. The same tools are required for embryo biopsy with the exception of the micropipette, which has a larger inner diameter of 30–35 µm

development, follow-up studies have been carried out to investigate possible detrimental effect. Following PGD and ICSI, zygotes with two pronuclei were observed in 1,192 (81.8%) of 1,458 oocytes, compared to 30,972 (77.3%) of 40,092 in a routine non-PGD cycle, which suggested no difference in fertilization rate observed after PB1 in comparison to nonbiopsied oocytes. Also there was no difference in blastocyst formation of the embryos resulting from the biopsied oocytes. We compared the blastocyst formation of embryos resulting from biopsied oocytes observed in 1,653 (50.2%) of 3,293 embryos, which was not different from 49.8% (9,726 of 19,529 nonbiopsied 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 [51]. As will be seen below, there was no difference either after sequential PB1, PB2, and blastomere sampling.

The PB approach will have an increasing impact on those ethnic groups in which PGD may be done only before fertilization, such as in Austria, Germany, Switzerland, and Malta. In these countries, the testing may be limited to PB1, which, as mentioned above, might not be sufficient to predict embryo genotype, unless PB2 is tested before fusion of pronuclei, which may be combined with freezing of the oocytes at the pronuclear stage. After the analysis, in a subsequent menstrual cycle, only the oocytes predicted as having the normal maternal allele may be thawed and cultured to allow fusion of the pronuclei, embryo development, and the embryo transfer.

In fact, it is presently possible to complete the testing of PB2 in approximately 9 h after removal, so there is no need for freezing the oocytes free of mutation or aneuploidy, which may be cultured as usual and replaced on day 3 or day 5, while the abnormal oocytes are frozen at the pronuclear stage [52]. Since zygotes are not considered to be embryos until pronuclear fusion, and no abnormal oocytes may be thawed and cultured, obviating the establishment of the affected embryo, this technique may be ethically more acceptable to many couples. Therefore, the technique may allow creating a new class of genetic diagnosis, which may be called pre-embryonic genetic diagnosis, pushing the frontier of genotyping to an even earlier stage, as shown in pre-embryonic diagnosis of sickle cell and Sandhoff disease (SHD) presented below.

### 2.1.2 Pre-embryonic Genetic Diagnosis (PEGD)

#### 2.1.2.1 PEGD with Freezing at Pronuclear Stage

A 33-year old woman and her spouse at risk for producing a child with sickle cell disease referred for PGD to avoid a possible termination of a pregnancy following prenatal diagnosis. A standard

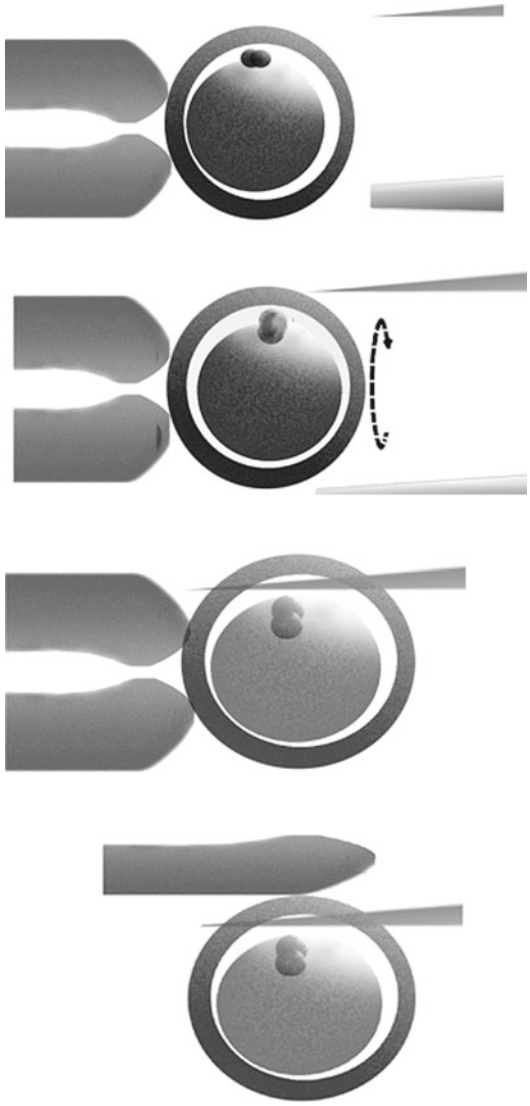

**Fig. 2.3** First polar body removal prior to ICSI. (*Step 1*) The oocyte is secured by gentle suction from the holding pipette and the oocyte is rotated so that the PB1 is visualized at 12 o'clock. (*Step 2*) The oocyte is rotated horizontally, slightly forward, so that it faces the operator. (*Step 3*) The microneedle is passed through the zona pellucida at the 1–2 o'clock position and passed tangentially through the perivitelline space and out at the 10–11 o'clock position. (*Step 4*) The oocyte is released from the holding pipette and held by the microneedle. The microneedle is brought to the bottom of the holding pipette and pressed to it, pinching a portion of the zona pellucida. By gently rubbing against the holding pipette with a sawing motion, the cut is accomplished and the oocyte is released

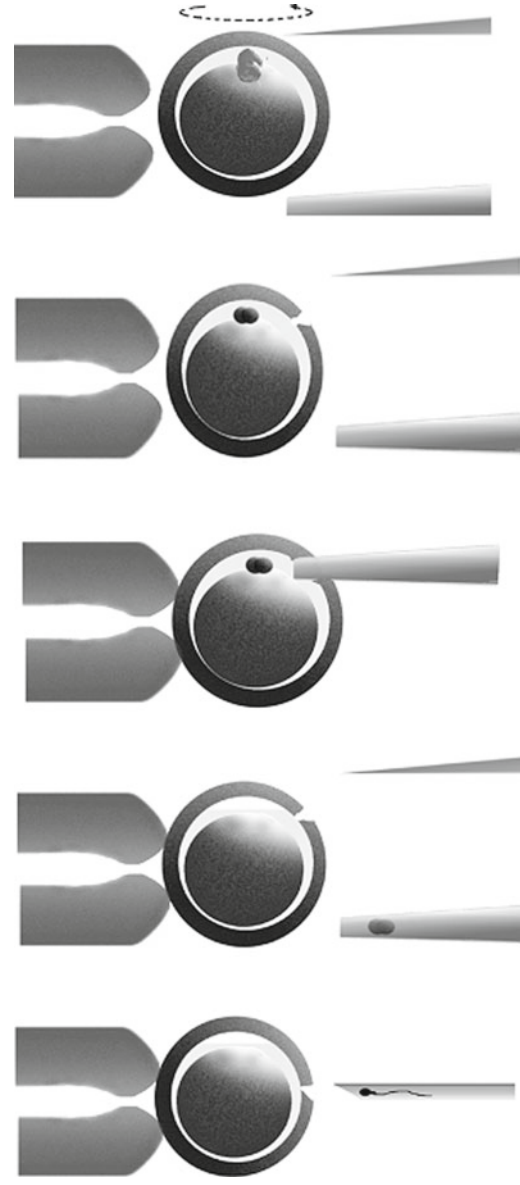

**Fig. 2.4** First polar body removal continued (see Fig. 2.3). (*Step 5*) The oocyte is brought to the holding pipette by moving the microscope stage and is rotated vertically using the microneedle until the slit opening is visualized at the 2 o'clock position. (*Step 6*) Once again it is held in place by gentle suction from the holding pipette, making sure the slit opening is at the 2 o'clock position and is in focus. (*Step 7*) The micropipette is brought into the same focal plane as the slit opening and PB1. The micropipette is passed under the zona pellucida to PB1. (*Step 8*) Using gentle suction created by the hydraulic microsyringe system, PB1 is aspirated into the micropipette and then deposited in a separate microdrop of medium. (*Step 9*) The oocyte is transferred to another micromanipulation dish for intracytoplasmic sperm injection (ICSI), which is performed by passing the microtool through the slit opening

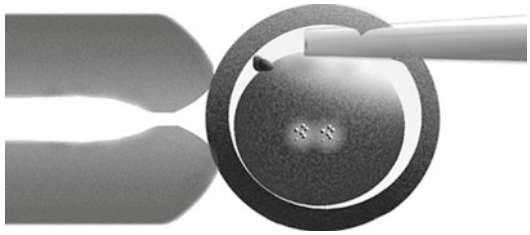

**Fig. 2.5** Second polar body removal on day 1 after fertilization assessment. The second polar body (PB2) is removed following fertilization assessment. The same microtools are required as with PB1 removal. The zygote is held in place by gentle suction from the holding pipette and is rotated using the microneedle. The same slit opening can be used or if PB2 has been extruded away from the opening, a second intersecting slit (3D-PZD) (see Fig. 2.12) can be made in order to have easier access to the second polar body

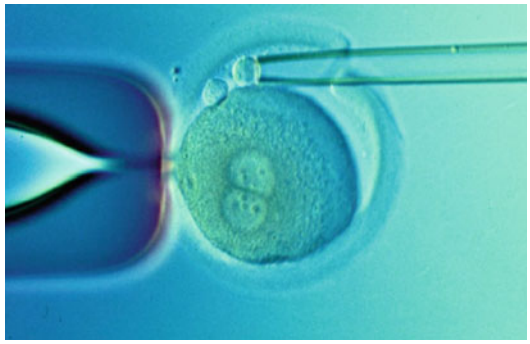

**Fig. 2.6** Simultaneous removal of the first and second polar bodies. Both PB1 and PB2 are removed following fertilization assessment, which is used for aneuploidy testing. The same microtools are required as with PB1 removal. The zygote is held in place by gentle suction from the holding pipette and is rotated using the microneedle. The same slit opening can be used as above (see Fig. 2.5), allowing sufficient opening to remove both PB1 and PB2

IVF protocol was initiated but the patient suffered from hyperstimulation syndrome, which precluded transfer of embryos in that cycle. Twenty-eight mature oocytes were aspirated and placed in culture medium. Of the 28 aspirated oocytes, 14 extruded PB1 that 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 embryos were frozen. The PB1 and PB2 were analyzed by multiplex nested PCR to avoid allele dropout (ADO), which occurs in approximately 5–10% of PB analyses. This involved 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 thyrosin 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 pregnancy, three additional non-linked STRs were amplified, including STR at the 5 untranslated region of human coagulation factor A subunit gene (HUMF13A01), STR for von Willebrand disease (vWD), and an STR for chromosome 21 (D21S11). The list of primer sequences, reaction conditions, and details of the nested PCR were described earlier [48, 53].

The pronuclear-stage oocytes predicted to be normal were thawed, cultured to develop into the cleaving embryos of the 6–8-cell stage, 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. Of 28 aspirated oocytes, 14 extruded their PB1 and were studied for the presence of sickle cell mutation. Following intracytoplasmic sperm injection PB2s were extruded from 13 of them and studied. Results of both PB1 and PB2 were available in 12 of these 13 oocytes. Overall, 6 oocytes were predicted to contain a normal allele, based on the heterozygous status of PB1 and the hemizygous mutant status of PB2.

In one of the oocytes, although the sickle cell analysis of PB1 showed only the normal allele in agreement with the 5 -STR, it was heterozygous for the THO-STR, suggesting that this is a case of ADO. Therefore, without simultaneous amplification of linked STRs, a misdiagnosis of the heterozygous oocyte as homozygous due to ADO would have occurred leading to a misdiagnosis of the maternal contribution to the zygote. In this particular instance, the error would have caused an unaffected zygote to be misdiagnosed as affected, but the reverse could also occur.

Subsequently, the patient was prepared for a frozen embryo transfer. In the first frozen cycle, four zygotes determined to have the maternal

unaffected allele were thawed and cultured. Three developed into cleaving embryos of acceptable quality, and were transferred, resulting in a singleton pregnancy, which was spontaneously aborted [53]. In the second frozen cycle, two unaffected embryos were transferred and resulted in a singleton pregnancy and birth of an unaffected child, following confirmation of PB diagnosis by chorionic villus sampling (CVS). The results of the application of non-linked markers allowed not only the exclusion of a possible DNA contamination but also the identification of the embryo that was implanted yielding a clinical pregnancy. All the remaining oocytes predicted to contain an abnormal gene were not further cultured, but exposed directly to PCR analysis at the pronuclear stage for confirmation of diagnosis as frozen sample, and shown to be abnormal as predicted by PB analysis.

#### 2.1.2.2 PEGD Without Pronuclear-Stage Freezing

A 32-year old woman and her spouse at risk for producing a child with SHD requested PGD to be performed without any possible discard of embryos even if affected [52]. As seen from the pedigree shown in Fig. 2.7, 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 the hexaminidase B gene (HEXB) on chromosome 5, which consists of 14 exons distributed over 40 Kb of DNA (MIM 268800; 606873). Mutation in this gene causes beta-hexaminidase deficiency, resulting in the lysosomal storage disease GM2-gangliosidosis. The same condition is caused also by Tay–Sachs disease resulting from the defect of the hexaminidase 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 (Fig. 2.8). 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 16 Kb 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. The maternal and paternal haplotypes, established by family studies and PB analysis, are presented in Fig. 2.7, while primer sequences are listed in Table 2.1.

A single PGD cycle was initiated, which was performed according to the following modification of the timetable of the applied procedures of sequential PB1 and PB2 analysis described above. PB1 was removed as usual 3.5 h after aspiration, followed by ICSI. PB2 was removed soon after it was extruded, approximately within 6.5 h after ICSI, to allow sufficient time for completion of the DNA analysis before pronuclei fusion (see Fig. 2.9). DNA analysis is currently done in less than 9 h overall (see procedure description below), making it realistic to freeze the oocytes predicted to contain the deleted HEXB allele before syngamy (within 24 h after aspiration or 12 h 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 day 3 blastomere biopsy.

Of 18 oocytes available for testing in a single PGD cycle, 16 were with conclusive PB1 and PB2 results, of which 8 contained the maternal 16Kb deletion and were frozen at the pronuclear stage (Fig. 2.7). 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. 2.7b).

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 4 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.

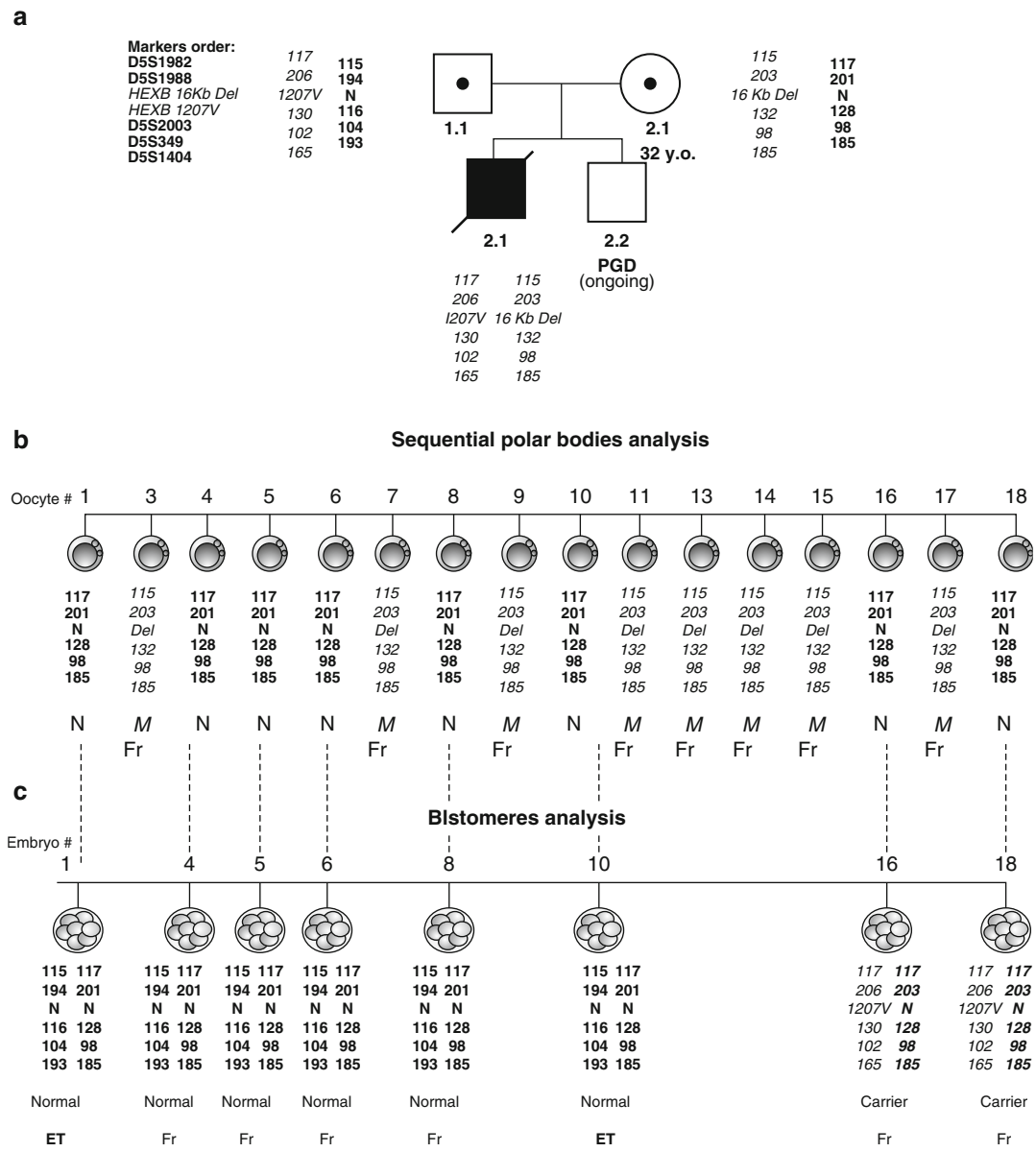


**Fig. 2.7** Pre-embryonic diagnosis of Sandhoff disease. (**a**) Family pedigree with mutation and haplotype analysis based on parental (1.1 and 1.2) and affected child's (2.1) genomic DNA testing. The markers' order is presented on the *upper left* for the father and *upper right* for the mother. Maternal and paternal mutations and the linked markers are shown in *non-bold*, while normal alleles and their linked markers are shown in *bold*. (**b**) Results of sequential first and second polar body analysis of 16 oocytes, showing 8 normal (*bold*) and 8 mutant oocytes (*non-bold*) which were frozen prior to syngamy. (**c**) Blastomere analysis of embryos, resulting from the mutation-free oocytes, which confirms the polar body diagnosis. Two of these embryos, #1 and #10, free of both maternal and paternal mutations, were transferred, resulting in the birth of an unaffected child. The remaining 6 embryos, two of which were heterozygous, were frozen for future use by the couple

A follow-up blastomere analysis of the embryos deriving from the oocytes predicted to be free of maternal deletion showed the complete correspondence to the PB diagnosis. Six of these embryos appeared to contain also a normal paternal allele (embryos 1, 4, 5, 6, 8, and 10), while only 2

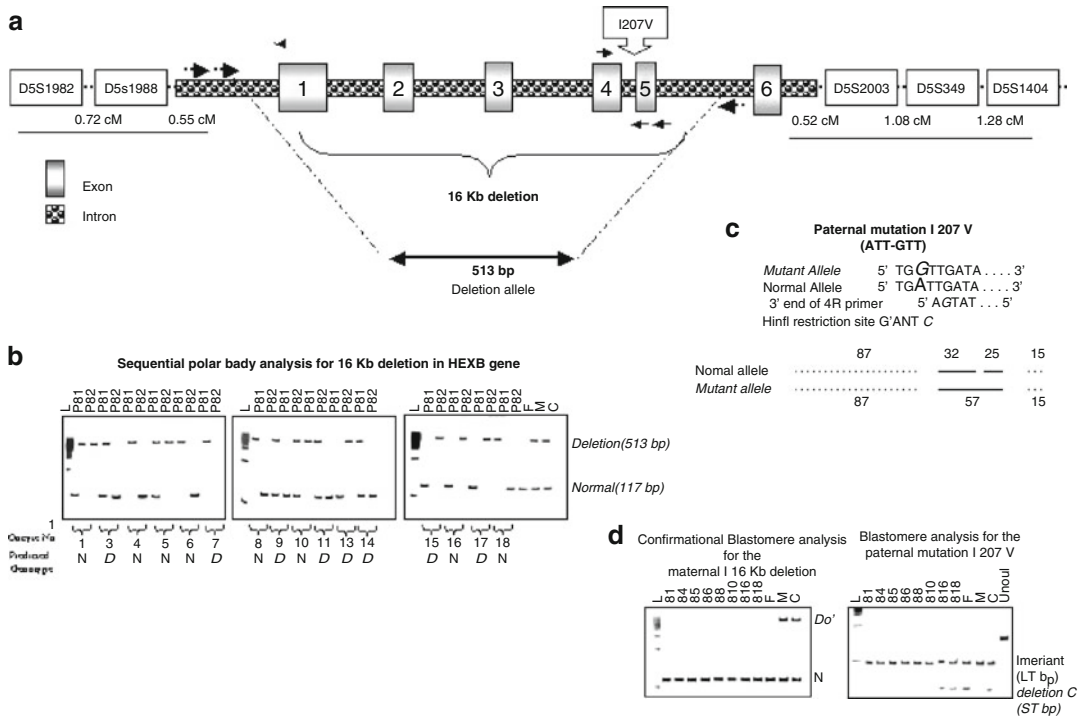


**Fig. 2.8** Map of human HEXB gene and results of maternal and paternal mutation testing in polar bodies and blastomeres. (**a**) Schematic presentation of maternal and paternal mutations and linked polymorphic markers. (**b**) Polar body analysis of the maternal 16 kb deletion (*N* normal, *D* deletion). (**c**) Restriction map: HhaI enzyme created two fragments in normal gene, leaving the paternal mutation I 207 V uncut. (**d**) Blastomere analysis for maternal deletion and paternal mutation, confirming the PB diagnosis

(embryos 16 and 18) inherited the paternally derived mutant allele, confirmed by all five linked polymorphic markers tested (Figs. 2.7 and 2.8).

Two of these embryos (embryos #1 and #10), with both maternal and paternal normal alleles, were transferred, resulting in a singleton pregnancy and the birth of an unaffected child. The remaining six unaffected embryos were frozen to be available for the couple in the future, while eight mutant oocytes were frozen at the pronuclear stage.

The presented results show that PEGD is a realistic option for those couples that cannot accept traditional PGD, because of their objection to any micromanipulation and potential discard of the tested embryos. In contrast to the previous PEGD described above, involving the freezing of all the tested oocytes at the pronuclear stage immediately after ICSI and extrusion of PB2, the presented case is realized without freezing the mutation-free oocytes, which were detected well before the pronuclei fusion, after which the embryo discard could not be avoided. Although all the oocytes could have been frozen irrespective of the DNA diagnosis, as described in the previous case, not all frozen pronuclear-stage oocytes could potentially be successfully recovered, which may incidentally include also a few preselected unaffected embryos for transfer and could have affected the PEGD outcome. The realization of PEGD 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 h.

This approach may currently be applied to autosomal-recessive, X-linked, and maternally derived dominant and chromosomal mutations, detectable by sequential PB1 and PB2 analysis. To perform PEGD for paternally derived dominant and chromosomal mutations, a technique for

**Table 2.1** Primers for the detection of the 16 Kb deletion and I 207 V mutation in the hexaminidase B gene (HEXB) causing Sandhoff disease, and the linked markers in heminested PCR analysis

| Gene/polymorphism | Accession no. | Heterozygosity index | No alleles | Upper primer | Lower primer | Annealing temperature (°C) |
|---|---|---|---|---|---|---|
| 16 kb deletion (heminested) amplifies only deleted sequence | ENST00000261416 (Ensembl) | NA | NA | Outside: 5 ACCTCTTTA TGGCTGGCTCC 3 | 5 AATTATGGGATG ACTGCCTATT 3 | 62–45 |
| | | | | Inside: 5 AGACACGGCA AGATTAGAGTAATAT 3 | 5 AATTATGGGATG ACTGCCTATT 3 | 55 |
| Exon 4-Exon 5 amplifies only normal sequence | ENST00000261416 (Ensembl) | NA | NA | Outside: 5 TAGAGACCTT TAGCCAGTTAGTTTA 3 | 5 GCTAAGACAAAT ATCTGGGGAAA 3 | 62–45 |
| | | | | Inside: 5 TAGAGACCTTTA GCCAGTTAGTTTA 3 | 5 CTAAACAGGTTAC ATTTTTTTCTAT 3 | 55 |
| I 207 V (heminested) (Hinf I cuts normal sequence) | ENST00000261416 (Ensembl) | NA | NA | Outside: 5 AATAGATTTAG TCTTCATTGAGTTC 3 | 5 ATTACTTACCAGA GTTTTAAGAATA 3 | 62–45 |
| | | | | Inside: 5 AATAGATTTAGT CTTCATTGAGTTC 3 | 5 GCAGATAATGTCT GGATGTATGA 3 | 55 |
| D5S1982 (heminested) | Z52566 | 0.70 | 7 | Outside: 5 AGAGTTTGGG CAAGGCGTA 3 | 5 GGAAAGACATTTA ACCCTTTCTCT 3 | 62–45 |
| | | | | Inside: 5 GATGAGAATGA AGGTTAAAAAGTCC 3 | 5 GGAAAGACATTTA ACCCTTTCTCT 3 | 55 |
| D5S1988 (heminested) | Z52691 | 0.87 | 14 | Outside: 5 AGCTTACTTCA CTTGGCATAA 3 | 5 AAGAAATGGAAG CAACCTAAG 3 | 62–45 |
| | | | | Inside: 5 AGCTTACTTCA CTTGGCATAA 3 | 5 GTCCACCGATGG ATGAATG 3 | 55 |
| D5S2003 (heminested) | Z52980 | 0.81 | 9 | Outside: 5 AGCCTAAGTGA CAAAGTGAGACA 3 | 5 CTCACAGAGGGT GTGTTATAATAGA 3 | 62–45 |
| | | | | Inside: 5 AGCCTAAGTGA CAAAGTGAGACA 3 | 5 TAGAGTCCTTTTC ATTGCCAA 3 | 55 |
| D5S349 (heminested) | M87741 | 0.81 | 8 | Outside: 5 ATATTTGGTTT CCATAGAATCTGAG 3 | 5 CCACCAGATTAAG CGTGAATC 3 | 62–45 |
| | | | | Inside: 5 ATATTTGGTTT CCATAGAATCTGAG 3 | 5 CCTCTAGAAAATG GTAGTTGGG 3 | 55 |
| D5S1404 (heminested) | L30346 | 0.81 | 10 | Outside: 5 GCCAATTTCT TGTCTATTCCTTAG 3 | 5 TAATTTACCCACTG TATCAGTCAGG 3 | 62–45 |
| | | | | Inside: 5 GCCAATTTCTT GTCTATTCCTTAG 3 | 5 GGTTCCATGAGAA GTAAGAGATCTA 3 | 55 |

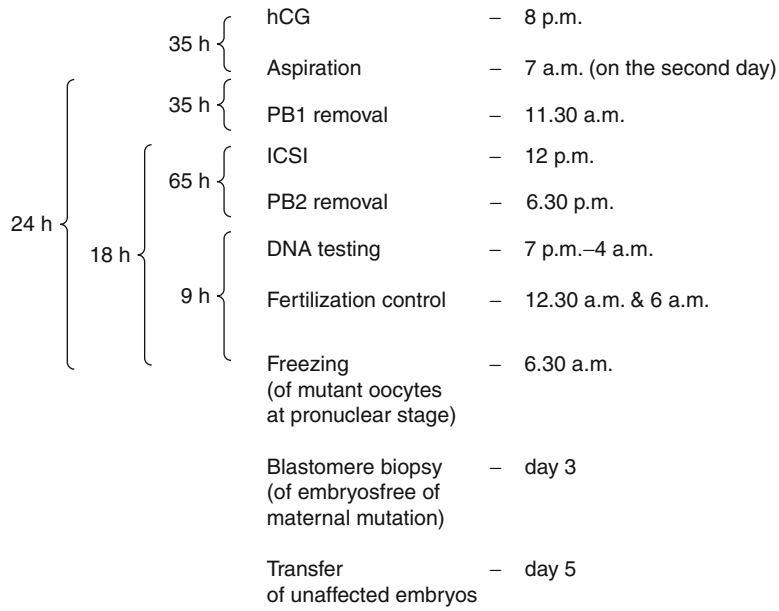


**Fig. 2.9** Timeframe for preembryonic diagnosis of Sandhoff disease (see explanation in the figure)

sperm duplication prior to genetic analysis may be required, shown below to be feasible through sperm nuclear transfer into anucleated metaphase II oocytes. The technique will allow genetic analysis of one of the sperm duplicates, using the other one for fertilization and transfer of the resulting embryos if the corresponding duplicate shows normal genotype. In this way, the establishment and discard of any embryo containing a paternal mutation may be avoided. However, more data might be necessary to work out special conditions supporting the faithful replication of human sperm genome, to ensure that the haploid cell pairs obtained from sperm duplication are identical.

As mentioned, PEGD may be applied for aneuploidy testing, as the majority of chromosomal disorders deriving from the female meiosis can be tested 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 presented results, the detection of the second meiosis errors is currently feasible within the time available prior to pronuclei fusion, so PEGD for chromosomal disorders may in future be also applied in those countries where PGD is still not acceptable because of the potential discard of the affected embryos with the currently used methods.

With the addition of the PEGD approach, the presently available techniques allow offering a greater variety of methods for predicting and avoiding not only the birth, but also conception or implantation, of the affected embryos. This provides the at-risk couples with any possible option for avoiding the offspring with genetic and chromosomal disorders, independent of their attitudes to oocyte or embryo micromanipulation and testing.

Presented data demonstrate feasibility of performing PEGD for single-gene disorders, which resulted in obtaining unaffected pregnancies and birth of healthy children. Of course PGD for single-gene disorders may be performed by the use of PB1 analysis alone, as described in the first case of PGD 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.

The data also show that to avoid discard of the preimplantation embryos reaching the cleavage stage by the time the PB genotyping results were obtained, freezing of oocytes may be used immediately after fertilization and extrusion of PB2 and prior to fusion of the male and female pronuclei – the actual point which is considered

to be the beginning of the embryonic period of development [54]. In fact, freezing presently may be omitted entirely, as the recent developments in PCR analysis allow completing the genetic diagnosis before pronuclei fusion. This opens a possibility for the application of PGD for couples unable to accept any intervention and discard of the human embryos.

### 2.1.3 Preconception Testing for Paternally Derived Mutations by Sperm Duplication

As seen above, the genetic composition of oocytes may reliably be tested through removal and testing of PB1 and PB2. On the other hand, no method has yet been available for testing the outcome of male meiosis, as genetic analysis destroys the sperm, making it useless for fertilization. To overcome this problem, a new technique has been introduced, allowing duplicating a sperm before genetic analysis, so one of the duplicated sperms can be used for testing and the other for fertilization and consequent transfer of the resulting embryos, provided that the genetic analysis of the corresponding duplicate shows normal genotype [55]. To demonstrate the reliability of the technique, over 100 human sperms from chromosomally normal donors, as well as from translocation carriers, were injected into the enucleated mouse oocytes, and the duplicated cells resulting from an overnight culture were tested by FISH to compare the chromosomal status of both daughter cells. All but 3% of the haploid cell pairs derived from the normal donors were identical for the chromosomes tested, while, as expected, a high proportion of the paired nuclei derived from sperm of translocation carriers were chromosomally unbalanced, suggesting that ooplasm from mature mouse eggs can support the faithful replication of any human sperm genome, irrespective of the genotype.

A similar technique was developed to duplicate human sperm using human oocytes (Fig. 2.10), however, showing that the duplication of sperm may be done faithfully in only half of the cases, in contrast to the use of murine oocytes, so the technique has still to be tested further before applying clinically, with the expected important practical implications for PGD of paternally derived

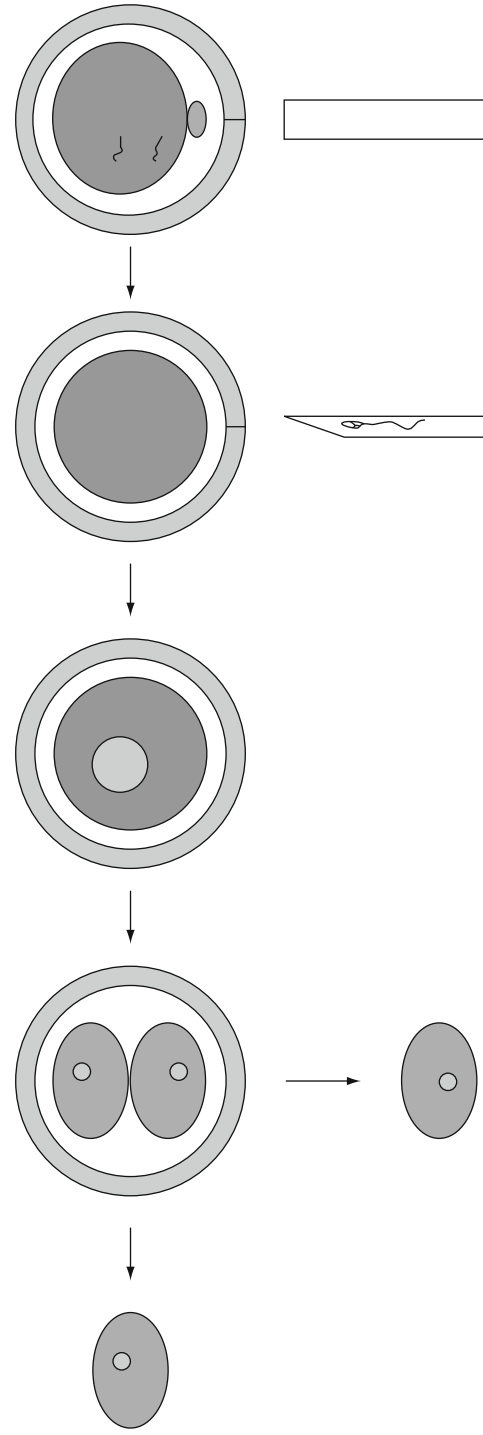

**Fig. 2.10** Flowchart of sperm duplication in human MII cytoplast: (*Step1*) enucleation of human metaphase II oocyte; (*Step 2*) injection of single human sperm into the cytoplast; (*Step 3*) reconstructed androgenic embryo with one pronucleus; (*Step 4*) development of this pronucleus into 2-cell embryos; (*Step 5*) testing of one of the cells, with the other one available for further zygote construction of known male contribution

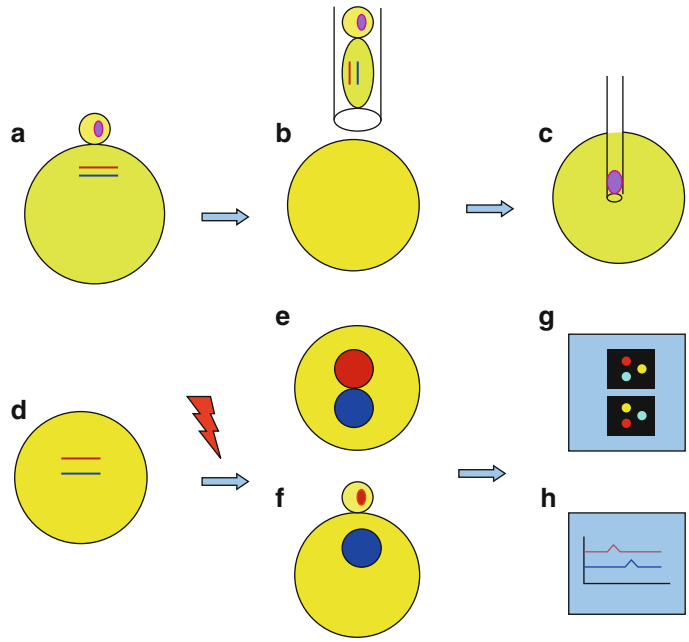


**Fig. 2.11** Flowchart of haploidization procedure for obtaining gametes from somatic cells: (**a**) in vitro matured MII oocyte with extruded first polar body (PB1); (**b**) both PB1 and metaphase II chromosomes are removed (*shown in pipette*), to prepare a recipient enucleated ooplast; (**c**) single diploid cumulus cells were prepared as a nuclei donor, from which a nucleus was mechanically isolated, for the introduction into ooplast; (**d**) reconstructed oocyte converts somatic cell nucleus into metaphase chromosomes; (**e**) two pronuclei; or (**f**) pronucleus and PB1 obtained after electrostimulation using electrofusion device RGI-4 (Chicago, IL); (**g**) fluorescent in situ hybridization analysis; or (**h**) PCR-based chromosomal analysis, confirming the formation of two haploid sets of chromosomes in both (**e**) and (**f**) scenarios

conditions, such as translocations, known to produce as much as 70% of abnormal sperm on an average.

The technique has also potential for research purposes, as shown in preliminary work devoted to the study of mosaicism nature [56]. Following duplication of human sperm in cow oocytes, a series of 31 resulting embryos were cultured up to the 8-cell stage, and tested by probes specific to chromosomes 13, 16, 18, 21, and 22. As many as 16% of the resulting sperm duplicates appeared to be not identical, which may further be related to the genetic differences between the donors involved. In fact, one of the three sperm donors for the above experiment produced mostly mosaic embryos in two PGD cycles. However, the rate of mosaicism in sperm duplicates of the three donors involved in this small series was similar, indicating that the generation of mosaic embryos, at least in the patients previously tested by PGD, may not be related to the sperm genotype, but to the sperm centrosome [56].

The genotype of the sperm may also be tested following the testicular biopsy culture and promotion of the developmental progression of spermatocytes through meiosis in vitro, providing the possibility of meiosis outcome analysis to infer the genotype of the resulting sperm to be used for fertilization. However, this is still a theoretical possibility, which has not been realized.

Preconception diagnosis may be realized also in the future by the development of human gametes, using the techniques of haploidization, which is described below.

### 2.1.4 Development of Artificial Human Gametes In Vitro

Attempts were undertaken for creating both female and male gametes, both demonstrating a strong morphological evidence for haploidization [57, 58]. The technique is based on inducing nuclei of mitotic somatic cells to skip the S-phase of the cell cycle and undergo haploidization when introduced into oocytes, which allows obtaining artificial gametes from somatic cells through the process of haploidization. We showed that the efficiency of haploidization of donor cumulus cell nuclei differs depending on the stage of development of the enucleated recipient oocyte [59, 60]. This may be tested using the extruded PBs, or generated pronuclei, which also allow investigating the correctness of chromosomal segregation. As seen from the flowchart in Fig. 2.11, the first step involves enucleation of in vitro matured MII oocytes under the

control of UV luminescence, which is important to ensure the accuracy of chromosome analysis of the resulting haploid nuclei. Then the cumulus cell nuclei, which are at the G0 of the cell cycle, are introduced into ooplasts by injection and the oocytes are activated by electrostimulation delivered by the electrofusion device (XRONOS, RGI-4 (Chicago, IL)). Following the oocyte activation, the chromosomes of the transferred nuclei segregate with the extrusion of polar bodies, or form two pronuclei both evidencing the formation of artificial gametes through somatic cell haploidization [59, 60]. The FISH analysis and DNA fingerprinting of PB1 and pronuclei resulting from the haploidization procedure showed the haploid chromosomal set, with the resulting DNA originating from the donor nuclei, so the extruded PB1 may be used to investigate the genetic contents of the corresponding pronucleus. However, preliminary data showed that as many as 90% of these haploid nuclei appeared to be with chromosomal aneuploidies. This suggests that the use of the resulting haploid nuclei in the gamete reconstruction procedure may not be acceptable at the present time.

To determine if an incubation time of nuclei in ooplast improves chromosomal segregation, two groups of a total of 122 reconstructed MII oocytes were studied, one activated 5–7 h after the nuclear transfer, and the other after 12–21 h. However, an aneuploidy rate as high as approximately 90% was observed irrespective of incubation time, with the majority being of a complex nature, suggesting no improvement of the accuracy of chromosomal segregation with the prolonged incubation time [59, 60]. So, although haploidization of somatic cells may be achieved using MII oocyte cytoplasm, the aneuploidy rate is much higher than in normal meiosis, which currently makes the techniques not acceptable for clinical practice.

### 2.1.5 Embryo Biopsy

Embryo biopsy is performed as soon as the embryo reaches a minimum of six cells or more so as not to cause a considerable decrease in cell number at the later stages of development. A mechanical opening of zona pellucida has been developed, called 3D-PZD, allowing the creation

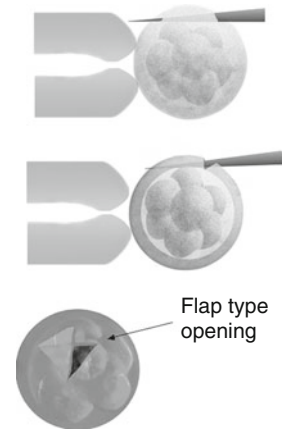


**Fig. 2.12** Three-dimensional partial zona dissection (3D-PZD) for embryo biopsy. (*Step 1*) The embryo is held in position by gentle suction from the holding pipette. The embryo is rotated using the microneedle so that a blastomere with a well-visualized nucleus is present at 12 o'clock. The embryo is then rotated horizontally so that the blastomere of choice is facing the operator. The microneedle is passed tangentially under the zona pellucida through the perivitelline space moving from the 1–2 o'clock position to the 10–11 o'clock position. A first slit is created as described in Fig. 2.3. (*Step 2*) The embryo is rotated so that the slit opening is at the 1–2 o'clock position and a second intersecting slit is made by passing the microneedle through the first slit opening and out at the 10–11 o'clock position. The cut is accomplished as with all partial zona dissection previously described in Fig. 2.3. This second intersecting slit creates a larger flap-type opening for the purpose of accessing PB2 as mentioned in Fig. 2.5 or to utilize the required larger micropipette for embryo biopsy

of a V-shaped triangular flap or square flap opening, sufficient in size for a micropipette to pass through in order to remove a blastomere(s) [48]. Micromanipulation dishes are prepared the same way as for PB1 removal, except for sucrose use, which is eliminated. The micromanipulation setup is the same as for PB1 removal with one exception – the micropipette has a larger diameter of 25–30 μm. The embryo biopsy procedure is shown and described in Fig. 2.12.

With the current tendency for blastocyst transfer, there has been a renewed interest in the development of methods for blastocyst biopsy, which has resulted in successful PGD cycles performed by blastocyst biopsy for genetic and chromosomal disorders, yielding unaffected clinical pregnancies and births of healthy children [61]. Blastocyst biopsy may be performed by mechanical methods,

as the trophectoderm is beginning to herniate through the zona pellucida [48]. Several trophectoderm cells are removed at this stage by smooth aspiration with a biopsy pipette with an internal diameter of 30 µm. However, most centers are currently using a microlaser procedure, which is applied to break down the tight junctions between trophectoderm cells, followed by the aspiration of 5–6 trophectoderm cells.

Gradually, blastocyst biopsy is becoming a method of choice in many centers, and has currently a growing potential with the improvement of freezing techniques, and particularly vitrification. It has also special implication with the introduction of microarray technology, which has much more accurate and reliable results when performed on blastocyst biopsy [23, 28, 29]. In addition to using blastocyst biopsy as the method of choice, it is also used as additional testing required for confirming PB or blastomere diagnosis.

The follow-up studies of embryos after blastomere biopsy did not show any detrimental effect. No increase in congenital malformation has been reported among thousands children born following PB or blastomere biopsy, although further systematic study will be needed to monitor the clinical outcomes of PGD using PB1 and PB2 sampling or embryo biopsy and to collect further data on the safety of the procedures used in PGD for single-gene and chromosomal disorders. However, according to the data presented in Fig. 2.13, there seems to be no difference in the developmental potential of embryos following single, double, or triple biopsy procedures, compared to the rate of blastocyst formation following ICSI (control group). As can be seen from Fig. 2.13, no significant differences were observed in blastocyst formation between the embryos with the embryo biopsy procedure of a single blastomere biopsy and the embryos with two biopsy procedures, including simultaneous PB1 and PB2 removal on day 1, followed by the removal of a single blastomere on day 3. There was no decrease, but even an increase in the rate of blastocyst formation when compared to the control group, for the group of embryos in which three biopsy procedures were performed, including PB1 removal prior to ICSI, PB2 removal at the time of fertilization assessment, and a single blastomere removal on day 3. This is in agreement with the pregnancy

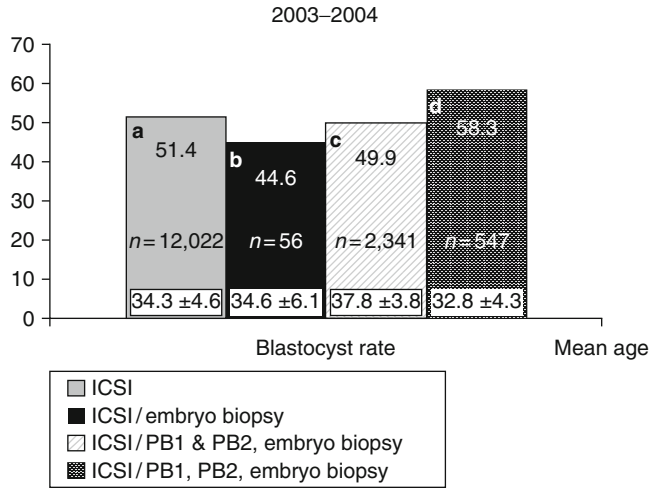


**Fig. 2.13** Effect of micromanipulations on embryo development. Bar graph demonstrating nondetrimental effect of biopsy procedures on the rate of blastocyst formation following intracytoplasmic sperm injection (control group). No significant difference was seen between embryos after (1) embryo biopsy procedure of a single blastomere and also embryos in which (2) biopsy procedures were performed in which the first and second polar bodies were removed simultaneously on day 1 (pronuclear stage) followed by the removal of a single blastomere on day 3, when compared to the control group. There was no decrease, but a significant increase in the rate of blastocyst formation when compared to the control group, for the group of embryos in which three biopsy procedures were performed. The first polar body was removed prior to ICSI, the second polar body was removed at the time of fertilization assessment, and a single blastomere was removed on day 3

outcome data, which will be presented in (Chap. 3), showing a 40% pregnancy rate in approximately 1,000 PGD cycles performed for single-gene disorders by sequential PB1 and PB2 analysis, followed by blastomere biopsy.

Therefore, accumulated experience allows concluding that the biopsy procedures involved in PGD do not seem to have significant detrimental effect on embryo viability, although further randomized control studies are needed to obtain more detailed information. At present, preliminary data of available randomized control studies are controversial. In one of them, the effect was so significant that it was probably caused by the lack of sufficient experience in biopsy procedures [62].

## 2.2 Single-Cell Genetic Analysis

Single-cell genetic analysis includes single-cell DNA and FISH analysis, which have become important tools for the application of PGD in assisted reproduction and genetic services, providing an important option for couples at genetic risk to avoid the birth of an affected offspring and have a healthy child of their own.

### 2.2.1 DNA Analysis

Because PGD for single-gene disorders is based on single-cell genetic analysis, its accuracy depends largely on the limitations of single-cell DNA analysis, which may potentially cause misdiagnosis. One of the key contributors to misdiagnosis is the phenomenon of preferential amplification, also known as allele-specific amplification failure (allele drop out, ADO), requiring the application of special protocols to ensure the highest ADO detection rate [48, 63, 64]. A few previously reported misdiagnoses, involving PGD for beta-thalassemia, myotonic dystrophy (DM), fragile-X syndrome (XMR1), and cystic fibrosis (CF), might have been due to this phenomenon, which has not initially been fully realized [39–41, 46, 65].

It has been demonstrated that ADO rates in single cells might be different for different types of heterozygous cells [66]. The ADO rate may exceed 20% in blastomeres compared to the ADO rate in single fibroblasts and PB1, which was shown to be under10%. A high rate of ADO in blastomeres may lead to an obvious misdiagnosis, especially in compound heterozygous embryos. As mentioned, most misdiagnoses, especially those at the initial stage of application of PGD for single-gene disorders, were in the cases of blastomere biopsy from apparently compound heterozygous embryos.

The reliability of PGD depends on the controlling ADO. We have previously demonstrated that ADO rates in single-cell PCR may vary with different lysis procedures, cell types, and loci analyzed [48, 63, 67]. Therefore, reliable methods are needed to detect potential ADO, avoiding misdiagnosis in PGD for single-gene disorders. Our experience demonstrated feasibility of detection of ADO by a sequential analysis of oocytes, using PB1 and PB2, and by simultaneous amplification of mutant genes with linked polymorphic markers [48, 64, 67], which is described in brief below.

The biopsied single cells are placed directly into a lysis solution, consisting of 0.5 mcl 10×PCR buffer, 0.5 mcl 1% Tween 20, 0.5mcl 1% Triton×100, 3.5 mcl H2O, and 0.05 mcl Proteinase K (20 mg/ml in a 0.5 ml PCR tube). After spinning down at a low speed in a microfuge for a few seconds, the samples are covered with one drop of mineral oil and incubated at 45°C for 15 min in a thermal cycler. Proteinase K was then inactivated at 96°C for 20 min, which is also the beginning of the hot start of the first-round PCR. Lower stringency and longer annealing time are used in the first-round PCR, with the introduction of the mixture of all outside primers for both mutant genes and polymorphic markers. Following the first-round PCR, separate aliquots are amplified in the second-round PCR with specific inside primers for each site, using a higher stringency. Such a dual or multiple amplification reaction allows detection of most of the ADO cases. If there are pseudogenes, to eliminate false priming, the first-round primers are designed to anneal to the regions of nonidentity with pseudogenes [48]. In addition to short tandem repeats (STR) linked to the genes studied, STRs located on other chromosomes are also studied for testing of a possible contamination by

extraneous DNA, and identification of the origin of individual embryos in the established pregnancies. A list of STRs, their sequences, and PCR reaction conditions for their analysis are presented in each respective section.

Fluorescent PCR (F-PCR) is used for a direct fragment-size analysis of a PCR product [68]. F-PCR is useful also for a direct sequencing of the PCR product in the detection of point mutations and for distinguishing preferential amplification from ADO. A considerable proportion of ADO is detected by sequential analysis of PB1 and PB2, which may be demonstrated by data on sequential PCR analysis of 26 alleles in PB1 and PB2 obtained from 1,047 oocytes, which showed that 32 of 53 of all ADOs in mutation analysis are detectable simply by sequential analysis of PB1 and PB2, avoiding misdiagnosis due to ADO when no informative polymorphic markers are available. As mentioned, a high rate of ADO is observed especially in blastomeres, leading to an obvious misdiagnosis in compound heterozygous embryos, which was the case at the initial stage of application of PGD for single-gene disorders based on blastomere biopsy obtained from apparently compound heterozygous embryos [39, 46]. This is now avoided by testing two or more linked polymorphic markers if available, making DNA testing in PB or single blastomeres a highly reliable procedure. Contrary to expectation, the application of F-PCR does not sufficiently improve detection of potential misdiagnoses in PGD of single-gene disorders. Testing of 148 single fibroblasts by both conventional and F-PCR provided minor contribution to ADO detection rates. Based on this observation, it was postulated that simultaneous amplification of single cells for any causative gene, together with one linked polymorphic marker, reduces the ADO rate by more than half, irrespective of the use of conventional or F-PCR. With the additional second marker in multiplex PCR, the ADO rate may further be reduced by half, being completely absent if three or more linked markers are simultaneously amplified, as shown above.

We previously reported the results of 114 PGD cycles for couples at high risk for having children with single-gene disorders, resulting in preselection and transfer of a sufficient number of mutation-free oocytes in almost all cycles. Of 1,047 oocytes with DNA results, 672 (64.1%) had heterozygous PB1, that is, with both normal and mutant genes amplified, which is therefore ideal for further testing, although their potential transfer depended entirely on the identification of mutant genes in the sequential analysis of PB2. Thus, priority in preselection of embryos for transfer was given to the embryos resulting from the oocytes with heterozygous PB1 because in the absence of DNA contamination this indicates the absence of ADO of either the normal or mutant allele. Although most of the transferred embryos were preselected using this particular strategy, some preselected embryos still originated from homozygous normal oocytes, inferred from the homozygous mutant status of PB1 and hemizygous normal status of PB2. These embryos were accepted for transfer only if ADO could have been excluded using linked polymorphic marker analysis. Otherwise, such embryos were excluded from transfer and exposed to follow-up confirmation analysis of the resulting embryos.

The follow-up analysis of the embryos excluded from transfer either because they were affected or because there was insufficient information to preselect them for transfer provided the data for evaluating the proportion of undetected ADO. Overall, 82 (7.8%) ADOs were observed, which included 75 detected and 7 undetected ones, suggesting that not 970 but actually 1,052 oocytes were heterozygous. The genotype of six embryos appeared to be different from that predicted by mutation analysis, and one by STR, due to ADO in PB1, which were diagnosed as homozygous instead of their actual heterozygous status. The data indicated to 98% accuracy, which is quite acceptable for clinical use of PGD for single-gene disorders.

As can be seen from these data, to avoid a misdiagnosis due to preferential amplification, a simultaneous detection of the mutant gene together with up to three or more highly polymorphic markers, closely linked to the gene tested, may be required [48, 63]. Each additional linked marker may reduce the misdiagnosis rate by half, so with one linked marker amplified together with mutation, a misdiagnosis risk in blastomere analysis may be reduced from 20% to 10%, with two

from 10% to 5%, and with three from 5% to practically zero. So a *multiplex nested PCR* analysis is performed, with the initial PCR reaction containing all the pairs of outside primers, so that following the first-round PCR, separate aliquots of the resulting PCR product may be amplified using the inside primers specific for each site. Only when the polymorphic sites and the mutation agree are embryos transferred. So multiplex amplification allows detecting ADO and prevents the transfer of misdiagnosed affected embryos.

Our data show that one of the most efficient approaches for avoiding misdiagnosis is a sequential genetic analysis of the PB1 and PB2 in PGD for maternally derived mutations. Detection of both mutant and normal alleles in the heterozygous PB1, together with the mutant allele in the corresponding PB2, leaves no doubt that the resulting maternal contribution to the embryo is normal, even without testing for the linked markers as a control. However, it will be ideal to test simultaneously at least for one linked marker to confirm the diagnosis. Alternatively, the mutation-free oocytes is also predicted when the corresponding PB1 is a homozygous mutant, in which scenario the corresponding PB2 should be a hemizygous normal, similar to the resulting maternal pronucleus. However, the genotype of the resulting maternal contribution may be quite opposite, that is, mutant, if the corresponding PB1 is in fact heterozygous, but erroneously diagnosed as homozygous normal because of ADO of the normal allele. In the above scenario, the extrusion of the normal allele with PB2 would lead to the mutant allele left in the resulting oocyte. Therefore, the embryos resulting from the oocytes with homozygous mutant PB1 cannot be acceptable for transfer, unless the heterozygous status of PB1 is excluded by the use of linked markers as described. The example of misdiagnosis, due to ADO of the normal allele in PB1, has been described earlier in a PGD cycle performed for FMR1 [65]. To completely avoid misdiagnosis, a sequential PB1 and PB2 may be required to combine with multiplex PCR to exclude the possibility of an undetected ADO in heterozygous PB1. As described above, the analysis of more than 1,000 oocytes tested by sequential PB1 and PB2 analysis showed that more than half of the ADOs were detected by sequential analysis of PB1 and PB2, with the remaining cases detected by multiplex PCR. The accuracy of this approach may be demonstrated by the reports of PGD for thalassemia and familial dysautonomia (FD) (see below), resulting in the transfer of three unaffected embryos in each case, which were confirmed by the birth of two sets of triplets free from thalassemia and FD [69, 70].

The other method with the proved potential for detecting and avoiding misdiagnosis due to preferential amplification is fluorescence PCR (F-PCR), which may allow detection of some of the heterozygous PB1 or blastomeres misdiagnosed as homozygous in conventional PCR, and therefore having the potential of reducing the ADO rates at least to some extent [64]. In addition, the method also allows a simultaneous gender determination, DNA fingerprinting, and detection of common aneuploidies. F-PCR combined with a multiplex system and sequential PB1 and PB2 analysis in cases of maternally derived mutations allows excluding almost completely the risk for misdiagnosis due to preferential amplification.

The accuracy of PGD has been further improved with the application of fluorescent PCR with the expand long template (ELT) kit, which enabled reducing the ADO rate from as high as 30–35% in both conventional and fluorescent PCR to as low as 5% in testing for DM [71]. Another development in improving the accuracy of single-cell PCR analysis involves the application of real-time PCR, which was found to reduce the ADO rate almost by half, compared to conventional or fluorescent PCR (Fig. 2.14). The application of these approaches together with simultaneous testing for the causative mutation along with at least one or two linked markers allows avoiding reliably the risk for misdiagnosis.

Finally, because of the high rate of mosaicism at the cleavage stage, testing for the chromosome, in which the gene in question is mapped, is of an obvious value, to exclude the lack of mutant allele due to monosomy of this chromosome in the biopsied blastomere. As mentioned, aneuploidy testing is technically feasible and is done by adding primers for chromosome-specific microsatellite

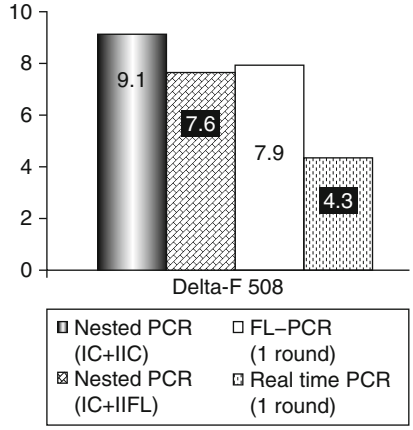


**Fig. 2.14** Reduction of allele dropout (ADO) rates by real time PCR. ADO rates are shown for nested conventional PCR (*left bar*), nested combined PCR (first-round conventional, second-round fluorescent: *middle–left bar*), one-round fluorescent PCR (*middle right bar*), and one-round real-time PCR (*right bar*) with single human fibroblasts heterozygous for deltaF508 mutation in the CFTR gene. Comparable ADO rates are seen in the first three types of PCR, while application of one round of real-time PCR reduces ADO in single fibroblasts almost by half, demonstrating that real-time PCR is the most sensitive for detection of ADO, although it cannot completely prevent misdiagnosis

markers to the multiplex PCR protocols worked out for specific genetic disorder [72]. The development of multiplex nested PCR systems also allows performing PGD for different conditions simultaneously, as attempted with PGD for CFTR mutation together with XMR1 or gender determination [73, 74], and will be also described in Sect. 4. The PCR-based strategy for aneuploidy testing is shown in Fig. 2.15, listing polymorphic markers used for identification of the copy number of chromosomes 13, 15, 16, 17, 18, 21, 22, and X. Y. However, this may currently be performed by microarray analysis in the same whole-genome amplification product, combining testing for mutations and polymorphic markers with 24 chromosome aneuploidy testing [18–29].

Due to the need for the development of a custom-made PGD design for each mutation and each couple, preparatory work has become an integral part of PGD for single-gene disorders to ensure avoiding potential misdiagnosis. For example, in some cases, a particular set of outside primers has to be designed to eliminate false priming to the pseudogene, as described in PGD for long-chain 3-hydroxyacyl-Coa dehydrogenase deficiency [75]. Also, the preparatory work may frequently involve a single sperm typing needed for establishing paternal haplotypes, so that linked marker analysis could be performed in addition to mutation testing, especially in cases of paternally derived dominant conditions or PGD combined with preimplantation HLA matching (see below). The use of haplotyping for PGD without direct mutation testing is presently used as a more universal procedure, called preimplantation genetic haplotyping (PGH) [76]. This also improves the accuracy of PGD, as the availability of the parental haplotypes, irrespective of whether the mother or the father is a carrier, allows not only confirming the absence of the mutant gene but also the presence of both maternal and paternal wild alleles in PGD by blastomere analysis, especially when only one informative marker is available (Chap. 3).

### 2.2.2 FISH Analysis

PGD for the age-related aneuploidies is currently done by FISH analysis, using commercially available chromosome-specific probes (Vysis, Downers Groves, IL, USA). It was first applied in 1991 for gender determination using DNA probes specific either for the X or Y chromosome [8]. Since testing for only one of the sex chromosomes could lead to misdiagnosis of gender due to a possible failure of hybridization, a dual FISH was introduced, involving the simultaneous detection of X and Y, each in a different color [9]. Further, the dual FISH analysis was combined with a ploidy assessment to improve the accuracy, by adding a centromeric probe specific for chromosome-18 [10, 11]. Testing was then extended up to five autosomes, including chromosomes 13, 16, 21, and 22 [48, 77, 78], although it is currently possible to analyze up to a dozen chromosomes, using additional rounds of re-hybridization.

As mentioned, the overall experience of preimplantation FISH analysis currently involves more than 50,000 clinical cycles, resulting in an improved pregnancy rate in poor-prognosis IVF patients [2, 3, 79–82], despite the present

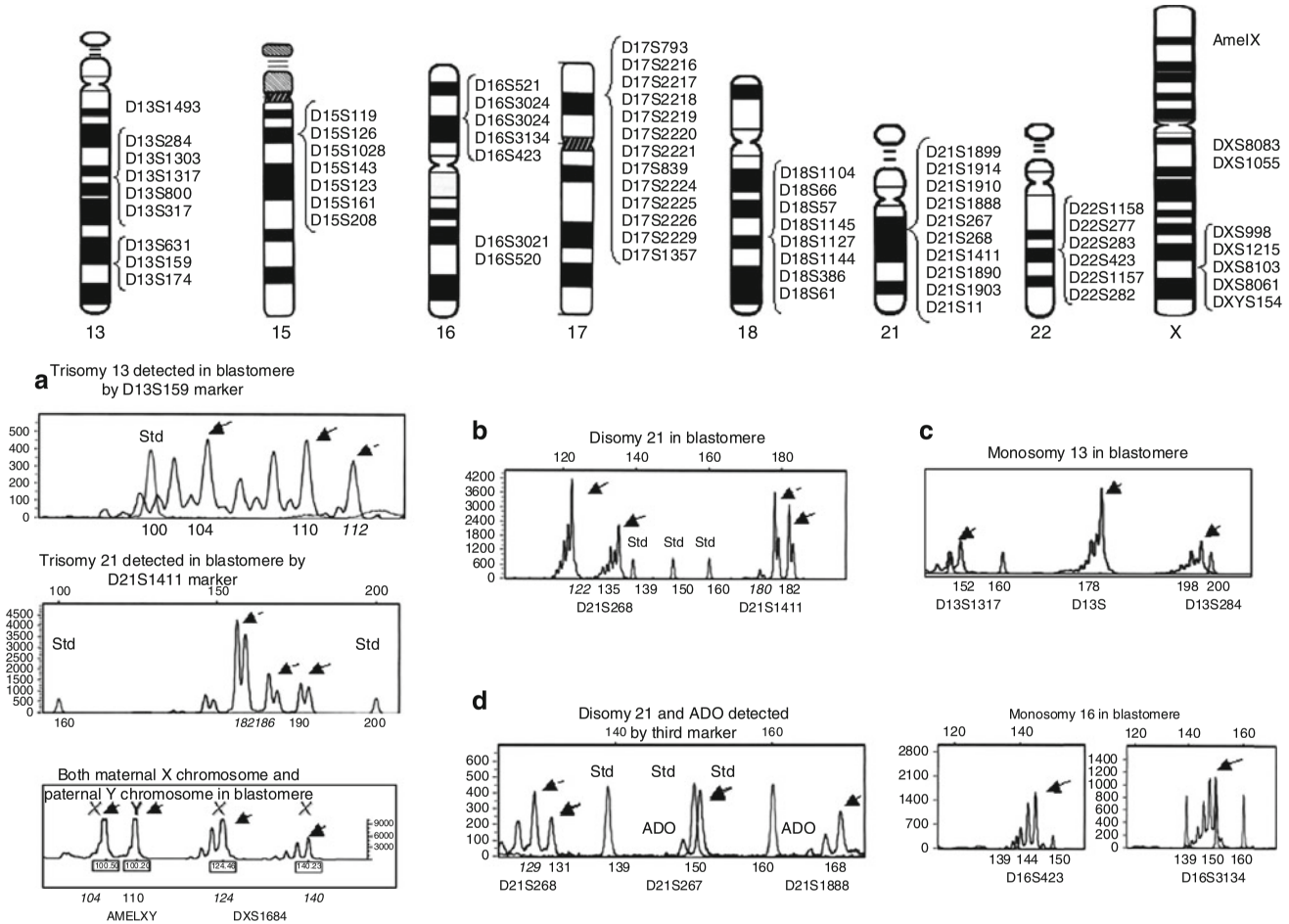


**Fig. 2.15** PCR-based aneuploidy testing. (*Top*) Positions of polymorphic markers applied for the PCR-based aneuploidy testing for selected chromosomes. To differentiate between allele dropout (ADO) and the absence of the whole chromosome (monosomy), at least three informative polymorphic markers for each chromosome are tested. (*Bottom*) Examples of single-blastomere PCR results with (**a**) trisomy13, 21, and XXY; (**b**, **d**) normal for chromosome 21; (**c**) monosomy 13 and 16; and (**d**) ADO, distinguished from monosomy 21 by the use of three linked markers

controversy on this issue. The majority of these cycles were performed by FISH analysis of blastomeres, while approximately one-fifth was done by FISH analysis of PB1 and PB2, resulting in thousands of unaffected pregnancies and healthy children born at the present time. The follow-up confirmation studies of the preselected abnormal embryos, and the babies born following the procedure, demonstrated an acceptable accuracy of the FISH analysis, which is described below.

The reliability of the FISH technique for aneuploidy detection in blastomeres has been extensively studied [1, 13, 31, 35, 83, 84]. By comparing the FISH results in the cleaving embryos to morphological abnormalities and maternal age, it was established that the observed chromosomal abnormalities were indeed not related to the limitations of the FISH technique, but were due to the embryo variables [13, 83]. However, a high rate of mosaicism was observed at the cleavage stage [12, 13, 83, 84], which was particularly high in slow embryos, exhibiting an arrested development. Initially, an overall 12% mosaicism rate was suggested in cleaving embryos [15], but it has now been shown to occur in as many as half of preimplantation embryos, representing one of the major limitations of the FISH analysis for aneuploidies, performed at this stage [1, 35]. Clearly mosaicism will affect the accuracy of the diagnosis, except for those cases when PGD detects the abnormal cell from a mosaic embryo, which will not be transferred. It was also shown that mosaicism may present diagnostic problems at the blastocyst stage [85], despite the initial prediction that the abnormal cells are deviated mainly to trophectoderm.

The first attempt to use FISH analysis for testing PB1 was undertaken in 1994. In this work, 130 unfertilized MII oocytes were tested simultaneously with their PB1, using X-chromosome and chromosome 18 specific probes. It was demonstrated that PB1 FISH data allow an exact prediction of the chromosome set in the corresponding oocytes [86, 87]. Each chromosome in PB1 was represented by double dots (signals), corresponding to two chromatids in each univalent (Fig. 2.16). The data suggested that the number of signals (chromatids) in PB1 reliably predicts the corresponding number of signals (chromatids) in the MII oocytes, therefore, providing an excellent tool for the genetic preselection of oocytes. It was also of interest that, in addition to a normal distribution of signals in PB1 and the corresponding MII oocytes, meiotic

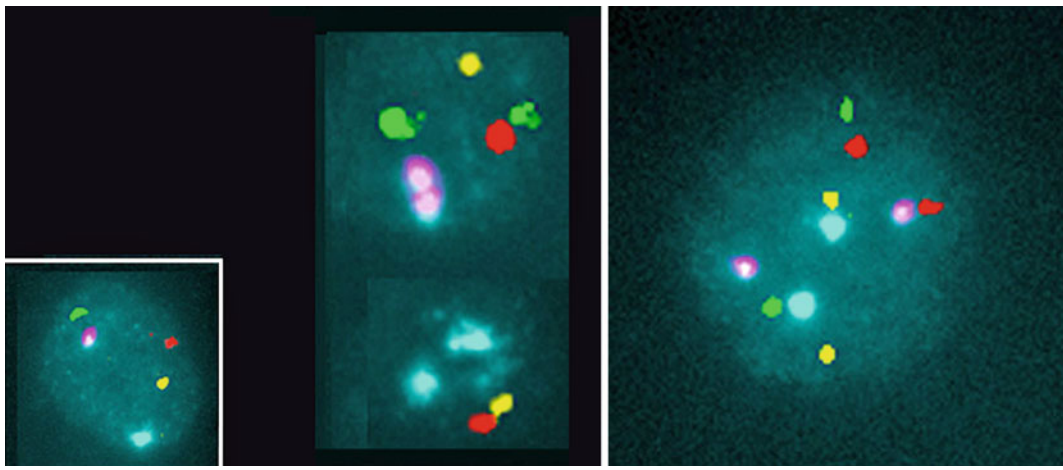

**Fig. 2.16** Normal pattern of FISH images of PB1 and PB2 and blastomeres after a 3-h hybridization with MultiVysion PB panel probe for autosomes 13 (*red*), 16 (*aqua*), 18 (*violet blue*), 21 (*green*), and 22 (*gold*). PB1 and PB2 were removed simultaneously on day 1 at the pronuclear stage of development following fertilization assessment. (*Middle panel*) PB1 chromosomes showing a normal number of single dot signals (two per chromosome, representing each chromatid). (*Lower left*) A normal number of signals (one per chromosome (chromatid)) are present in the PB2 interphase nucleus inferring a normal chromosome complement in the oocyte and resulting embryo. (*Right panel*) Normal FISH images of interphase nuclei isolated from blastomeres, resulting from the oocytes shown on the left, after embryo biopsy on day 3 of embryo development (two signals for each of the autosomes tested)

errors were also detected, confirming the accuracy of PB1 diagnosis for predicting the genotype of the corresponding oocyte. For example, in one PB1 four signals for chromosome 18 were detected, perfectly in accordance with the lack of the chromosome 18 signals in the corresponding MII oocyte (chromosome 18 non-disjunction). This suggested that the chromosomal complements of the oocyte could be inferred from the testing of PB1, which can be removed following its extrusion from the mature oocyte, with no potential influence on the embryo viability. Another interesting phenomenon was the observation of chromatid malsegregation as a possible cause of chromosomal aneuploidy in the resulting mature oocytes. In four oocytes, instead of the expected two signals, three were found in the MII oocytes, which perfectly corresponded to a single signal in the corresponding PB1. Similar results were reported by another group, confirming diagnostic significance of the PB1 FISH analysis for predicting the genotype of the preimplantation embryo [88].

PB1 testing was one of the first approaches used for PGD of translocations (16), based on the fact that PB1 never forms an interphase nucleus and consists of metaphase chromosomes. It is known that PB1 chromosomes are recognizable when isolated 2–3 h after *in vitro* culture, with degeneration beginning 6–7 h after extrusion. Therefore, whole-chromosome painting or the chromosome segment-specific probes could be applied for testing of maternally derived chromosomal translocations in PB1. Although the method resulted in a significant reduction of spontaneous abortions in the patients carrying translocations, yielding unaffected pregnancies and births of healthy children, it has shown to be sensitive to malsegregation and/or recombination between chromatids, requiring a further follow-up analysis of PB2, in order to accurately predict the meiotic outcome following the second meiotic division (Chap. 5).

In contrast to PB1, PB2 is the by-product of the second meiotic division, extruded following fertilization of an oocyte. The need for FISH analysis of PB2 for PGD of aneuploidies was first proposed in 1995, when it was demonstrated that PB1 testing alone does not allow predicting the resulting genotype of the oocyte [89]. It was shown that in contrast to the paired dots in PB1, each chromosome in PB2 was represented by a single dot (see normal pattern of FISH signals in Fig. 2.16), so the lack of or addition of a signal for a particular chromosome provided evidence of a second meiotic division error. Although only 19 of 55 oocytes in this first study were tested by both PB1 and PB2, evidence for errors was observed not only in meiosis I, detected by PB1 analysis, but also in meiosis II, which may be detected by PB2 testing. These data suggested that some oocytes selected as normal, based on the PB1 FISH analysis, still could have been abnormal following nondisjunction in the second meiotic division. Therefore, FISH analysis for both PB1 and PB2 has become the basic requirement for PGD of aneuploidies, which allows detecting errors in both the first and second meiotic divisions. Currently, more than 25,000 oocytes have been analyzed by FISH analysis, showing the accuracy and reliability of PB1 and PB2 testing for predicting the karyotype of the embryo, resulting from the corresponding oocyte [24]. As will be described below, more than 50% of oocytes from IVF patients of advanced reproductive age are abnormal, resulting from the errors in both the first and second meiotic divisions, in contrast to the previously believed concept that aneuploidies mainly originate from meiosis I.

As mentioned, PB2 testing is also an important component of PGD for maternally derived translocations. However, this is still done on interphase as, despite the progress in transforming PB2 into metaphase chromosomes via electrofusion of PB2 nucleus with foreign one-cell human embryo, the proportion of metaphase plates obtained was not sufficient to be useful in clinical practice [90]. Much higher efficiency was observed in conversion of interphase nuclei of blastomeres, which is described below.

Visualization of chromosomes of individual blastomere nuclei requires the application of nuclear transfer technique, which was initially attempted for the conversion of interphase nuclei of PB2 into metaphase [91]. The original design was to fuse individual blastomeres with enucleated human oocytes. Although metaphases were obtained from two-thirds of blastomeres treated by this method, its efficiency was not high enough to be applicable to PGD. This was due to the inability

of a replicating nucleus to form metaphase chromosomes after the induction of premature chromosome condensation (PCC). However, because biopsied blastomeres may be at any stage of the cell cycle at the time of biopsy, there was a need to control the timing of mitosis of blastomere nuclei, which can be achieved by the introduction of a blastomere into the cytoplasm of a cell at a known cell cycle [92]. To achieve such reprogramming, the individual blastomeres were fused with intact or enucleated mouse zygotes at pronuclear stage, known to be at the S-phase of the cell cycle.

So the method involves the use of frozen mouse zygotes, which can be purchased from Charles River Laboratories (Wilmington, MA) as recipient cytoplast to induce the conversion of blastomere nucleus into metaphase. Also there is no need for enucleation of mouse oocytes, as mouse and human chromosomes may be clearly distinguished. Blastomere biopsy is performed in the same way as described above, except for choosing only intact blastomeres with clearly visible nuclei. Several precautions have also to be taken to ensure the integrity of the blastomere plasma membrane during biopsy procedure. Although intact blastomeres may be inserted microsurgically into perivitelline space, this appeared to be traumatic and was replaced by blastomere–zygote agglutination with phytohemagglutinin (Irvine Scientific, Santa Ana, CA). Before the procedure, the thawed mouse zygotes are freed of zonae pellucidae with acidic Tyrode's solution and pipetted through the flame-polished Pasteur pipettes with an internal diameter of 80 μm. Then, using the flame-polished Pasteur pipettes with an internal diameter of 100 μm, blastomere–zygote pairs are brought together and agglutinated in 300 μg/ml of phytogemagglutinin in protein-free human tubal fluid buffered with 20 mM of HEPES in a four-well plastic dish (Nunc).

Electrofusion is induced in the same way as mentioned in the above section, except for substitution of 0.5% polyvinylpirrolidone in the electrofusion medium by 0.1% with molecular weight 360.000 (kd). Blastomere–zygote pairs are oriented between electrodes by hand, with the final orientation achieved with alternating current (500 kHz; 0.2 kV/cm for 2 s). Cell fusion is induced with a single direct current pulse (1 kV/cm for 500 s), and the results are assessed in 20 min.

When human blastomeres are fused with intact mouse zygotes, the heterokaryons entering mitosis are identified under a dissecting microscope. Because of the transparency of mouse cytoplasm, the disappearance of pronuclei and the formation of the joint metaphase plate are clearly visible. The heterokaryons with persisting pronuclei are exposed for 1 h to 5 μM of OA in phosphate-buffered saline containing 3 mg/nL of bovine serum albumin and 0.5 μg/nL of cytochalasin D. After 10–15 min incubation in a hypotonic solution (0.1% sodium-citrate and 0.6% bovine serum albumin), the resulting mitotic heterokaryons are fixed in a cold 3:1 solution of methanol and acetic acid in a four-well plastic dish. When the cytoplasm clears, heterokaryons are transferred onto slides and air-dried. Chromosome plates are assessed by phase contrast and then used for standard chromosome analysis. For FISH analysis the slides are pretreated with formaldehyde and pepsin (Abbott Inc., Downers Grove, IL).

Overall success rate of the procedure is as high as 83%, with its efficiency improved with experience [36, 48]. Similar results were obtained by using bovine ooplasts for fusion with human blastomeres [92]. The data showed that some of the failures are simply due to the absence of the nucleus in biopsied blastomeres, or because the heterokaryons were fixed after they had already cleaved. It is also useful to perform blastomere biopsy not earlier than day 3 or day 4, to avoid biopsy of 2- and 4-cell embryos, leading to the accelerated heterokaryon cleavage. However, the success rate did not depend at all on whether mouse zygotes were enucleated before fusion with blastomeres. This allows simplifying the procedure by using intact mouse zygotes.

So, the procedure is quite simple and includes the following components. Mouse zygotes are thawed free of zonae pellucidae and PB2 1–2 h before electrofusion with human blastomeres. Four hours after fusion, heterokaryons are monitored for signs of the disappearance of pronuclei, and fixed at mitosis following hypotonic treatment. To avoid monitoring and a possible miss of mitosis the heterokaryons may be cultured in the presence of microtubuli inhibitors, vinblastine, or

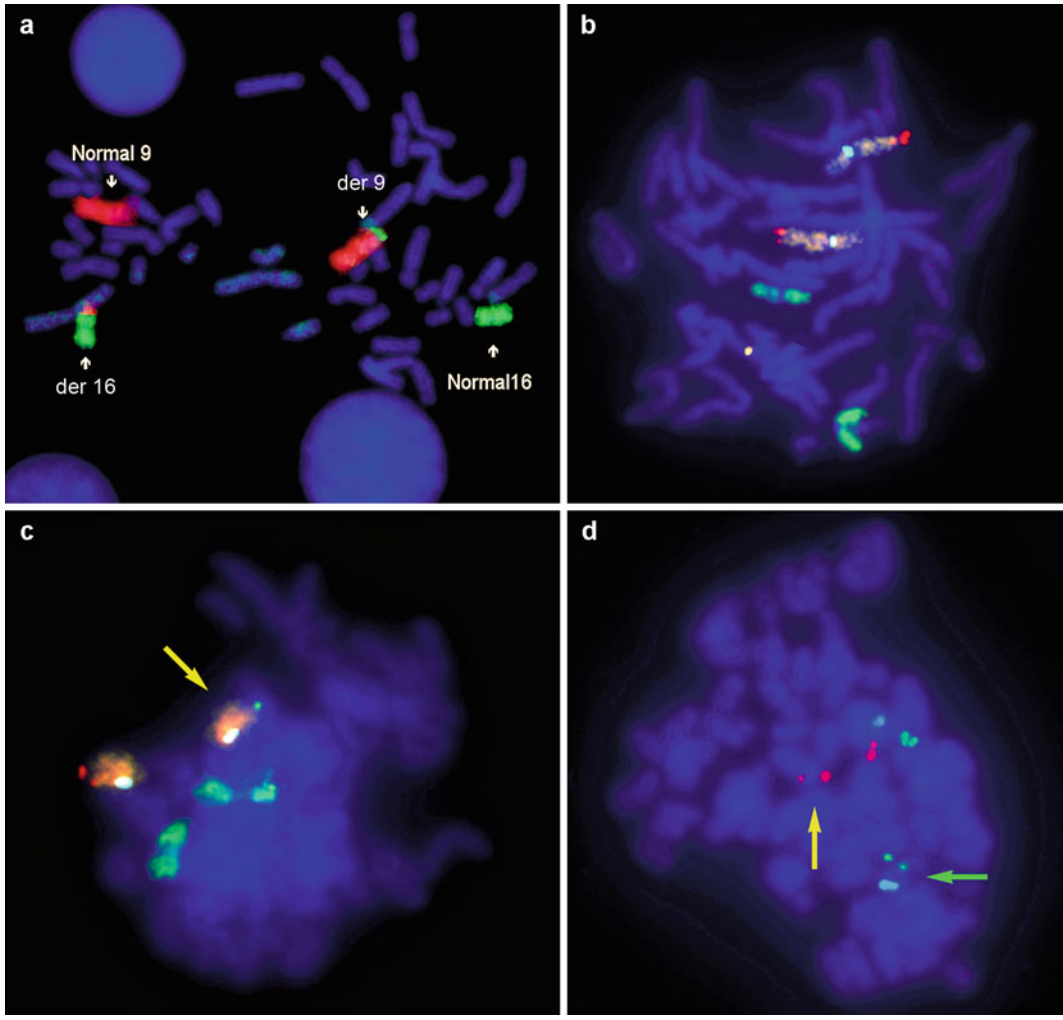


**Fig. 2.17** Blastomere nuclear conversion to metaphase for PGD of a maternally derived reciprocal translocation [46,XX, t(9;16)(q34.3;p13.1)]. (**a**) FISH analysis of metaphase chromosomes of a peripheral blood lymphocyte from the carrier. Chromosome 9 is identified with WCP in orange (seen through a red single bandpass filter) in conjunction with Tel 9q in *red*. Chromosome 16 is identified with WCP in *green* in conjunction with Tel 16p in *green*. Sub-telomeric probes were added to this probe cocktail since the translocated portion of each chromosome is small. Derivatives are seen with both *red* and *green* fluorescence. (**b**) WCP in *orange* for chromosome 9 in conjunction with CEP 9 in *aqua* and Tel 9q in *red* along with WCP 16 (*green*) in conjunction with Tel 16p (*green*) on karyotypically normal, peripheral blood. (**c**) Analysis by embryo biopsy and blastomere nucleus conversion to metaphase chromosomes. Unbalanced chromosome complement [9,der (9),16,16] in which derivative (9) is present, evident by the presence of *red* and *green* fluorescence from Tel 16p (*yellow arrow*). This embryo was omitted from transfer. (**d**) Analysis by embryo biopsy and blastomere nucleus conversion to metaphase chromosomes. FISH analysis utilizing locus-specific and sub-telomeric probes revealed a balanced chromosome complement by the presence of both derivatives. Derivative 9 is distinguished from the normal chromosome 9 by the presence of a CEP signal in *aqua* in conjunction with a Tel 16p signal in *green* (*green arrow*) and derivative 16 is distinguished from normal chromosome 16 by a Tel 9 q signal (only) in *red* with the absence of a CEP 9 *aqua* signal (*yellow arrow*). This corresponding embryo was suitable for transfer barring any developmental problems

podophyllotoxin. All the embryos, left in the culture by the ninth hour after fusion, are fixed following 1 h pretreatment with OA. The example of blastomere nucleus conversion applied for PGD of reciprocal translocation 46,XX, t(9;16)(q34.3;p13.1) is shown in Fig. 2.17.

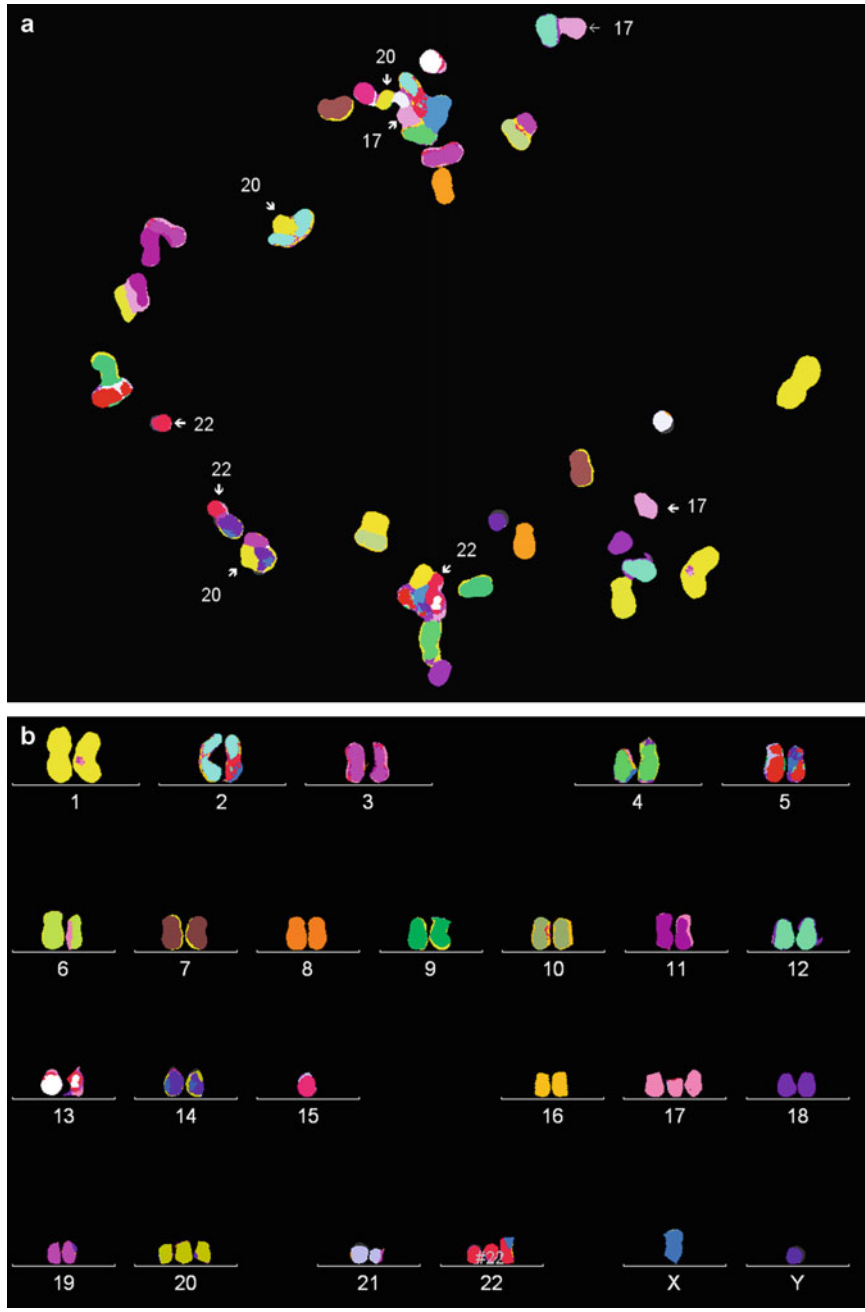


**Fig. 2.18** Karyotype of blastomere obtained without conversion technique. Embryo follow-up analysis after polar body analysis revealed second meiotic division error for chromosome 22 indicating a trisomy 22 embryo. (**a**) Testing was performed by embryo biopsy and "nonconversion" techniques used to obtain metaphase chromosomes from a single blastomere nucleus. Afterward, FISH was performed on the metaphase spread utilizing Metasystems' XCyte mFISH probe, which uses five fluorescent dyes to detect different painting probes at the same time, allowing for identification of all 24 different chromosomes with a single hybridization. Each chromosome is distinguished by a separate or combination of different fluorophores that are separated by appropriate filter sets. Based on the specific fluorochrome combination the chromosomes are given a pseudo-color according to the 24X Cyte labeling scheme. This allows for the analysis of complex numerical aberrations even when there is slight chromosome overlap present as seen in this metaphase spread. (**b**) Karyotype established by utilization of the Isis program revealing complex numerical aberrations consisting of trisomies 17, 20, and 22 and monosomy15

The method has been applied for PGD of paternally derived reciprocal translocations and for confirmation of PGD of chromosomal abnormalities performed by PB1 and PB2 FISH analysis. With the current success rate of blastomere nucleus conversion into metaphase it was applied for clinical cases, which will be described in Sect. 5.

It has recently been reported that the blastomere metaphase can be also obtained without the application of a specific conversion method [93]. To obtain analyzable chromosomes, the embryos were monitored closely the second day after ICSI, to identify the blastomere with nuclear breakdown, which was biopsied and fixed within 1 h. This method was currently modified by 1-h culture of the biopsied blastomere in medium with vinblastine, which resulted in harvest of metaphase chromosomes of good quality (Fig. 2.18).

To get more reproducible results this method was further improved by using chemical agents, which involves morphological selection for biopsy of the largest blastomere with 1–2 large nucleoli within the cell nucleus. Upon embryo biopsy, each blastomere is contained in microdrops of Global culture medium (LifeGlobal, USA) supplemented with Plasmanate (Bayer Biological, USA) 10% vol:vol., containing caffeine (Sigma) (1 mmol/l) and a low dose of colcemid (Sigma) (<0.1 μg/ml) under mineral oil [33, 94]. Blastomeres are incubated at 37°C in an atmosphere of 6% $CO_2$ and air for approximately 2–3 h or until nuclear membrane breakdown is observed. Afterward, the blastomeres are treated in a hypotonic solution followed by fixation using a cold solution of methanol and glacial acetic acid, 3:1. Careful attention is paid so as not to overspread the chromosomes in order to avoid chromosome loss during fixation. Consequently, not all metaphases are suitable for cytogenetic investigation by G-banding; however, they are suitable for FISH analysis to identify structural rearrangements (Fig. 2.19).

### 2.2.3 Microarray Analysis

A major recent breakthrough has been in the development and application of microarray technology for PGD of chromosomal disorders, allowing for a highly improved detection of chromosomally abnormal oocytes and embryos [18–29]. Although there are different platforms for 24-chromosome testing, the most adequate for the purpose of PGD at this time is 24sure technology, developed by BlueGnome Ltd, Cambridge, UK, because it can be applied to all the biopsy materials, including PB1, PB2, blastomeres, and blastocyst, allowing completion of the test within 12 h, and providing accurate results in over 90% samples. The protocol consists of at least 6 steps, including amplification (2 h), labeling (2.5 h), hybridization (3.5 h), washing (30 min), scanning (30 min), and data analysis (1 h). The technique tests for all 24 chromosomes for any gain or loss with the bacterial artificial chromosome (BAC) pooling strategy, which, coupled with the uniquely designed software, enables obtaining straightforward results on aneuploidy in a single cell. Currently, two 24sure array formats are used for two applications. BACs spotted on the 24sure array are selected on the basis of having little variations in over 5,000 hybridizations, to deliver the highest level of reproducibility and sensitivity in aneuploidy testing.

To apply the technique for additional analysis for chromosomal rearrangements, 24sure + array format is used, with greater coverage of genome, including subtelomeric and pericentromeric regions, enabling accurate characterization of arm-level aneuploidy and other large-scale structural abnormalities. Both the formats are used with Sure Ref reference DNA, providing a hybridization reference, well matched for quality, to an amplified single cell. Also a sex-mismatched design is used to provide useful reference in interpreting the results, such as the use of male reference in the hybridization, mismatching with X chromosome in the analysis of polar bodies.

One of the critical steps of the procedure is whole-genome amplification with the Super Plex Single-Cell Whole-Genome Amplification Kit (BlueGnome Ltd), which is performed according to the manufacturer's instructions. Specific quality control criteria for sample quality and quantity are used to ensure that only specific amplifications are labeled. The fluorescent labeling system

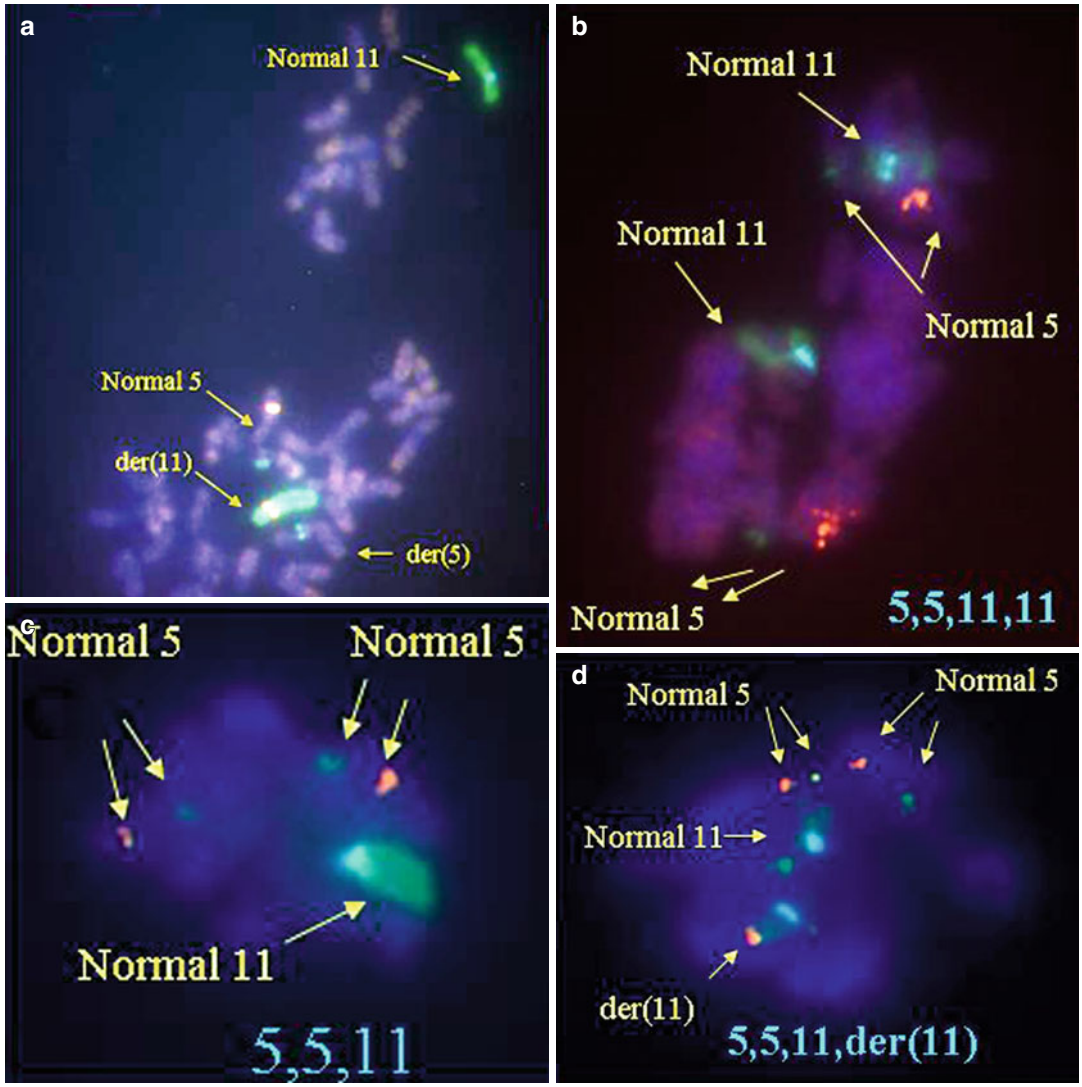


**Fig. 2.19** Preimplantation diagnosis for maternally derived rearrangement 46,XX,ins(11;5)(q22.2;q31.1q34) by chemical conversion distinguishing normal from unbalanced embryos. Fluorescent in situ hybridization (FISH) was performed for chromosome 11 using whole chromosome paint 11 (WCP 11) in *green* and the centromeric enumeration probe for chromosome 11 (CEP 11) in *aqua*. Chromosome 5 is identified by a dual probe which targets a region of the p arm LSI D5S23, D5S721 (5p15.2) in *green* and the q arm LSI EGR1 (5q31) in *orange*. The remaining chromosomes are seen in *blue* after the application of DAPI counterstain. (**a**) FISH image of a metaphase from a peripheral blood lymphocyte from the female carrier. Normal chromosome 11 is seen with WCP 11 in *green* and CEP 11 in *aqua*. Derivative 11 is identified by a combination of WCP 11 *green*, CEP 11 *aqua*, and 5q31 in *orange* identifying the inserted segment of chromosome 5 into derivative 11. Normal chromosome 5 is identified by the presence of both 5p15.2 in *green* and 5q31 in *orange*. Derivative 5 is identified by 5p15.2 in *green only* since it lacks the 5q31 region, which has been inserted into the q arm of chromosome 11. (**b**) FISH image of condensed chromosomes isolated from a blastomere from a normal 8-cell embryo showing two normal chromosomes 5 and 11. (**c**) FISH image of condensed chromosomes in close proximity to one another indicating an unbalanced chromosome complement. Two normal chromosomes 5 (*top*) and one normal chromosome 11 (*middle*), and a derivative chromosome 11 (*bottom*) are present. (**d**) FISH image of condensed chromosomes from an unbalanced 8-cell embryo, which shows two normal chromosomes 5 (*top left* and *right*) and one normal chromosome 11 (*lower right*)

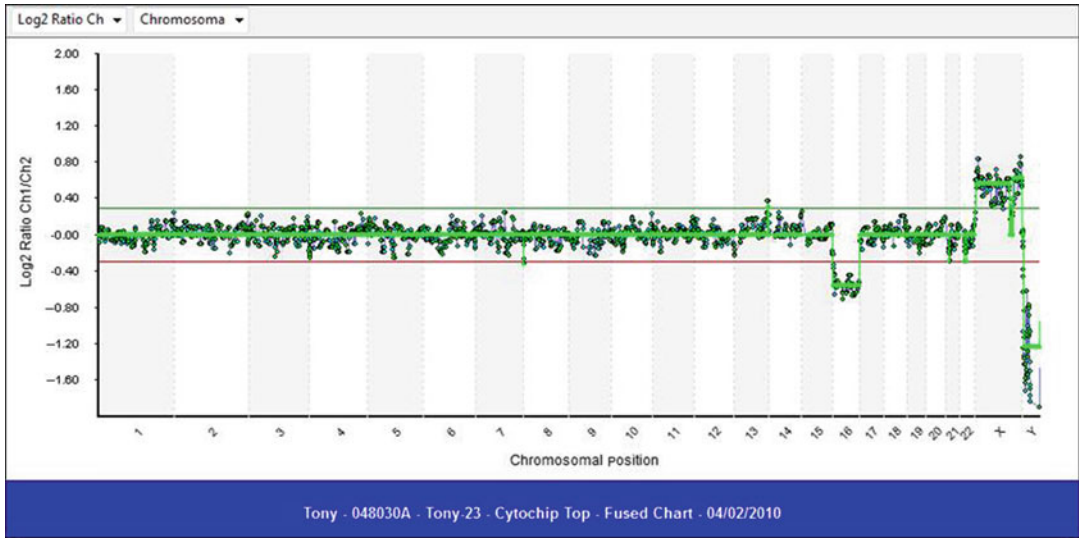


**Fig. 2.20** Results from a PB1 carrying a loss of chromatid 16, obtained by array-CGH analysis

(BlueGnome Ltd) is used for the labeling of the amplified samples of biopsy materials, as well as labeling of a commercially available reference DNA. Test and reference DNA co-precipitation, their denaturation, array hybridization, and the post-hybridization washes are done according to protocol provided by the manufacturer.

A laser scanner is used to excite the hybridized fluorophores, and to read and store the resulting images of hybridization, using the special software provided by Bluegnome. Chromosome profiles are examined for gain or loss, or for structural rearrangement. Figure 2.20 shows the results of PB1 analysis, demonstrating trisomy 16.

Preliminary data on array-CGH (24sure by BlueGnome) testing of the first and second polar bodies, and the resulting oocytes, were consistent with FISH results, suggesting that array-CGH may be the method of choice for PGD of aneuploidies [24–27]. An ESHRE Task Force pilot study demonstrated an accurate identification of chromosomal status in 89% of oocytes tested, based on microarray analysis of PB1 and PB2 [26, 27]. When combined with embryo biopsy to detect paternally derived aneuploidies and those originating from mitotic errors, this could provide an improved detection and avoidance of aneuploid embryos from transfer. However, as evidenced from inconsistencies between the predicted and observed types of errors in cleaving embryos [24] and by the high prevalence of mosaicism at this stage, blastocyst biopsy may appear a better choice, taking also into consideration the recent reports on a higher accuracy and improved clinical outcome following the application of 24-chromosome testing coupled with blastocyst biopsy [23, 28, 29].

This approach has opened the possibility of combining aneuploidy testing for 24 chromosomes, with PGD for single-gene disorders, which was first performed for $G_{M1}$ gangliosidosis, which is an autosomal-recessive lysosomal storage disorder [95]. Of 10 embryos tested by SNP Microarray on blastocyst biopsy, 7 were euploid and 3 aneuploid, while 2 embryos were determined as affected with $G_{M1}$ gangliosidosis, 5 embryos were mutation carriers, and 3 were normal. By combining the results of these tests, 5 embryos were found to be suitable for transfer. This novel approach was then extended to a variety of genetic conditions, and also applied together with preimplantation HLA typing, to investigate feasibility of a combined test for 24-chromosome aneuploidy, with PGD for single-gene disorders and preimplantation HLA typing. The list included gangliosidosis GM1,

isolated hypertrophic cardiomyopathy, cystic fibrosis, Fragile –X, neurofibromatosis type 1, Nieman Pick disease, and congenital gangliosidosis. A total of 77 embryos were tested, including 43 by blastocyst biopsy in 6 and 34 by blastomere biopsy in 4 cycles. Of 77 embryos tested, 30 (39%) were predicted to be aneuploidy-free and also unaffected. Overall, such embryos were available for transfer in all but one cycle, and transferred without freezing in the majority of cycles performed by blastomere biopsy (unpublished data).

At the present time, this combined approach is applied in approximately 100 cycles, including aneuploidy testing together with Mendelian disorders and HLA typing, as described above, and also together with PGD for translocations [96], demonstrating the possibility of testing for multiple indications in a single comprehensive test, avoiding additional biopsy procedures. This also improves the efficiency of PGD, as the transfer of aneuploid embryos is excluded in cycles from patients of advanced reproductive age.

So, presented data show that the PB approach, based on PB1 and PB2 analysis, as well as genetic analysis of single blastomeres or blastocyst cells, may presently be applied for PGD of single-gene disorders, aneuploidies, and translocations. Based on this analysis, the embryos resulting from unaffected oocytes are transferred back to patients within the implantation window, while those predicted as affected are followed up by PCR or FISH analysis to confirm the PB or blastomere diagnosis. The results of genetic analysis using both sources of biopsy material will be presented below, showing the accuracy and reliability of PGD for single-gene and chromosomal disorders.

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# Preimplation Diagnosis for Single-Gene Disorders

3

Single-gene disorders are the first group of indications for which preimplantation genetic diagnosis (PGD) was originally introduced 21 years ago, with the purpose of performing genetic testing before pregnancy, in order to establish only unaffected pregnancies and avoid the need for pregnancy termination, which is the major limitation of traditional prenatal diagnosis [1, 2]. Despite the requirement for ovarian hyperstimulation and in vitro fertilization (IVF), needed to perform genetic testing of oocyte or embryo prior to transfer, PGD has been accepted in most parts of the world [3, 4]. At least 10,000 PGD cycles were performed for single-gene disorders and, as will be shown below, is presently offered for some indications that have never been practiced in prenatal diagnosis, such as late-onset diseases with genetic predisposition, and preimplantation HLA typing, making PGD not only an alternative but also a complement to prenatal diagnosis [5–8]. The progress of PGD has been extensively reviewed, so the present book will mainly concentrate on those aspects of PGD that are useful for reproductive medicine and genetics practices, including available PGD approaches for different groups of genetic disorders, their accuracy, and major indications compared to prenatal diagnosis, and present practical details useful for the realization of PGD for each of the conditions described.

Indications for PGD were initially similar to those practiced in prenatal diagnosis and applied for those at-risk couples that could not accept pregnancy termination, expected in 25–50% of cases following prenatal diagnosis, depending on the mode of inheritance. However, these indications have been extended beyond those for prenatal diagnosis, and currently include the conditions with a low penetrance, late-onset disorders with genetic predisposition, and HLA typing with or without testing for causative genes [8]. The list of disorders, for which PGD has been applied, according to our experience, now comprises close to 300 conditions (Table 3.1), with most frequent ones still being cystic fibrosis (CFTR), hemoglobin disorders, and some of the dynamic mutations, such as myotonic dystrophy. Initially, the choice between prenatal diagnosis and PGD mainly depended on the patient's attitude to termination of pregnancy, which is strongly influenced by social and religious factors, but steadily it is becoming a part of family planning for couples at risk to ensure having only unaffected pregnancy, especially when there is a risk of having offspring with severe late-onset common disorders with a strong genetic predisposition [8]. However, the majority of patients are still unaware of the availability of PGD, due to a relative novelty of the procedure. So there is an obvious need for increasing awareness of PGD both for couples at risk and the medical profession, who require information about benefits, accuracy, safety, and expected risks of the procedure.

Tables 3.2 and 3.3 present our overall data of 2,158 PGD cycles for single-gene disorders, which is the world's largest experience in one center. A total of 938 of these cycles were performed by PB approach, involving the retrieval and testing

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**Table 3.1** List of diseases and genes for which PGD was performed

| Disease | MIM number | Inheritance | Gene name/ symbol | Protein name | Location |
|---|---|---|---|---|---|
| Achondroplasia (ACH) | 100800 | AD | FGFR3 | Fibroblast growth factor receptor 3 [Precursor] | 4 p16.3 |
| Acyl-CoA dehydrogenase, medium-chain, deficiency | 201450 | AR | ACADM | Acyl-CoA dehydrogenase, medium-chain specific, mitochondrial [Precursor] | 1p31 |
| Acyl-CoA dehydrogenase, very long-chain (ACADVL) | 609575 | AR | ACADVL | Acyl-coenzyme A dehydrogenase, very long chain | 17p13-p11 |
| Adenosine deaminase deficiency (ADA) | 102700 | AR | ADA | Adenosine deaminase | 20q13.11 |
| Adenomatous polyposis of the colon (APC) | 175100 | AD | APC | Adenomatous polyposis coli protein | 5q21-q22 |
| Adrenoleukodystrophy (ALD) | 300100 | XL | ABCD1 | Adrenoleukodystrophy protein | Xq28 |
| Agammaglobulinemia, X-linked (XLA) | 300755 | XL | BTK | Bruton agammaglobuline-mia tyrosine kinase | Xq21.3-q22 |
| Aicardi-Goutieres syndrome 1 (AGS1) | 225750 | AR | TREX1 | Three prime repair exonuclease 1 | 3p21.31 |
| Aicardi-Goutieres syndrome 5 (AGS5) | 612952 | AR | SAMHD1 | SAM domain and HD domain 1 | 20pter-q12 |
| Albinism, ocular, type I (OA1) | 300500 | XL | OA1 | G-protein coupled receptor 143 | Xp22.3 |
| Alopecia universalis congenita (ALUNC) | 203655 | AR | HR | Hairless protein | 8p21.2 |
| Alpers diffuse degeneration of cerebral gray matter with hepatic cirrhosis | 203700 | AR | POLG | Mitochondrial DNA polymerase gamma | 15q25 |
| Alpha 1 antitrypsin deficiency (AAT) | 107400 | AR | SERPINA1 | Alpha-1-antitrypsin [Precursor] | 14q32.1 |
| Alport syndrome, X-linked (ATS) | 301050 | XL | AMMECR1 | AMME syndrome candidate gene 1 protein | Xq22.3 |
| Alzheimer disease 3 | 607822 | AD | PSEN1 | Presenilin 1 | 14q24.3 |
| Alzheimer disease 4 | 606889 | AD | PSEN2 | Presenilin 2 (Alzheimer disease 4) | 1q31-q42 |
| Amyloidosis I, hereditary neuropathic | 176300 | AD | TTR | Transthyretin [Precursor] | 18q11.2-q12.1 |
| Amyotrophic lateral sclerosis 1 (ALS1) | 105400 | AD | SOD1 | Superoxide dismutase 1, soluble | 21q22.11 |
| Androgen receptor (AR) (testicular feminization; spinal and bulbar muscular atrophy; Kennedy disease) | 313700 | XL | AR | AR protein | Xq11-q12 |
| Aneuploidies by STR genotyping | | | | | |
| Angelman syndrome | 105830 | AD | UBE3A | Ubiquitin protein ligase E3A | 15q11.2 |
| Angioedema, hereditary (HAE) | 106100 | AD | SERPING1 | Plasma protease C1 inhibitor precursor | 11q11-q13.1 |
| Argininosuccinic aciduria | 207900 | AR | ASL | Argininosuccinate lyase | 7cen-q11.2 |
| Arthrogryposis, distal, type 2B (DA2B) | 601680 | AD | TNNT3 | Troponin T type 3 (skeletal, fast) | 11p15.5 |

**Table 3.1** (continued)

| Disease | MIM number | Inheritance | Gene name/ symbol | Protein name | Location |
|---|---|---|---|---|---|
| Ataxia-telangiectasia (AT) | 208900 | AR | ATM | Serine-protein kinase ATM | 11q22-q23 |
| Basal cell nevus syndrome (BCNS; Gorlin) | 109400 | AD | PTCH | Patched protein homolog 1 | 9q22.1-31 |
| Beta-hydroxyisobutyryl CoA deacylase, deficiency | 250620 | AR | HIBCH | 3-Hydroxyisobutyryl-coenzyme A hydrolase | 2q32.2 |
| Blepharophimosis, ptosis, and epicanthus inversus (BPES) | 110100 | AD | FOXL2 | Forkhead box protein L2. | 3 q23 |
| Blood group – Kell-Cellano system | 110900 | AD | KEL | Kell blood group glycoprotein | 7q33 |
| Brachydactyly, type B1 (BDB1) | 113000 | AD | ROR2 | Receptor tyrosine kinase-like orphan receptor 2 | 9q22 |
| Brain tumor, posterior fossa of infancy, familial | 601607 | AD | SMARCB1 | SWI/SNF related, matrix associated, actin dependent regulator of chromatin subfamily B member | 22q11.2 |
| Breast cancer, familial | 113705 | AD | BRCA1 | Breast cancer type 1 susceptibility protein | 17q21 |
| Breast-ovarian cancer, familial, susceptibility to | 612555 | AD | BRCA2 | Breast cancer 2 | 13q12.3 |
| Canavan disease | 271900 | AR | ASPA | Aspartoacylase | 17pter-p13 |
| Carbamoyl phosphate synthetase I (CPS I) deficiency | 237300 | AR | CPS1 | Carbamoyl-phosphate synthase 1, mitochondrial | 2q35 |
| Cardioencephalomyopathy, fatal infantile, due to cytochrome c oxidase deficiency | 604377 | AR | SCO2 | SCO cytochrome oxidase deficient homolog 2 (yeast) | 22q13.33 |
| Cardiomyopathy, dilated, 1A (CMD1A) | 115200 | AD | LMNA | Lamin A/C | 1q21.2 |
| Cardiomyopathy, dilated, 1DD (CMD1DD) | 613172 | AD | RBM20 | RNA binding motif protein 20 | 10q25.2 |
| Cardiomyopathy, familial hypertrophic, 1 (CMH1) | 192600 | AD | MYH7 | Myosin, heavy chain 7, cardiac muscle, beta | 14q12 |
| Cardiomyopathy, familial hypertrophic, 4 (CMH4) | 115197 | AD | MYBPC3 | Myosin binding protein C, cardiac | 11p11.2 |
| Cardiomyopathy, familial hypertrophic, 7 (CMH7) | 613690 | AD | TNNI3 | Troponin I type 3 (cardiac) | 19q13.4 |
| Carnitine deficiency, systemic primary (CDSP) | 212140 | AR | SLC22A5 | Solute carrier family 22 (organic cation/carnitine transporter), member 5 | 5q31 |
| Carnitine deficiency, systemic primary (CDSP) | 212140 | AR | SLC2A10 | Solute carrier family 2 (facilitated glucose transporter), member 10 | 20q13.1 |
| Carnitine palmitoyltransferase II deficiency, lethal neonatal | 608836 | AR | CPT2 | Carnitine palmitoyltransferase 2 | 1p32 |
| Ceroid lipofuscinosis, neuronal 2, late infantile (CLN2) | 204500 | AR | CLN2 | Tripeptidyl-peptidase I [Precursor] | 11p15 |

(continued)

**Table 3.1** (continued)

| Disease | MIM number | Inheritance | Gene name/ symbol | Protein name | Location |
|---|---|---|---|---|---|
| Charcot-Marie-Tooth disease, axonal, type 2E | 607684 | AD | NEFL | Neurofilament triplet L protein | 8p21 |
| Charcot-Marie-Tooth disease, demyelinating, type 1A (CMT1A) | 118220 | AD | PMP22 | Peripheral myelin protein 22 | 17p12 |
| Charcot-Marie-Tooth disease, demyelinating, type 1B (CMT1B) | 118200 | AD | MPZ | Myelin P0 protein [Precursor] | 1q23.3 |
| Charcot-Marie-Tooth disease, X-linked, 1 (CMTX1) | 302800 | XL | GJB1 | Gap junction beta-1 protein | Xq13.1 |
| Cholestasis, progressive familial intrahepatic 2 | 603201 | AR | ABCB11 | ATP-binding cassette, sub-family B (MDR/TAP), member 11 | 2q24 |
| Chondrodysplasia punctata 1, X-linked recessive (CDPX1) | 302950 | XL | ARSE | Arylsulfatase E | Xp22.3 |
| Choroideremia (CHM) | 303100 | XL | CHM | Rab proteins geranylgeranyltransferase component A 1 | Xq21.2 |
| Ciliary dyskinesia, primary, 3 (CILD3) | 608644 | AR | DNAH5 | Dynein, axonemal, heavy chain 5 | 5p15.2 |
| Citrullinemia, classic | 215700 | AR | ASS1 | Argininosuccinate synthase 1 | 9q34.1 |
| Coenzyme Q10 deficiency | 607426 | AR | COQ2 | Coenzyme Q2 homolog, prenyltransferase | 4q21.23 |
| Cohen syndrome (COH1) | 216550 | AR | VPS13B | Vacuolar protein sorting 13 homolog B | 8q22.2 |
| Collagen, type IV, alpha-5 (COL4A5) | 303630 | XL | COL4A5 | Collagen, type IV, alpha 5 | Xq22.3 |
| Colorectal cancer, hereditary nonpolyposis, type 1 (HNPCC1) | 120435 | AD | MSH2 | DNA mismatch repair protein Msh2 | 2p 2-p21 |
| Colorectal cancer, hereditary nonpolyposis, type 1 (HNPCC1) | 600678 | AD | MSH6 | mutS homolog 6 (*E. coli*) | 2p16 |
| Colorectal cancer, hereditary nonpolyposis, type 2 (HNPCC2) | 609310 | AD | MLH1 | DNA mismatch repair protein Mlh1 | 3 p21.3 |
| Congenital adrenal hyperplasia (CAH) | 201910 | AR | CYP21A2 | Cytochrome P450 XXIB | 6 p21.3 |
| Congenital disorder of glycosylation, type I (CDG1A) | 212065 | AR | PMM2 | Phosphomannomutase 2 | 16p13.3-p13.2 |
| Corneal dystrophy, Avellino type (CDA) | 607541 | AD | TGFB1 | Keratoepithelin | 5q31 |
| Craniofacial dysostosis, type I (CFD1) | 123500 | AD | FGFR2 | Fibroblast growth factor receptor 2 [Precursor] | 10q26.13 |
| Currarino syndrome | 176450 | AD | HLXB9 | Homeobox protein HB9 | 7q36 |
| Cutis laxa, autosomal recessive, type I | 219100 | AR | FBLN4 | EGF-containing fibulin-like extracellular matrix protein 2 | 11q13 |

**Table 3.1** (continued)

| Disease | MIM number | Inheritance | Gene name/ symbol | Protein name | Location |
|---|---|---|---|---|---|
| Cystic fibrosis (CF) | 219700 | AR | CFTR | Cystic fibrosis transmembrane conductance regulator | 7q31.2 |
| Cystinosis, nephropathic (CTNS) | 219800 | AR | CTNS | Cystinosin | 17p13 |
| Darier-White disease (DAR) | 124200 | AD | ATP2A2 | Sarcoplasmic/endoplasmic reticulum calcium ATPase 2 | 12q23-q24.1 |
| D-bifunctional protein deficiency | 261515 | AR | HSD17B4 | Hydroxysteroid (17-beta) dehydrogenase 4 | 5q21 |
| Deafness, neurosensory, autosomal recessive 1 (DFNB1) | 220290 | AR | GJB2 | Gap junction protein connexin-26 | 13q11-q12 |
| Diamond-Blackfan anemia (DBA) | 105650 | AD | RPS19 | 40S ribosomal protein S19 | 19q13.2 |
| Dihydroxyadenine urolithiasis | 102600 | AD | APRT | Adenine phosphoribosyltransferase | 16q24 |
| Donohue syndrome | 246200 | AR | INSR | Insulin receptor | 19p13.3-p13.2 |
| Dyskeratosis congenita, autosomal dominant, 1 (DKCA1) | 127550 | AD | TINF2 | TERF1 (TRF1)-interacting nuclear factor 2 | 14q12 |
| Dystonia 1, torsion, autosomal dominant (DYT1) | 128100 | AD | TOR1A | Torsin family 1, member A (torsin A) | 9q34 |
| Dystrophia myotonica 1 | 160900 | AD | DMPK | Myotonin-protein kinase | 19q13.2-q13.3 |
| Early-onset familial Alzheimer disease | 104760 | AD | APP | Amyloid beta A4 protein [Precursor] | 21q21.3 |
| Ectodermal dysplasia, anhidrotic | 224900 | AR | EDAR | Tumor necrosis factor receptor superfamily member EDAR [Precursor] | 2q 11-q13 |
| Ectodermal dysplasia, hypohidrotic, X-linked (XHED) | 305100 | XL | EDA | Ectodysplasin A | Xq12-q13.1 |
| Ectrodactyly, ectodermal dysplasia, and cleft lip/palate syndrome 1 (EEC1) | 129900 | AD | p63 | Tumor protein 63 | 7q11.2-q21.3 |
| Ehlers-Danlos syndrome, type I | 130000 | AD | COL5A1 | Collagen, type V, alpha 1 | 9q34.2-q34.3 |
| Ehlers-Danlos syndrome, type IV | 130050 | AD | COL3A1 | Collagen, type III, alpha 1 | 2q31 |
| Ehlers-Danlos syndrome, type VI | 225400 | AR | PLOD1 | Procollagen-lysine 1, 2-oxoglutarate 5-dioxygenase 1 | 1p36.22 |
| Ehlers-Danlos syndrome, type VIIC | 225410 | AR | ADAMATS2 | ADAM metallopeptidase with thrombospondin type 1 motif, 2 | 5q35.3 |
| Emery-Dreifuss muscular dystrophy, autosomal recessive (EDMD3) | 604929 | AR | LMNA | Lamin A/C | 1q21.2 |
| Emery-Dreifuss muscular dystrophy, X-linked (EDMD) | 310300 | XL | EMD | Emerin | Xq28 |
| Epidermolysis bullosa dystrophica, Pasini type | 131750 | AR | COL7A1 | Collagen alpha 1(VII) chain [Precursor] | 3 p21.3 |

(continued)

**Table 3.1** (continued)

| Disease | MIM number | Inheritance | Gene name/ symbol | Protein name | Location |
|---|---|---|---|---|---|
| Epidermolysis bullosa letalis | 226650 | AR | LAMB3 | Laminin, beta 3 | 1q32 |
| Epidermolysis bullosa simplex with pyloric atresia | 612138 | AR | PLEC1 | Plectin 1, intermediate filament binding protein 500kDa | 8q24 |
| Epidermolysis bullosa, junctional, Herlitz type | 226700 | AR | LAMA3 | Laminin, alpha 3 | 18q11.2 |
| Epileptic encephalopathy, early infantile, 2 | 300672 | XL | CDKL5 | Cyclin-dependent kinase-like 5 | Xp22 |
| Epiphyseal dysplasia, multiple, 1 (EDM1) | 132400 | AD | COMP | Cartilage oligomeric matrix protein [Precursor] | 19p13.1 |
| Exostoses, multiple, type I | 133700 | AD | EXT1 | Exostosin-1 | 8q24.11-q24.13 |
| Exostoses, multiple, type II | 133701 | AD | EXT2 | Exostosin 2 | 11p12-p11 |
| Fabry disease | 301500 | XL | GLA | Alpha-galactosidase A [Precursor] | Xq22 |
| Facioscapulohumeral muscular dystrophy 1a (FSHMD1a) | 158900 | AD | FRG1 | FRG1 protein | 4 q35 |
| Familial Mediterranean fever gene (MEFV) | 608107 | AR | MEFV | Mediterranean fever protein | 16p13 |
| Fanconi anemia, complementation group C (FANCC) | 227645 | AR | FANCC | Fanconi anemia group C protein | 9q22.3 |
| Fanconi anemia, complementation group D2 (FANCD2) | 227650 | AR | FANCD2 | Fanconi anemia, complementation group D2 | 3p26 |
| Fanconi anemia, complementation group E (FANCE) | 600901 | AR | FANCE | Fanconi anemia, complementation group E | 6p22-p21 |
| Fanconi anemia, complementation group F (FANCF) | 603467 | AR | FANCF | Fanconi anemia group F protein | 11p15 |
| Fanconi anemia, complementation group G | 602956 | AR | FANCG | DNA-repair protein XRCC9 | 9p13 |
| Fanconi anemia, complementation group I (FANCI) | 609053 | AR | FANCI | Fanconi anemia, complementation group I | 15q26.1 |
| Fanconi anemia, complementation group J | 609054 | AR | BRIP1 | Fanconi anemia group J protein | 17q22 |
| Fancony anemia, complementation group A; FANCA | 227650 | AR | FANCA | Fanconi anemia group A protein | 16q24.3 |
| Fragile site mental retardation 1 | 309550 | XL | FMR1 | Fragile X mental retardation 1 protein | Xq27.3 |
| Fragile site, folic acid type, rare, FRA(X)(q28) (FRAXE) | 309548 | XL | FMR2 | Fragile X mental retardation 2 protein | Xq28 |
| Fraser syndrome | 219000 | AR | FRAS1 | Fraser syndrome 1 protein | 4q21.21 |
| Friedreich ataxia 1 (FRDA) | 229300 | AR | FRDA | Frataxin, mitochondrial precursor | 9q13 |
| Galactosemia | 230400 | AR | GALT | Galactose-1-phosphate Uridylyltransferase | 9p13 |
| Gangliosidosis, generalized GM1, type I | 230500 | AR | GLB1 | Galactosidase, beta 1 | 3p21.33 |

**Table 3.1** (continued)

| Disease | MIM number | Inheritance | Gene name/ symbol | Protein name | Location |
|---|---|---|---|---|---|
| Gastric cancer, hereditary diffuse (HDGC) | 137215 | AD | CDH1 | Cadherin 1, type 1, E-cadherin | 16q22.1 |
| Gaucher disease, type I | 230800 | AR | GBA | Glucosylceramidase [Precursor] | 1q21 |
| Geroderma osteodysplasticum (GO) | 231070 | AR | SCYL1BP1 | SCY1-like 1 binding protein 1 | 1q24.2 |
| Gerstmann-Straussler disease (GSD) | 137440 | AD | PRNP | Prion protein | 20p13 |
| Glaucoma 3, primary congenital, A (GLC3A) | 231300 | AR | CYP1B1 | Cytochrome P450, family 1, subfamily B, polypeptide 1 | 2p21 |
| Glucose transport defect, blood–brain barrier | 606777 | AD | SLC2A1 | Solute carrier family 2 (facilitated glucose transporter), member 1 | 1p35-p31.3 |
| Glucose-6-phosphate dehydrogenase (G6PD) | 305900 | XL | G6PD | Glucose-6-phosphate dehydrogenase | Xq28 |
| Glutaric acidemia I | 231670 | AR | GCDH | Glutaryl-coenzyme A dehydrogenase | 19p13.2 |
| Glycogen storage disease I | 232200 | AR | G6PC | Glucose-6-phosphatase, catalytic subunit | 17q21 |
| Glycogen storage disease II | 232300 | AR | GAA | Glucosidase, alpha; acid | 17q25.2-q25.3 |
| Glycogen storage disease type VI | 232700 | AR | PYGL | Glycogen phosphorylase, liver form | 14q21-q22 |
| Granulomatous disease, chronic | 233710 | AR | NCF2 | Neutrophil cytosolic factor 2 | 1q25 |
| Granulomatous disease, chronic, X-linked (CGD) | 306400 | XL | CYBB | Cytochrome b-245, beta polypeptide | Xp21.1 |
| Griscelli syndrome with hemophagocytic syndrome, type 2 (GS2) | 607624 | AR | RAB27A | RAB27A, member RAS oncogene family | 15q15-q21.1 |
| Hemochromatosis (HFE) | 235200 | AR | HFE | Hemochromatosis | 6p21.3 |
| Hemoglobin–alpha locus 1 (HBA1) | 141800 | AR | HBA1 | Hemoglobin alpha chain | 16pter-p13.3 |
| Hemoglobin–alpha locus 2 (HBA2) | 141850 | AR | HBA2 | Hemoglobin alpha subunit | 16pter-p13.3 |
| Hemoglobin–beta locus (HBB) | 141900 | AR | HBB | Hemoglobin beta chain | 11p15.5 |
| Hemophagocytic lymphohistiocytosis, familial, 2 | 603553 | AR | PRF1 | Perforin 1 [Precursor] | 10q22 |
| Hemophagocytic lymphohistiocytosis, familial, 3 (FHL3) | 608898 | AR | UNC13D | unc-13 homolog D | 17q25.1 |
| Hemophagocytic lymphohistiocytosis, familial, 4 (FHL4) | 603552 | AR | STX11 | Syntaxin 11 | 6q24.2 |
| Hemophilia A | 306700 | XL | F8 | Coagulation factor VIII [Precursor] | Xq28 |
| Hemophilia B | 306900 | XL | F9 | Coagulation factor IX [Precursor] | Xq27.1-q27.2 |
| Hereditary motor and sensory neuropathy VI | 601152 | AD | MFN2 | Mitofusin 2 | 1p36.22 |

(continued)

**Table 3.1** (continued)

| Disease | MIM number | Inheritance | Gene name/ symbol | Protein name | Location |
|---|---|---|---|---|---|
| HLA matching genotyping | | | | | 6 q21.3 |
| Holt-Oram syndrome (HOS) | 142900 | AD | TBX5 | T-box 5 | 12q24.1 |
| Homocystinuria due to deficiency of N(5,10)-methylenetetrahydrofolate reductase activity | 236250 | AR | MTHFR | Methylenetetrahydrofolate reductase | 1p36.3 |
| Hoyeraal-Hreidarsson syndrome (HHS) | 300240 | XL | DKC1 | H/ACA ribonucleoprotein complex subunit 4 | Xq28 |
| Huntington disease (HD) | 143100 | AD | HTT | Huntingtin | 4 p16.3 |
| Hurler syndrome | 607014 | AR | IDUA | Alpha-L-iduronidase [Precursor] | 4 p16.3 |
| Hyalinosis, infantile systemic | 236490 | AR | ANTXR2 | Anthrax toxin receptor 2 | 4q21 |
| Hydrocephalus, X-linked (L1CAM) | 308840 | XL | L1CAM | Neural cell adhesion molecule L1 [Precursor] | Xq28 |
| Hyperglycinemia, nonketotic (NKH) | 605899 | AR | AMT | Aminomethyltransferase | 3p21.2-p21.1 |
| Hyperglycinemia, nonketotic (NKH) | 605899 | AR | GLDC | Glycine dehydrogenase | 9p22 |
| Hyper-IgE recurrent infection syndrome | 147060 | AD | STAT3 | Signal transducer and activator of transcription 3 (acute-phase response factor) | 17q21.31 |
| Hyperinsulinemic hypoglycemia, familial, 1 (HHF1) | 256450 | AR | ABCC8 | ATP-binding cassette, sub-family C (CFTR/MRP), member 8 | 11p15.1 |
| Hypomagnesemia, renal, with ocular involvement | 248190 | AR | CLDN16 | Claudin 16 | 3q28 |
| Hypophosphatasia, infantile | 241500 | AR | ALPL | Alkaline phosphatase, tissue-nonspecific isozyme [Precursor] | 1p36.1-34 |
| Hypophosphatemic rickets, X-linked dominant | 307800 | XL | PHEX | Phosphate regulating endopeptidase homolog | Xp22.2-p22.1 |
| Ichthyosis follicularis, atrichia, and photophobia syndrome | 308205 | XL | MBTPS2 | Membrane-bound transcription factor peptidase, site 2 | Xp22.13 |
| Ichthyosis, lamellar, 1 (LI1) | 190195 | AD | TGM1 | Transglutaminase 1 (K polypeptide epidermal type I, protein-glutamine gamma-glutamyltransferase) | 14q11.2 |
| Ichthyosis, lamellar, 2 (LI2) | 601277 | AR | ABCA12 | ATP-binding cassette, sub-family A (ABC1), member 12 | 2q34 |
| Immunodeficiency due to defect in CD3-zeta | 610163 | AR | CD247 | CD247 molecule | 1q22-q23 |
| Immunodeficiency with hyper-IgM, type 1 (HIGM1) | 308230 | XL | CD40LG | Tumor necrosis factor ligand superfamily member 5 | Xq26 |
| Immunodysregulation, polyendocrinopathy, and enteropathy, X-linked (IPEX) | 304790 | XL | FOXP3 | Forkhead box P3 | Xp11.23-q13.3 |
| Incontinentia pigmenti (IP) | 308300 | XL | IKBKG | NF-kappa-B essential modulator | Xq28 |

**Table 3.1** (continued)

| Disease | MIM number | Inheritance | Gene name/ symbol | Protein name | Location |
|---|---|---|---|---|---|
| Isovaleric acidemia (IVA) | 243500 | AR | IVD | Isovaleryl coenzyme A dehydrogenase | 15q14-q15 |
| Joubert syndrome 3 (JBTS3) | 608629 | AR | AHI1 | Abelson helper integration site 1 | 6q23.3 |
| Joubert syndrome 6 (JBTS6) | 610688 | AR | TMEM67 | Transmembrane protein 67 | 8q22.1 |
| Juvenile myelomonocytic leukemia (JMML) | 607785 | AD | NRAS | Neuroblastoma RAS viral (v-ras) oncogene homolog | 1p13.2 |
| Kallmann syndrome 2 | 147950 | AD | FGFR1 | Fibroblast growth factor receptor 1 | 8p11.2-p11.1 |
| Krabbe disease | 245200 | AR | GALC | Galactocerebrosidase [Precursor] | 14q31 |
| Leber congenital amaurosis 2 (LCA2) | 204100 | AR | RPE65 | Retinal pigment epithelium-specific protein 65kDa | 1p31 |
| Leber congenital amaurosis 6 | 605446 | AR | RPGRIP1 | Retinitis pigmentosa GTPase regulator interacting protein 1 | 14q11 |
| Leigh syndrome (LS) | 185620 | AR | SURF1 | Surfeit locus protein 1 | 9q34.2 |
| Leri-Weill dyschondrosteosis (LWD) | 127300 | XL | SHOX | Short stature homeobox | Xp22.33;Yp11.3 |
| Leukoencephalopathy with vanishing white matter (VWM) | 603896 | AR | EIF2B2 | Translation initiation factor eIF-2B beta subunit | 14q24 |
| Leukoencephalopathy with vanishing white matter (VWM) | 603896 | AR | EIF2B4 | Eukaryotic translation initiation factor 2B, subunit 4 delta, 67kDa | 2p23.3 |
| Leukoencephalopathy with vanishing white matter (VWM) | 603896 | AR | EIF2B5 | Eukaryotic translation initiation factor 2B, subunit 5 epsilon, 82kDa | 3q27.1 |
| Li-Fraumeni syndrome 1 (LFS1) | 151623 | AD | TP53 | Cellular tumor antigen p53 | 17p13.1 |
| Lipoid congenital adrenal hyperplasia | 201710 | AR | STAR | Steroidogenic acute regulatory protein | 8p11.2 |
| Loeys-Dietz syndrome (LDS) | 609192 | AD | TGFBR2 | Transforming growth factor, beta receptor II (70/80kDa) | 3p22 |
| Long-chain 3-hydroxyacyl-CoA dehydrogenase deficiency (HADHA) | 600890 | AR | HADHA | Trifunctional enzyme alpha subunit, mitochondrial [Precursor] | 2p 3 |
| Lymphedema-distichiasis syndrome | 153400 | AD | FOXC2 | Forkhead box C2 | 16q24.1 |
| Machado-Joseph disease (MJD) | 109150 | AD | ATX3 | Machado-Joseph disease protein 1 | 14q24.3-q31 |
| Marfan syndrome (MFS) | 154700 | AD | FBN1 | Fibrillin 1 [Precursor] | 15q21.1 |
| Meckel syndrome, type 4 (MKS4) | 611134 | AR | CEP290 | Centrosomal protein 290kDa | 12q21.32 |
| Meckel syndrome, type 6 (MKS6) | 612284 | AR | CC2D2A | coiled-coil and C2 domain containing 2A | 4p15.32 |
| Metachromatic leukodystrophy | 250100 | AR | ARSA | Arylsulfatase A [Precursor] | 22q13.31-qter |
| Metaphyseal chondrodysplasia, Schmid type (MCDS) | 156500 | AD | COL10A1 | Collagen, type X, alpha 1 | 6q21-q22 |

(continued)

**Table 3.1** (continued)

| Disease | MIM number | Inheritance | Gene name/ symbol | Protein name | Location |
|---|---|---|---|---|---|
| Methylmalonic aciduria | 251000 | AR | MUT | Methylmalonyl CoA mutase | 6p12.3 |
| Microcephaly 3, primary, autosomal recessive (MCPH3) | 604804 | AR | CDK5RAP2 | CDK5 regulatory subunit associated protein 2 | 9q33.2 |
| Microcoria-congenital nephrosis syndrome | 609049 | AR | LAMB2 | Laminin beta-2 | 3p21 |
| Microphthalmia, isolated 2 (MCOP2) | 610093 | AR | VSX2 | Visual system homeobox 2 | 14q24.3 |
| Microtubule-associated protein tau (MAPT) | 157140 | AD | MAPT | Microtubule-associated protein tau | 17q21.1 |
| Migraine, familial hemiplegic, 1 (FHM1) | 141500 | AD | CACNA1A | Calcium channel, voltage-dependent, P/Q type, alpha 1A subunit | 19p13.2-p13.1 |
| Morquio syndrome, nonkeratosulfate-excreting type | 252300 | AR | GALNS | Galactosamine (N-acetyl)-6-sulfate sulfatase | 16q24.3 |
| Mosaic variegated aneuploidy syndrome 1 (MVA1) | 257300 | AR | BUB1B | Budding uninhibited by benzimidazoles 1 homolog beta | 15q15 |
| Mucopolysaccharidosis type II (Hunter) Hunter-McAlpine craniosynostosis syndrome | 309900 | AD | IDS | Iduronate 2-sulfatase [Precursor] | Xq28 |
| Mucopolysaccharidosis type IIIa | 252900 | AR | SGSH | N-sulfoglucosamine sulfohydrolase | 17q25.3 |
| Mucopolysaccharidosis type VI | 253200 | AR | ARSB | arylsulfatase B | 5q11-q13 |
| Multiple acyl-CoA dehydrogenase deficiency (MADD) | 231680 | AR | ETFA | Electron-transfer-flavoprotein, alpha polypeptide | 15q23-q25 |
| Multiple endocrine neoplasia, type I (MEN1) | 131100 | AD | MEN1 | Multiple endocrine neoplasia I | 11q13.1 |
| Multiple endocrine neoplasia, type IIA (MEN2A) | 171400 | AD | RET | Ret proto-oncogene | 10q11.2 |
| Muscular dystrophy, becker type (BMD) | 300376 | XL | DMD | Dystrophin | Xq21.2 |
| Muscular dystrophy, congenital merosin-deficient, 1A (MDC1A) | 607855 | AR | LAMA2 | Laminin, alpha 2 | 6q22-q23 |
| Muscular dystrophy, Duchenne type (DMD) | 310200 | XL | DMD | Dystrophin | Xq21.2 |
| Myoclonic epilepsy of Lafora | 254780 | AR | NHLRC1 | E3 ubiquitin-protein ligase | 6p22.3 |
| Myopathy, myofibrillar, desmin-related | 601419 | AD | DES | Desmin | 2q35 |
| Myotonia congenita, autosomal dominant | 160800 | AD | CLCN1 | Chloride channel 1, skeletal muscle | 7q32-qter\|7q35 |
| Myotonic dystrophy 2 (DM2) | 602668 | AD | CNBP | CCHC-type zinc finger, nucleic acid binding protein | 3q21 |

**Table 3.1** (continued)

| Disease | MIM number | Inheritance | Gene name/ symbol | Protein name | Location |
|---|---|---|---|---|---|
| Myotubular myopathy 1 (MTM1) | 310400 | XL | MTM1 | Myotubularin | Xq28 |
| N-acetylglutamate synthase deficiency | 237310 | AR | NAGS | N-acetylglutamate synthase | 17q21.31 |
| Nail-patella syndrome (NPS) | 161200 | AD | LMX1B | LIM homeobox transcription factor 1, beta | 9q34 |
| Nephrogenic syndrome of inappropriate antidiuresis | 300539 | XL | AVPR2 | Arginine vasopressin receptor 2 | Xq28 |
| Nephrosis 1, congenital, Finnish type (NPHS1) | 256300 | AR | NPHS1 | Nephrosis 1, congenital, Finnish type (nephrin) | 19q13.1 |
| Neuraminidase deficiency | 256550 | AR | NEU1 | Sialidase 1 (lysosomal sialidase) | 6p21.3 |
| Neurofibromatosis, type I (NF1) | 162200 | AD | NF1 | Neurofibromin | 17q11.2 |
| Neurofibromatosis, type II (NF2) | 101000 | AD | NF2 | Merlin | 22q12.2 |
| Neuropathy, hereditary sensory and autonomic, type I (HSAN1) | 162400 | AD | SPTLC1 | Serine palmitoyltransferase, long chain base subunit 1 | 9q22.1-q22.3 |
| Neuropathy, hereditary sensory and autonomic, type III (HSAN3) | 223900 | AR | IKBKAP | Kinase complex-associated protein | 9q31 |
| Niemann-Pick disease, type A | 257200 | AR | SMPD1 | Sphingomyelin phosphodiesterase 1, acid lysosomal | 11p15.4-p15.1 |
| Niemann-Pick disease, type C1 (NPC1) | 257220 | AR | NPC1 | Niemann-Pick C1 protein | 18q11-q12 |
| Noonan syndrome 1 (NS1) | 163950 | AD | PTPN11 | Protein tyrosine phosphatase, non-receptor type 11 | 12q24 |
| Noonan syndrome 3 (NS3) | 609942 | AD | KRAS | v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog | 12p12.1 |
| Noonan syndrome 4 (NS4) | 610733 | AD | SOS1 | Son of sevenless homolog 1 | 2p22-p21 |
| Norrie disease (NDP) | 310600 | XL | NDP | Norrin | Xp11.4-p11.3 |
| Oculocutaneous albinism, type I (OCA1) | 203100 | AR | TYR | Tyrosinase [Precursor] | 11q14-q21 |
| Oculocutaneous albinism, type II (OCA2) | 203200 | AD | OCA2 | P protein | 15q11.2-q12 |
| Omenn syndrome | 603554 | AD | RAG1 | V(D)J recombination-activating protein 1 | 11p13 |
| Optic atrophy 1 (OPA1) | 165500 | AD | OPA1 | Dynamin-like 120 kDa protein, mitochondrial [Precursor] | 3 q28-q29 |
| Ornithine transcarbamylase deficiency | 311250 | XL | OTC | Ornithine carbamoyltransferase, mitochondrial [Precursor] | Xp21.1 |
| Osteogenesis imperfecta congenita (OIC) | 166200 | AD | COL1A1 | Collagen alpha 1(I) chain [Precursor] | 17q21.31-q22 |
| Osteogenesis imperfecta congenita (OIC) | 166200 | AD | COL1A2 | Collagen alpha 2(I) chain [Precursor] | 7q22.1 |

(continued)

**Table 3.1** (continued)

| Disease | MIM number | Inheritance | Gene name/ symbol | Protein name | Location |
|---|---|---|---|---|---|
| Osteogenesis imperfecta, type IX | 259440 | AR | PPIB | Peptidylprolyl isomerase B (cyclophilin B) | 15q21-q22 |
| Osteopetrosis, autosomal recessive | 259700 | AR | TCIRG1 | Vacuolar proton translocating ATPase 116 kDa subunit a isoform 3 | 11q13.4-q13.5. |
| Pachygyria with mental retardation, seizures | 600176 | AR | WDR62 | WD repeat domain 62 | 19q13.12 |
| Pancreatitis, hereditary (PCTT) | 167800 | AD | PRSS1 | Protease, serine, 1 (trypsin 1) | 7q32-qter\|7q34 |
| Pelizaeus-Merzbacher-like disease (PMLD) | 311601 | XL | PLP1 | Myelin proteolipid protein | Xq22 |
| Periodic fever, familial, autosomal dominant | 142680 | AD | TNFRSF1A | Tumor necrosis factor receptor superfamily | 12p13.2 |
| Peutz-Jeghers syndrome (PJS) | 175200 | AD | STK11 | Serine/threonine kinase 11 | 19p13.3 |
| Pfeiffer syndrome | 101600 | AD | FGFR1 | Fibroblast growth factor receptor 1 | 8p11.2-p11.1 |
| Phenylketonuria | 261600 | AR | PAH | Phenylalanine-4-hydroxylase | 12q22-q24.2 |
| Polycystic kidney disease 1 (PKD1) | 601313 | AD | PKD1 | Polycystin 1 precursor | 16P13.3 |
| Polycystic kidney disease 2 (PKD2) | 173910 | AD | PKD2 | Polycystin 2 | 4 q22.1 |
| Polycystic kidney disease, autosomal recessive (ARPKD) | 263200 | AR | PKHD1 | Polycystic kidney and hepatic disease 1 [Precursor] | 6 p12.3 |
| Popliteal pterygium syndrome (PPS) | 119500 | AD | IRF6 | Interferon regulatory factor 6 | 1q32-q41 |
| Porphyria, congenital erythropoietic | 263700 | AR | UROS | Uroporphyrinogen III synthase | 10q26.2 |
| Propionic acidemia | 232000 | AR | PCCA | Propionyl coenzyme A carboxylase, alpha polypeptide | 13q32 |
| Propionic acidemia | 606054 | AR | PCCB | Propionyl coenzyme A carboxylase, beta polypeptide | 3q21-q22 |
| Prosaposin deficiency (PSAPD) | 611721 | AR | PSAP | Prosaposin | 10q21-q22 |
| Pseudohypoparathyroidism, type IA (PHP1A) | 103580 | AD | GNAS | Guanine nucleotide binding protein (G protein) | 20q13.3 |
| Pseudohypoparathyroidism, type IB (PHP1B) | 603233 | AD | GNAS | GNAS complex locus | 20q13.3 |
| Pseudovaginal perineoscrotal hypospadias | 264600 | AR | SRD5A2 | Steroid-5-alpha-reductase, alpha polypeptide 2 | 2p23.1 |
| Pyridoxamine 5-prime-phosphate oxidase deficiency | 610090 | AR | PNPO | Pyridoxamine 5′-phosphate oxidase | 17q21.32 |
| Pyruvate kinase deficiency of red cells | 266200 | AD | PKLR | Pyruvate kinase, liver and RBC | 1q21 |
| Restrictive dermopathy, lethal | 275210 | AR | ZMPSTE24 | Zinc metallopeptidase | 1p34 |

**Table 3.1** (continued)

| Disease | MIM number | Inheritance | Gene name/ symbol | Protein name | Location |
|---|---|---|---|---|---|
| Retinitis pigmentosa 4 (RP4); | 180380 | AD | RHO | Rhodopsin | 3q21-q24 |
| Retinitis pigmentosa 3 (RP3) | 300389 | XL | RPGR | Retinitis pigmentosa GTPase regulator | Xp21.1 |
| Retinoblastoma (RB1) | 180200 | AD | RB1 | Retinoblastoma-associated protein | 13q14.1-q14.2 |
| Retinoschisis 1, X-linked, juvenile (RS1) | 312700 | XL | RS1 | Retinoschisin 1 | Xp22.13 |
| Rett syndrome (RTT) | 312750 | XL | MECP2 | Methyl-CpG-binding protein 2 | Xq28 |
| Rhesus blood group, CcEe antigens (RHCE) | 111700 | AD | RHCE | CcEe antigens | 1p36.2-p34 |
| Rhesus blood group, D antigen (RHD) | 111680 | AD | RHD | D antigen | 1p36.11 |
| Saethre-Chotzen syndrome (SCS) | 101400 | AD | TWIST1 | Twist homolog 1 (Drosophila) | 7p21.2 |
| Sandhoff disease | 268800 | AR | HEXB | Beta-hexosaminidase beta chain [Precursor] | 5q13 |
| Severe combined immunodeficiency | 601457 | AR | RAG2 | Recombination activating gene 2 | 11p13 |
| Severe combined immunodeficiency, X-linked (SCIDX1) | 300400 | XL | IL2RG | Interleukin 2 receptor, gamma | Xq13.1 |
| Shwachman-Diamond syndrome (SDS) | 260400 | AR | SBDS | Shwachman-Bodian-Diamond syndrome protein | 7q11.21 |
| Sickle cell anemia | 603903 | AR | HBB | Hemoglobin beta chain | 11p15.5 |
| Smith-Lemli-Opitz syndrome (SLOS) | 270400 | AR | DHCR7 | 7-dehydrocholesterol reductase | 11q12-q13 |
| Sonic hedgehog (SHH) | 600725 | AD | SHH | Sonic hedgehog protein [Precursor] | 7q36 |
| Sotos syndrome | 117550 | AD | NSD1 | Nuclear receptor binding SET domain protein 1 | 5q35.2-q35.3 |
| Spastic paraplegia 3, autosomal dominant | 182600 | AD | ATL1 | Atlastin GTPase 1 | 14q22.1 |
| Spastic paraplegia 4, autosomal dominant (SPG4) | 182601 | AD | SPAST | Spastin | 2p24-p21 |
| Spinal muscular atrophy, distal, autosomal recessive | 604320 | AR | IGHMBP2 | Immunoglobulin mu binding protein 2 | 11q13.3 |
| Spinal muscular atrophy, type I (SMA1) | 253300 | AD | SMN1 | Survival motor neuron protein | 5q12.2-q13.3 |
| Spinocerebellar ataxia 1 (SCA1) | 164400 | AD | ATXN1 | Ataxin 1 | 6p23 |
| Spinocerebellar ataxia 2 (SCA2) | 183090 | AD | ATX2 | SCA2 protein | 12q24 |
| Spinocerebellar ataxia 6 (SCA6) | 183086 | AD | CACNA1A | Voltage-dependent P/Q-type calcium channel alpha-1A subunit | 19p13 |
| Spinocerebellar ataxia 7 (SCA7) | 164500 | AD | SCA7 | Ataxin-7 | 3 p21.1-p12 |
| Spinocerebellar ataxia, autosomal recessive 1 | 606002 | AR | SETX | Senataxin | 9q34.13 |

(continued)

**Table 3.1** (continued)

| Disease | MIM number | Inheritance | Gene name/ symbol | Protein name | Location |
|---|---|---|---|---|---|
| Stickler syndrome, type I (STL1) | 108300 | AD | COL2A1 | Collagen, type II, alpha 1 | 12q13.11-q13.2 |
| Stickler syndrome, type II (STL2) | 604841 | AD | COL11A1 | Collagen, type XI, alpha 1 | 1p21 |
| Succinic semialdehyde dehydrogenase deficiency | 271980 | AR | ALDH5A1 | Succinate semialdehyde dehydrogenase, mitochondrial [Precursor] | 6 p22 |
| Surfactant metabolism dysfunction, pulmonary, 3 (SMDP3) | 610921 | AR | ABCA3 | ATP-binding cassette, sub-family A (ABC1), member 3 | 16p13.3 |
| Symphalangism, proximal (SYM1) | 185800 | AD | NOG | Noggin [Precursor] | 17q22 |
| Tay-Sachs disease (TSD) | 272800 | AR | HEXA | Beta-hexosaminidase alpha chain [Precursor] | 15q23-q24 |
| Thrombasthenia of Glanzmann and Naegeli | 273800 | AR | ITGA2B | Integrin, alpha 2b | 17q21.32 |
| Thrombotic thrombocytopenic purpura, congenital (TTP) | 274150 | AR | ADAMTS13 | ADAM metallopeptidase with thrombospondin type 1 motif, 13 | 9q34 |
| Torsion dystonia 1, autosomal dominant (DYT1) | 128100 | AD | DYT1 | Torsin A [Precursor] | 9q34 |
| Treacher Collins-Franceschetti syndrome (TCOF) | 154500 | AD | TCOF1 | Treacle protein | 5q32-q33.1 |
| Tuberous sclerosis type 1 | 191100 | AD | TSC1 | Hamartin | 9q34 |
| Tuberous sclerosis type 2 | 191100 | AD | TSC2 | Tuberin | 16p13.3 |
| Tyrosinemia, type I | 276700 | AR | FAH | Fumarylacetoacetate hydrolase (fumarylacetoacetase) | 15q23-q25 |
| Ulnar-mammary syndrome (UMS) | 181450 | AD | TBX3 | T-box 3 | 12q24.1 |
| Von Hippel-Lindau syndrome (VHL) | 193300 | AD | VHL | Von Hippel-Lindau disease tumor suppressor | 3 p26-p25 |
| Waardenburg syndrome, type 2A (WS2A) | 193510 | AD | MITF | Microphthalmia-associated transcription factor | 3p14.2-p14.1 |
| Wiskott-Aldrich syndrome (WAS) | 301000 | XL | WAS | Wiskott-Aldrich syndrome protein | Xp11.23-p11.22 |
| Wolfram syndrome 1 (WFS1) | 222300 | AR | WFS1 | Wolframin | 4p16 |
| Wolman disease | 278000 | AR | LIPA | Lipase A, lysosomal acid, cholesterol esterase | 10q23.2-q23.3 |
| Zellweger syndrome (ZS) | 214100 | AR | PEX1 | Peroxisome biogenesis factor 1 | 7q21-q22 |
| Zellweger syndrome (ZS) | 214100 | AR | PEX3 | Peroxisomal biogenesis factor 3 | 6q24.2 |
| Zellweger syndrome (ZS) | 214100 | AR | PXMP3 | Peroxisomal membrane protein 3, 35kDa | 8q21.1 |

**Table 3.2** Clinical outcome of 2,158 PGD cycles for Mendelian disorders

| Cells tested | Patient | Cycle | Embryo transfers | Number of embryos transferred | Pregnancy | Birth |
|---|---|---|---|---|---|---|
| PB | 131 | 237 | 188 | 379 | 72 (38.3%) | 64 |
| PB + BL | 422 | 701 | 602 | 1,199 | 255 (41.4%) | 278 |
| *Subtotal* | *553* | *938* | *790* | *1,578* | *327* | *342* |
| *BL + BC* | *653* | *1,220* | *988* | *1,859* | *406* | *389* |
| *Total* | *1,206* | *2,158* | *1,778* | *3,437* | *733 (41.2%)* | *731* |

*PB* polar body, BL blastomere, *BC* blastocyst

**Table 3.3** Clinical outcome of PGD for Mendelian disorders performed by PB approach

| | Cycles | ET | # Embryos transferred | Pregnancy | Birth |
|---|---|---|---|---|---|
| *Autosomal-recessive* | | | | | |
| Polar bodies | 115 | 94 | 199 | 33 | 32 |
| Polar bodies + blastomere/blastocyst | 389 | 334 | 683 | 135 | 155 |
| Subtotal | 504 | 428 | 882 | 168 | 187 |
| *Autosomal-dominant* | | | | | |
| Polar bodies + | 46 | 37 | 78 | 20 | 17 |
| blastomere/blastocyst | 118 | 105 | 207 | 45 | 47 |
| Subtotal | 164 | 142 | 285 | 65 | 64 |
| *X-linked* | | | | | |
| Polar bodies + | 76 | 57 | 102 | 19 | 15 |
| blastomere/blastocyst | 194 | 163 | 309 | 75 | 76 |
| Subtotal | 270 | 220 | 411 | 94 | 91 |
| Total | 938 | 790 | 1,578 (1.99) | 327 (41.4%) | 342 |

of 9,036 oocytes, of which 7,653 (97.6%) were with both PB1 and PB2 available for analysis, with the results of sequential PB1 and PB2 testing obtained in 7,841 (97.6%) of these oocytes. This made it possible to preselect for transfer as many as 1,578 embryos originating from these oocytes (1.99 per transfer on an average) in 790 (84.2%) cycles, resulting in 327 pregnancies (41.4%) and the birth of 342 healthy children.

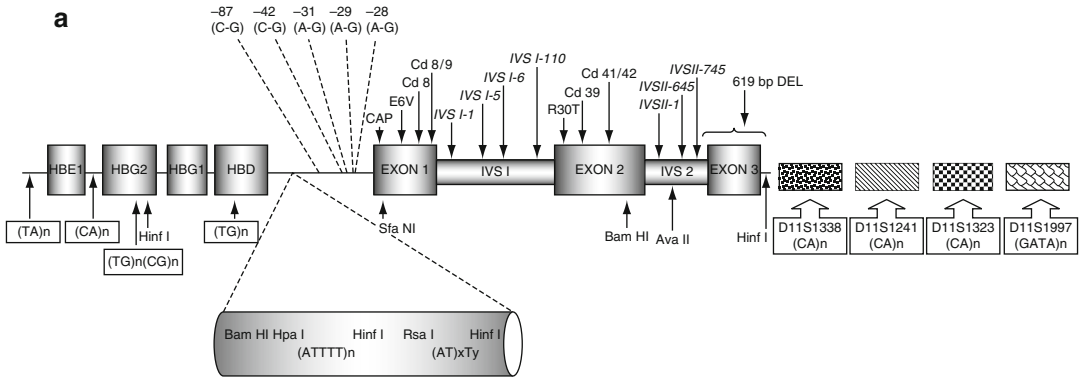
The remaining 1,220 PGD cycles were performed by blastomere or blastocyst biopsy, resulting in the transfer of 1,829 unaffected embryos in 988 cycles, yielding 406 clinical pregnancies and the birth of 389 healthy children. Overall, 2,158 cycles were performed for 1,206 patients at risk of producing offspring with single-gene disorders, which resulted in preselection and transfer of 3,437 unaffected embryos in 1,778 cycles (approximately two embryos per cycle), yielding 731 unaffected pregnancies (41.2% pregnancy rate per transfer) and the birth of 731 apparently healthy children.

## 3.1 Autosomal-Recessive Diseases

In our experience, more than half of the PGD cycles were performed for autosomal-recessive conditions, with 504 of them using the PB approach (Tables 3.2 and 3.3). The most common indications for PGD were *CFTR and hemoglobin disorders* (HBB), performed for an increasing number of mutations presented in Figs. 3.1 and 3.2.

### 3.1.1 Hemoglobinopathies

Testing for hemoglobin disorders currently represents the world's largest experience in PGD. For example, in some communities, such as in Cyprus, Greece, and Turkey, PGD is becoming a routine procedure for couples carrying thalassemia mutations who cannot accept prenatal diagnosis and termination of pregnancy [9–11]. Introduced for the first time in 1996 in Cyprus, only 40 PGD cycles for hemoglobin disorders

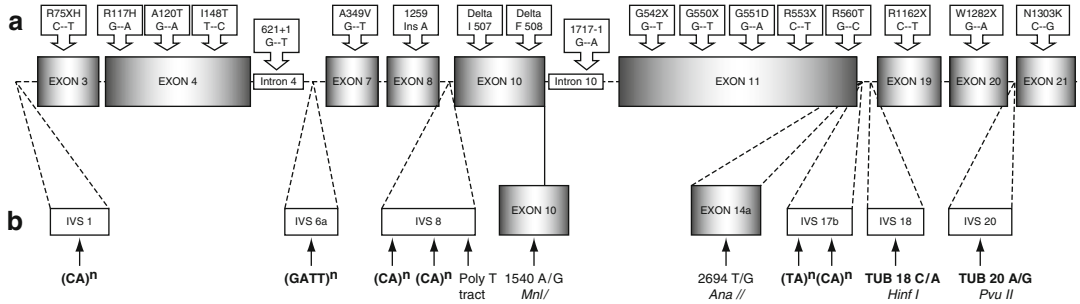



**b Polymorphic markers**

**List of mutations:**

| RNA Processing mutations: | Transcriptional mutations: | Nonfunctional mRNA: |
|---|---|---|
| IVSI–1 (G–A) | –87 (C–G) | Codon 8 (–AA) |
| IVSI–1 (G–T) | –42 (C–G) | Codon 39 (C–T) |
| IVSI–5 (G–C) | –31 (A–G) | Codon 41/42 (–CTTT) |
| IVSI–5 (G–T) | –29 (A–G) | |
| IVSI–5 (G–A) | –28 (A–G) | |
| IVSI–6 (T–C) | E 6V (Sickle cell anemia) | |
| IVSI–110 (G–A) | R30T | |
| IVS2–1 (G–A) | Deletion: | Cap site: |
| IVS2–654 (C–T) | 619 bp | +1 (A–C) |
| IVS2–745 (C–G) | | +2 (T–C) |

**Fig. 3.1** Mutations in beta-globin gene for which PGD was performed and polymorphic markers used in multiplex PCR analysis. Map of human beta-globin gene, showing sites and location of mutations (**a**), and linked polymorphic markers used for avoiding misdiagnosis (**b**). List of mutations is also presented in the *lower panel*




**a (Upper level). Mutations in CFTR gene**
**b (Bottom level). Polymorphic markers in CFTR gene**

**Fig. 3.2** Mutations in CFTR gene, for which PGD was performed and polymorphic markers used in multiplex PCR analysis. Map of CFTR gene, showing sites and location of mutations (**a**), and polymorphic markers used for avoiding misdiagnosis (**b**)

were performed before the year 2000, with hundreds of cycles performed since then. At the present time, the proportion of PGD cases for hemoglobin disorders in our overall PGD experience of over 2,158 PGD cycles for single-gene disorders is as high as one-quarter.

To improve accuracy of diagnosis of PGD for hemoglobin disorders a set of polymorphic markers, listed in Fig. 3.1, were used, which makes it realistic to select at least three closely linked informative markers in any case performed to analyze simultaneously with mutation testing.

**Table 3.4** Clinical outcome of PGD for hemoglobinopathies

| Mutation | Number of patients | Number of cycles | Number of transfers | Number of embryos transferred | Pregnancy | Births |
|---|---|---|---|---|---|---|
| IVS-I-1 (*G*>*A*) | 9 | 18 | 13 | 20 | 2 | 3 |
| IVS-I-5 (*G*>*C*) | 10 | 16 | 11 | 17 | 3 | 2 |
| IVS-I-6 (*T*>*C*) | 21 | 39 | 35 | 71 | 17 | 19 |
| IVS-I-110 (G>A) | 61 | 100 | 88 | 202 | 26 | 24 |
| IVS-II-1 (*G*>*A*) | 4 | 9 | 5 | 8 | 1 | 2 |
| IVS-II-745 (C>G) | 9 | 23 | 20 | 42 | 3 | 2 |
| Codon 6 (−A) | 1 | 1 | 1 | 3 | 0 | 0 |
| Codon 5 (−*CT*) | 3 | 3 | 2 | 3 | 1 | 1 |
| Codon 8 (−AA) | 12 | 20 | 17 | 26 | 3 | 4 |
| Codon 39 (C>T) | 10 | 16 | 14 | 26 | 6 | 7 |
| *HB O-Arab* [β121(GH4)Glu→Lys] | 1 | 1 | 1 | 2 | 0 | 0 |
| Codons 41/42 (−TCTT) | 7 | 1 | 4 | 10 | 1 | 1 |
| −29 (A>G) | 1 | 1 | 1 | 1 | 0 | 0 |
| −87 (C>G) | 1 | 2 | 2 | 2 | 0 | 0 |
| Cap +1(*A*>*C*) | 2 | 3 | 2 | 2 | 0 | 0 |
| *HB Monroe* [β30(B12)Arg→Thr] | 1 | 12 | 9 | 13 | 0 | 0 |
| HBB −619 bp deletion | 6 | 13 | 11 | 19 | 2 | 3 |
| *Sicilian delta-beta 0 Thal*-deletion of 13378 nts from the delta gene to beta gene | 1 | 4 | 4 | 9 | 1 | 2 |
| *Filipino beta 0* −45 kb deletion | 1 | 1 | 0 | 0 | 0 | 0 |
| Hb S [β6(A3)Glu→Val] | 59 | 90 | 80 | 169 | 32 | 32 |
| Hb E [β26(B8)Glu→Lys] | 1 | 2 | 1 | 1 | 0 | 0 |
| Hb C [β6(A3)Glu→Lys] | 1 | 1 | 1 | 2 | 1 | 1 |
| α-Thal (45 kb deletion) | 4 | 9 | 9 | 17 | 2 | 2 |
| Total | 226 | 395 | 331 | 665 (2%) | 102 (31%) | 105 |

A total of 395 PGD cycles were performed for 226 couples at risk of bearing children with hemoglobin (Hb) disorders. This included nine PGD cycles for alpha-thalassemia (α-thal), 296 for β-thal, and 90 for sickle cell disease. A total of 144 of these cycles were performed in combination with HLA typing to select unaffected embryos as potential donors for stem cell transplantation (see Chap. 4).

Of 395 clinical cycles performed, unaffected embryos for transfer were available in 331 (83.81%), resulting in 102 (30.8%) clinical pregnancies and the birth of 105 healthy children. Because the majority of cases as mentioned were done for Eastern Mediterranean patients, approximately half of the cases were performed for IVS I-110 mutation, which is the most common thalassemia mutation in the Mediterranean region (Table 3.4).

While PGD cycles were mainly performed for heterozygous carriers, eight cycles were done for couples with homozygous or compound heterozygous male or female patients at 50% risk of bearing an affected offspring. In these couples, PGD involved testing for either three different mutations or, in the majority of cases, for two different mutations or the same maternal and paternal mutation. Analysis in these cases was done either simultaneously or in sequence by testing the maternal mutation in PB1 and PB2 and the paternal one in blastomeres.

While beta-globin gene mutations were tested in the majority of cases, α-thal mutations involving a 45 kb deletion were tested in nine cycles performed for four couples, resulting in preselection and transfer of 17 unaffected embryos in nine cycles, yielding two clinical pregnancies and the birth of two unaffected children,

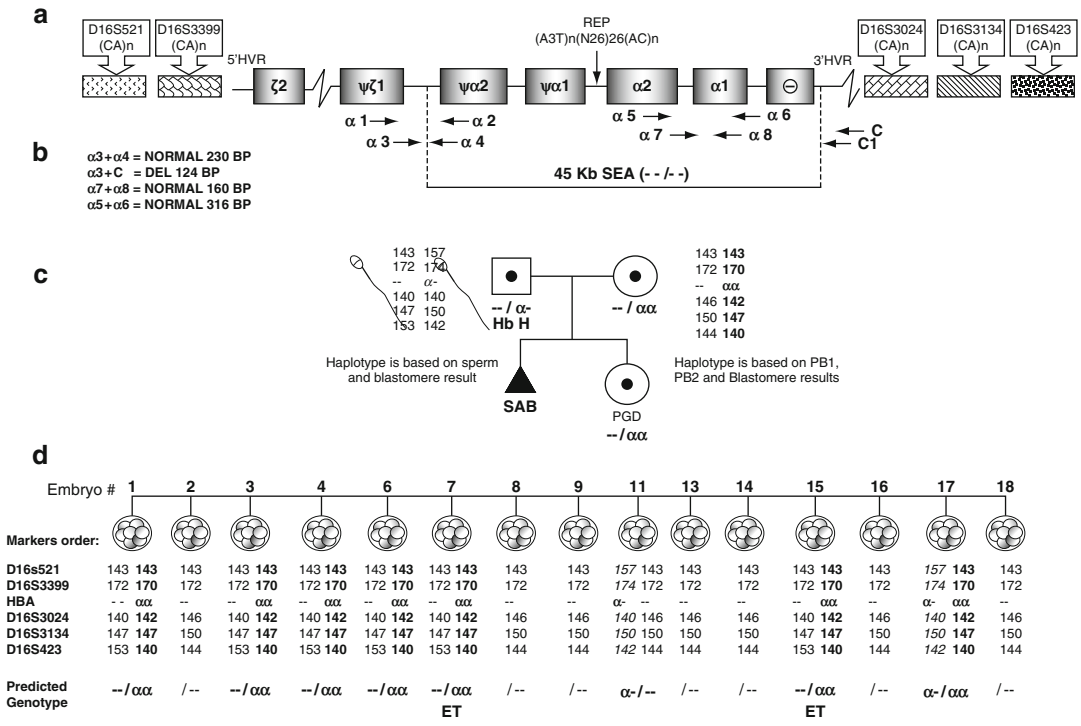



**Fig. 3.3** PGD for alpha thalassemia. (**a**) Map of human alpha-globin gene, showing the position of 45 Kb SEA deletion and polymorphic markers used in multiplex PCR analysis; (**b**) size of fragments; (**c**) family pedigree showing both parents carrying SEA deletion, the father also having HbH disease; parental haplotypes are also shown, with paternal haplotypes obtained from sperm testing, and maternal haplotypes obtained from PB1 and PB2 analysis; (**d**) results of testing of 15 embryos showing the presence of 6 heterozygous embryos for deletion, of which 2 were transferred back to the patients

confirmed to be free from hydrops fetalis. To avoid misdiagnosis, the haplotype analysis for five polymorphic markers was performed, with confirmatory testing on the nontransferred embryos showing a correct diagnosis.

The example of PGD for α-thalassemia is presented in Fig. 3.3. As seen from this figure, both parents are carriers of this mutation, with the father having also hemoglobin H disease. The couple had one previous pregnancy resulting in spontaneous abortion, caused by hydrops fetalis. To avoid misdiagnosis the haplotype analysis with at least five polymorphic markers involved was performed. Of 15 embryos tested, 8 were affected, with the remaining 7 carrying one copy of the deleted α-globin gene, of which 2 (embryos #7 and #15) were transferred, with the mutant embryos confirmed to be affected, showing the reliability of the approach.

### 3.1.2 Cystic Fibrosis (CFTR)

*CFTR* has been the major indication from the very beginning of the application of PGD [12–15], and this is currently a routine procedure, which has been done in our experience of approximately 400 cases, involving testing for more than two dozens of different mutations in the CFTR gene (Fig. 3.2). Testing for CFTR mutation is usually performed simultaneously with at least three strongly linked polymorphic markers, which may realistically be selected from a set of 11 available markers listed in Fig. 3.2. In our experience of PGD for CFTR, unaffected embryo transfer was possible in almost 90% of initiated cycles, resulting in 44% clinical pregnancies and the birth unaffected children.

Because of the high prevalence of CFTR mutations, there might be a need to test

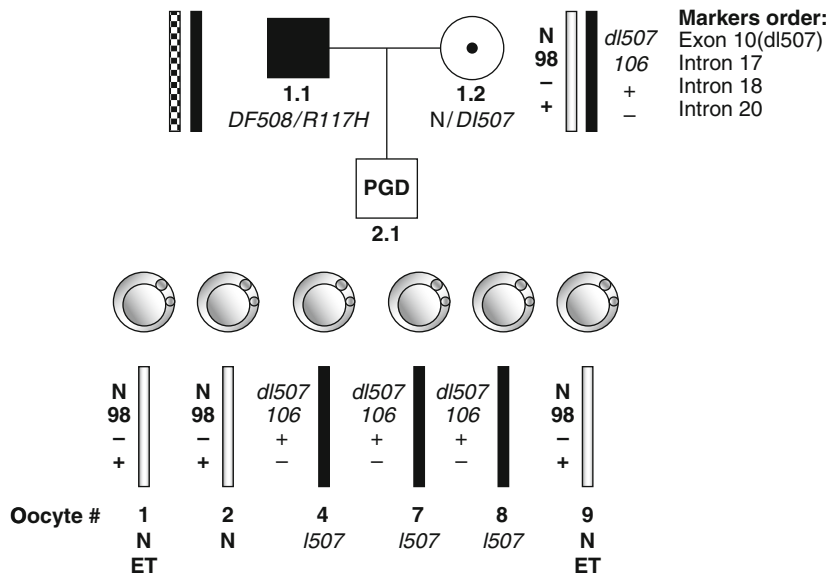



**Fig. 3.4** PGD for a couple with three different mutations in CFTR gene. (*Top*) The mother (1.2) is a carrier of the delta I507 mutation in CFTR gene. The father (1.1) is affected with *CF* and had delta F508 and R117H mutations in CFTR gene. (*Bottom*) PGD was performed by sequential PB1 and PB2 analysis. The mother is informative for three inside CFTR gene polymorphic markers (introns 17, 18, and 20). Multiplex heminested PCR of PB1 and PB2 revealed three normal (#1, #2, and #9) and three affected oocytes (#4, #7 and #8). Embryos #1 and #9 were transferred and a healthy boy was born

simultaneously for two or three CFTR mutations in the same reaction, as presented in Fig. 3.4. As can be seen from the pedigree, PGD for this couple seems to be the only choice, as the father is a double heterozygote for DF508 and R117H, and the mother is a carrier of the D1507 mutation in the CFTR gene. To avoid testing for three different mutations in blastomeres, taking into consideration approximately 20% risk for ADO for each of the three alleles, which may lead to a potential misdiagnosis, PB1 and PB2 testing was performed to limit the testing to the preselection of the mutation-free oocytes. As can be seen from Fig. 3.4, testing for DI507 maternal mutation, simultaneously with four closely linked markers, allowed the identification of three mutation-free oocytes from the six oocytes available for testing. Two of these embryos resulting from oocytes #1 and #9 with acceptable development potential were transferred, yielding a singleton pregnancy and the birth of a healthy boy, confirmed to be an unaffected carrier of the paternal mutation.

### 3.1.3 Familial Disautonomia (FD)

Although other autosomal-recessive disorders are much rarer (listed in Table 3.1), overall, they have become the established indicators for PGD in genetic practices. The practical implications of PGD for these rare recessive disorders may be demonstrated by the examples of PGD for familial dysautonomia (FD) and spinal muscular atrophy (SMA), presented below.

FD is an autosomal-recessive disorder, associated with the mutation affecting the donor splice site of intron 20 of the IKBKAP gene, assigned to chromosome 9q31 [16, 17]. It is the most common congenital sensory neuropathy present in 1/3,600 live births in Ashkenazi Jews. FD is present at birth with characteristic features, including the absence of fungiform papillae on the tongue, absence of flare after injection of intradermal histamine, decreased or absent deep-tendon reflexes, and absence of overflow of emotional tears. This is a devastating and debilitating disorder characterized by the poor development and progressive

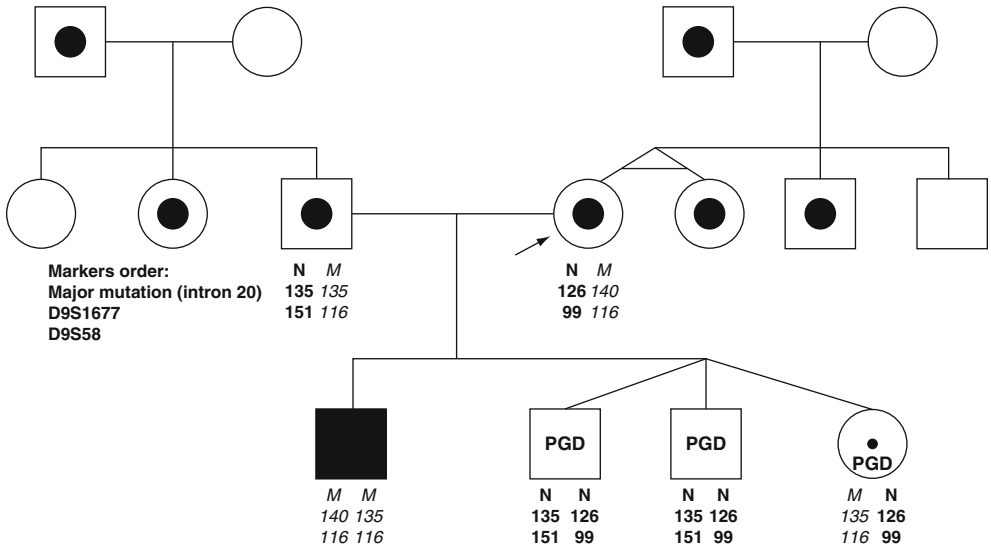



**Fig. 3.5** Family Pedigree of the couple undergoing PGD for familial dysautonomia (*FD*). The father is a carrier of mutation of the donor splice site of intron 20 of the IKBKAP gene, which is linked to 135 bp repeat of D9S1677, and 116 bp repeat of D9S58, while the normal allele is linked to 135 and 151 bp repeats of the same polymorphic markers respectively. The mother is also a carrier of the same mutation, linked to 140 and 116 bp repeats, the normal gene being linked to 126 and 99 bp repeats respectively. As seen from this panel, the paternal and maternal sisters and maternal brother are also carriers of the mutation, which was inherited from the paternal father and maternal mother, respectively. Reproductive outcomes of this couple, including one previous affected child with FD and unaffected triplets born following PGD, are shown at the *bottom*

degeneration of the sensory and autonomous nervous system, gastrointestinal and respiratory dysfunctions, vomiting crisis, excessive sweating, and postural hypotension. Despite a remarkable variability of the disease phenotype within and between families, expected to derive from different mutations producing inactivation of the gene, a single major mutation has been described [18]. It is also of interest that a single T→C change at the base pair 6 of the splice donor site, which is probably responsible for 99% of cases of FD, was shown to result in the skipping of exon 20 (74 bp) from the IKBKAP mRNA only in the brain tissue, with varying level of the gene expression in other tissues. This may explain the severe progressive degeneration of the sensory and autonomous nervous system, leading to continued neuronal depletion with age and early death. The product of the IKBKAP gene is a part of a multiprotein complex, hypothesized to play a role in general transcriptional regulation, so the complete inactivation of the gene might cause a lethal phenotype at any stage of embryonic development [18]. The remarkable variability of the disease phenotype may be explained by the presence of a partially functional gene product in some tissues, including the brain, because even a small amount of the encoded protein expressed at critical developmental stages might permit sufficient neuronal survival. In addition, a very rare minor FD missence mutation was described (G→C change at base pair 17 in exon 19 of the gene), which is associated with a mild phenotype in patients with heterozygous status for the major mutation [18].

Despite the above progress in understanding the nature and pathogenesis of the disease, FD is still fatal, with no effective management available at the present time, making PGD a useful option for those at-risk couples that cannot accept prenatal diagnosis and termination of pregnancy as an option for avoiding FD in their offspring. One of such couples presented for PGD with one previous child diagnosed to be affected with FD. Both parents have an Ashkenazi Jew ancestor and, as seen from the pedigree (Fig. 3.5), are carriers of the gene for FD, based on marker analysis, which has been available for all members of the extended family [19].

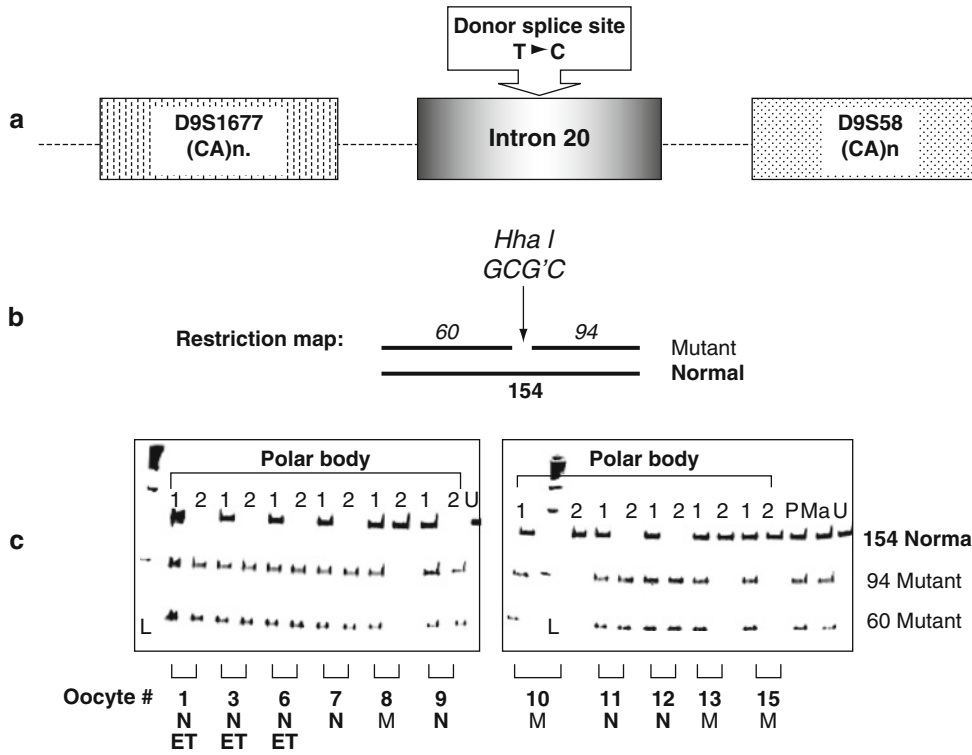



**Fig. 3.6** Preimplantation genetic diagnosis for major mutation in IKBKAP gene, causing FD. (**a**) Position of major splice donor mutation T– C in IKBKAP gene and linked markers. (**b**) Restriction map. Major mutation creates restriction site for *Hha*I enzyme. (**c**) PB analysis of normal and mutant sequences of IKBKAP gene. Of 11 oocytes tested, 7 were mutation-free based on heterozygous PB1 and affected PB2, of which oocytes #1, #3, and #6 were transferred resulting in unaffected triplets. *L* 100 bp ladder, *N* normal, *M* mutant, *ET* embryo transfer, *U* undigested PCR product, *Ma* maternal genotype, *P* paternal genotype

A PGD cycle was performed using a standard IVF protocol coupled with micromanipulation procedures for PB sampling, described above in Chap. 2. PB1 and PB2 were removed following maturation and fertilization of the oocytes, and tested by the multiplex nested PCR analysis, involving mutation testing simultaneously with different linked markers as described above. The mutation analysis involved the detection of T to C change in the donor splice site of intron 20, based on HhaI restriction digestion, which does not cut the normal allele, while creating two fragments of 60 and 94 bp in the mutant allele (Fig. 3.6).

As described in Chap. 2, there may be three genetic possibilities for the PB1 genotype from a heterozygous mother. If no crossover occurs, PB1 will be homozygous (either normal or mutant), but in the event of a crossover, PB1 will be heterozygous. If crossover does not occur and the PB1 is homozygous for the mutant gene, the oocyte must contain two copies of the normal gene and any embryo resulting from this oocyte can be transferred, but this was not the case in any of the oocytes shown in Fig. 3.6. If the PB1 is homozygous for the normal gene, the maternal contribution to the embryo must be the mutant gene, which was also not the case as seen from Fig. 3.6. In both of these occasions the extruded PB2 will have identical genotype to oocyte (opposite to genotype of PB1). In the event of crossover that has been observed in all cases shown in Fig. 3.6, PB1 is heterozygous and the analysis of PB2 is required to predict which maternal allele have been extruded with PB2 and which left in the maternal pronucleus following fertilization. Accordingly, if the normal gene is extruded with PB2 (e.g., PB2 is

hemizygous normal), the resulting maternal contribution to the embryos is the mutant gene, and in reverse if the mutant gene is extruded with PB2 (e.g., PB2 is hemizygous mutant), the resulting maternal contribution to the embryos is the normal gene. It is furthermore possible that even the oocytes predicted as mutant may further form unaffected heterozygous embryos, following fertilization by a mutation-free sperm. Therefore, with insufficient number of mutation-free oocytes, preselected by PB1 and PB2 sequential analysis, further testing of the resulting embryos may allow the identification of heterozygous unaffected carrier embryos for transfer.

The preselection of mutation-free oocytes was performed based on the simultaneous mutation detection and linked marker analysis, involving two strongly linked markers D9S58 and D9S1677, which were shown not to be involved in recombination in the analysis of 435 FD chromosomes [19]. Therefore, prior to PGD, a single sperm testing was performed to identify the paternal haplotypes, which were as follows: the mutant allele was linked to 116 bp, and the normal to 151 bp repeat of the D9S58 marker, while the D9S1677 marker was not informative. The maternal haplotypes were established based on PB analysis as follows: the mutant allele was linked to 116 bp repeat of the D9S58 marker, and 140 bp repeat of the D9S1677 marker, while the normal allele to 140 bp repeat of the D9S58 marker and 126 bp repeat of the D9S1677 marker (Fig. 3.5). Primer sequences and reaction conditions are presented in Table 3.5.

A single PGD cycle was performed, with 15 oocytes available for testing, of which 11 were with the information for both PB1 and PB2. Of these 11 oocytes, 4 were predicted to be mutant based on the heterozygous PB1 and hemizygous normal (mutation-free) PB2 (oocytes #8, #10, #13, and #15), while the remaining 7 oocytes were free of the mutant gene, as evidenced by the heterozygous PB1 and hemizygous mutant PB2. These results were in agreement with both markers, except for oocytes #7 and #11, in which ADO of D9S1677 allele linked to the mutant gene was observed. Three embryos resulting from the above seven oocytes (embryos #1, #3, #6; Fig. 3.6), with the mutation-free status confirmed by both markers, reaching the blastocyst stage, were transferred back to the patient, yielding a triplet pregnancy and the birth of three unaffected children, including two homozygous normal and one heterozygous carrier. Two of the other embryos resulting from normal oocytes did not form blastocysts and the other two were further tested because of ADO of one of the markers (oocytes #7 and #11).

Three of four embryos deriving from the mutant oocytes were shown to be heterozygous carriers of the mutant gene, and one homozygous mutant. One of the embryos deriving from normal oocytes was confirmed to be homozygous for normal gene and the other was a heterozygous carrier. These embryos, as well as other three embryos, which appeared to be heterozygous carriers, were frozen and are available for transfer in the future cycles, should the couple wish to have another unaffected child. Of course the above normal noncarrier embryo could be given preference in transfer, but because a possible detrimental effect of removing blastomeres from cleaving embryos cannot be completely excluded, we gave priority to the three non-biopsied embryos resulting from the mutation-free oocytes. However, when more data are collected on the possible effect of different biopsy procedures on the outcome of pregnancy, and the accuracy of blastomere analysis is further improved, the possibility for parents to choose implanting normal or carrier embryos should be explored.

These results and further similar cases performed by the present time demonstrate a diagnostic accuracy of PGD for FD by sequential PB1and PB2 analysis, as the follow-up analysis of the embryos, resulting from either mutant or normal (mutation-free) oocytes, was in agreement with the sequential PB1 and PB2 analysis in all the embryos tested, which is in accordance with the extensive data on the sequential PB1 and PB2 analysis, described above in Chap. 2. Although, as mentioned, ideally at least three linked markers are considered necessary to completely exclude the risk for misdiagnosis

**Table 3.5** Primers and PCR conditions used in PGD for FD

| Gene/polymorphism | Upper primer | Lower primer | Anneal $T_m$ (°C) |
|---|---|---|---|
| Intron 20 major mutation (T-C) *Hha I restriction* heminested PCR | Outside 5′ GTTGTTCATCATCGAGCCC 3′ | 5′ CTGATTGATGATATAGGTAATGAGG 3″ | 62–55 |
| | Inside 5′ GTTGTTCATCATCGAGCCC 3′ | 5′ GCTTTTCATAATTTTAAGTTCTCG 3′ | 55 |
| D9S1677 | Outside 5′ TGGCTGTTTTGAGAAGT 3′ | 5′ TGGGAGGATGAGTTGAG 3′ | 62–55 |
| | Inside 5′ Hex ATGTAACCTGTCTCCACTG 3′ | 5′ CTGGGCAACATAGCAAG 3′ | 57 |
| D9S58 | Outside 5′ AAGCAATCCTCGCACCTCAG 3′ | 5′ CCAGGAGTTTGAGACCAGCC 3′ | 62–55 |
| | Inside 5′ FamCCTGAGTAGCCGGGACTATA 3′ | 5′ TAGGCAACACATCAAGATCCT 3′ | 57 |

due to ADO, the use of the two linked markers or even one in the present study was reliable, because it was possible to follow both normal and mutant alleles through the first and second meiotic divisions in all but four oocytes, in which either no PB2 was available due to failure of fertilization or PB2 failed to amplify. In other words, the presence of both mutant and normal alleles in PB1 following meiosis I, and the detection of the mutant allele extruded with PB2 following meiosis II, leaves no doubt of the mutation-free status of the maternal pronucleus, even if no linked markers are available for testing. However, the testing of sufficient number of linked markers would be absolutely essential if PB1 appears to be homozygous mutant, to exclude the possible ADO of the normal allele, because the failure of detecting ADO could lead to the opposite interpretation of the results of PB2. With undetected ADO in PB1, the presence of normal allele in PB2 will erroneously suggest the normal (mutation-free) status of the resulting oocyte, which has apparently a mutant status.

The presented experience of PGD for FD demonstrates the clinical relevance of PGD in those couples that cannot accept prenatal diagnosis and termination of pregnancy [20]. Because of the high prevalence of FD in Ashkenazi Jews, with carrier frequency of 1 in 32, this approach may have practical implications, so the at-risk couples require information about the availability of PGD. The presented PGD design for FD may probably be applied without extensive preparatory work in different couples, due to the fact that a single major mutation is involved, although a sufficient number of informative linked markers should be selected, a variety of which are readily available.

As also shown by the above example of FD, it may be predicted that PGD may in future be applied for gene expression abnormalities, which might be limited to a particular tissue or particular stage of embryonic development. This may also allow preselecting the embryos with best potential to establish a viable pregnancy, based on the progress on the understanding of stage-specific gene expression.

### 3.1.4 Spinal Muscular Atrophy (SMA)

PGD for SMA still presents a real complexity [21, 22]. SMA is another relatively rare autosomal-recessive disorder with a newborn prevalence of 1/10,000 and a carrier frequency of 1 in 40–60 individuals. All types of SMA are caused by mutations in the survival motor neuron gene (SMN) locus mapped on chromosome 5q11.2–13 [16]. The SMN gene is present in two highly homologous copies, SMN1 and SMN2, of which homozygous loss of functional survival motor neuron 1 (SMN1) alleles results in SMA, while homozygous absence of SMN2 gives no clinical phenotype. Since 95% of SMA patients lack both copies of SMN1 in exon 7, PGD for SMA is based on the avoidance of SMN1 homozygous deletions, which is complex because of the sequence similarity between the SMN1 and SMN2 genes, requiring simultaneous linkage analysis involving STR markers. To test for paternal SMN1 and SMN2 deletion patterns on each chromosome by blastomere analysis, single sperm testing was required to establish the linkage between normal and deleted alleles with multicopy marker D15S1556 on the promoter region of both gene copies.

The example of PGD for SMA is presented in Fig. 3.7, which shows PGD for SMA performed for a couple with both parents carrying the deletion in the SMN1 gene. Paternal haplotypes were predicted by multiplex heminested PCR analysis in single sperm, and maternal ones by sequential PB1 and PB2 analysis. Of 16 embryos tested for deletion and 4 linked markers (primers are listed in Table 3.6), 2 embryos were mutant, 10 were predicted to be heterozygous for the deletion in SMN1 gene, and 2 with only one copy of the normal gene present. In addition, at least three STRs were amplified on each chromosome for aneupolidy testing of chromosomes 13, 16, 18, 21, and 22, which revealed five chromosomally abnormal embryos, including trisomy 13, monosomy 18, double trisomy 18 and 21, double monosomy 18 and 21, and triple monosomy 13, 16, and 22 (embryo #8, #10, #9, #14, and #13, respectively). Abnormalities predicted by PCR were confirmed

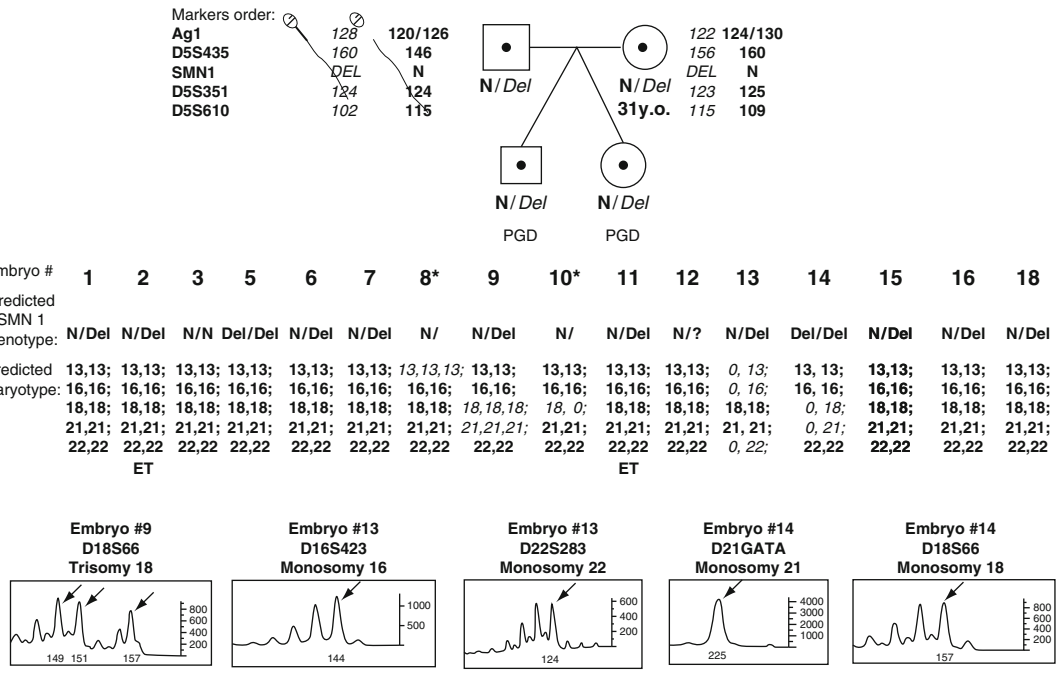



**Fig. 3.7** Combined PGD for spinal muscular atrophy (*SMA*) and aneuploidy. (*Top*) Pedigree of the family undergoing PGD for SMA. Both parents are the carriers of the deletion in SMN1 gene. Paternal haplotype was predicted by multiplex heminested single-sperm PCR analysis. Maternal haplotype was established by sequential PB1 and PB2 analysis. As a result of IVF–PGD cycle healthy twins were born. Positions of polymorphic markers linked to SMN1 gene and applied for improving accuracy of the mutation analysis are shown next to the paternal haplotypes. (*Middle*) Oocytes #3, #8, and #11 were predicted to be normal by polar body (*PB*) analysis. The embryos resulting from these oocytes were subjected to aneuploidy testing using five chromosome-specific probes. Trisomy for chromosome 13 was detected in embryo #8 and monosomy 18 in embryo #10. Blastomeres from the remaining 13 embryos were subjected to multiplex heminested PCR to perform simultaneous mutation, linked polymorphic marker, and aneuploidy analysis for chromosomes 13, 16, 18, 21, and 22. Of these, embryos #5 and #14 were affected, while embryos #1, #2, #6, #7, #9, #11, #13, #15, #16, and #18 were predicted to be heterozygous for the deletion in SMN1 gene. At least three short tandem repeats (STRs) were amplified on each chromosome for aneupolidy testing, which revealed monosomy13, 16, and 22 in embryo #13, monosomy 18 and 21 in embryo #14 and trisomy 18 and 21 in embryo # 9. Chromosomal abnormalities predicted by PCR were confirmed by whole embryo fixation and FISH analysis. Embryos #2 and #18 were transferred and healthy twins were born. All unaffected and chromosomally normal blastocysts were frozen for future cycles. (*Bottom*) Examples of chromosomal abnormalities (monosomy and trisomy) detected by PCR of different STRs. *N* normal allele, *Del* deletion, *ET* embryo transfer, *FISH* fluorescent in situ hybridization

by whole embryo fixation and FISH analysis. Embryos #2 and #11 were transferred, and healthy twins were born. All unaffected and chromosomally normal blastocysts were frozen for future family use.

The data also show the importance of simultaneous testing for aneuploidy, to avoid misdiagnosis and also to avoid the transfer of chromosomally abnormal embryos, destined to be lost in pre- or post-implantation development.

## 3.2 Autosomal-Dominant Disorders

Autosomal-dominant conditions are important candidates for PGD, as couples have a 50% risk of producing an affected child. PGD for autosomal-dominant disorders represents under one-fifth of our experience, which was extremely accurate and effective in detection and transfer of mutation-free embryos (Table 3.3).

**Table 3.6** Primers and PCR conditions used in PGD for SMA

| Gene/polymorphism | Upper primer | Lower primer | Annealing $T_m$ (°C) |
|---|---|---|---|
| SMN (detection of Exon 7 deletion by *Hinf I or Dra I digestion*) | Outside | | 62–45 |
| | 5′ TGCAGCCTAATAATTGTTTTCTTTG 3′ | 5′ CCAACCAGTTAAGTATGAGAATTCTAG 3′ | |
| | Inside for *Hinf I* : | | 55 |
| | 5′ CTTCCTTTTATTTTCCTTACAGGGATT 3′ | 5′ GTAGGGATGTAGATTAACCTTTTATCT 3′ | |
| | Inside for *Dra I* [3]: | Inside for *Dra I* [2]: | 55 |
| | 5′ AAATGTCTTGTGAAACAAAATGC 3′ | 5′ CCTTCCTTCTTTTTGATTTTGTTT 3′ | |
| SMN (detection of Exon 8 deletion by *Dde I digestion*) | Outside | | 62–45 |
| | 5′ GTGGAATGGGTAACTCTTCTTGA 3′ | 5′ TAAACTACAACACCCTTCTCACAG 3′ | |
| | Inside | | 48 |
| | 5′ TAATAACCAAATGCAATGTGAAAT 3′ | 5′ TAAACTACAACACCCTTCTCACAG 3′ | |
| D5S1556 promotor area (heminested) | Outside | | 62–45 |
| | 5′ TCCTTCCTGCCCCCATG 3′ | 5′ TCGGCATGTTGCTTAGGC 3′ | |
| | Inside | | 55 |
| | 5′ FamAAATTCCTAGTAGGAGCTTACATTTAC 3′ | 5′ TCGGCATGTTGCTTAGGC 3′ | |
| D5S435 (heminested) | Outside | | 62–45 |
| | 5′ TTCCTCCATTGACAACTCCTT 3′ | 5′ CCTTAGATAGGGTTGATTTACACA 3′ | |
| | Inside | | 55 |
| | 5′ HexGTCAAGAGCACAGTTTGGAGTG 3′ | 5′ CCTTAGATAGGGTTGATTTACACA 3′ | |
| D5S351 (nested) | Outside | | 62–45 |
| | 5′ GAGTTTGAAGACCAGTCTATGG 3′ | 5′ GAGCATTGCCACTTTAGCC 3′ | |
| | Inside | | 55 |
| | 5′ FamCACAGCGAGACCCCGTC 3′ | 5′ CCCGTGGAAGAAAGGCTAT 3′ | |
| D5S610 (heminested) | Outside | | 62–45 |
| | 5′ TCCAGTGAATTTTCATTTCAGATAC 3′ | 5′ CAAGTGACCCTCCCACCTT 3′ | |
| | Inside | | 55 |
| | 5′ TCCAGTGAATTTTCATTTCAGATAC 3′ | 5′ HexCCAGCCTAAACTGAACTTTCAAAG 3′ | |

Autosomal-dominant conditions for which PGD was performed are presented in Table 3.1, and the examples of two of them, early-onset *primary torsion dystonia* (*PTD*) and *Charcot-Marie-Tooth disease* (*CMT*), are described below.

### 3.2.1 Primary Torsion Dystonia (PTD)

Primary torsion dystonia (PTD) is caused in the majority of cases by a 3 bp deletion of the DYT1gene, located on chromosome 9q34 [16, 17, 23–25]. This is actually the most severe and common form of hereditary movement disorders, present in 1/15,000 live births, characterized by sustained twisting contractures that begin in an arm or leg between 4 and 44 years, spreading to other limbs within about 5 years. Although the phenotypic expression of the disease is similar in all ethnic populations, the highest prevalence was reported among Ashkenazi Jews. Despite a low penetrance (30–40%), the disease phenotype varies greatly between families. In contrast to other neurodegenerative disorders, PTD does not show any distinct neuropathology. A 3 bp deletion in the coding sequence of the DYT1 gene is believed to result in a loss of a pair of glutamic acid residues in a conserved region of an ATP-binding protein torsin A, which has resemblance to the heat-shock proteins, and may lead to imbalance of neuronal transmission in the basal ganglia implicated in dystonia. As low levels of dopaminergic metabolites in the cerebrospinal fluid of these patients show no response to dopa, it is probably caused by a defect in release rather than synthesis of dopamine.

The remarkable phenotypic variability of the disease may be explained by the interaction of the 3 bp deletion with modifying genetic, such as polymorphic, variations in torsin A or mutations in the associated proteins, or with environmental factors, such as trauma, high body temperature, or exposure to toxic agents. Although understanding these relationships may allow elucidating neuronal mechanisms underlying loss of movement control, there is no effective treatment as yet available. This makes PGD a useful option for those at-risk couples that cannot accept prenatal diagnosis and termination of pregnancy as an option for avoiding PTD in their offspring.

Two couple presented for PGD, both with the affected paternal partners carrying the DYT1 3 bp deletion. In one of the couples, the mutation was inherited from the paternal father (Fig. 3.8), whose four sons were affected. In the other, the male partner inherited the mutation from the mother, who did not have any other children (Fig. 3.9).

PGD cycles were performed using blastomere biopsy, and tested by the multiplex nested PCR analysis, involving the DYT1 mutation testing simultaneously with a set of linked polymorphic markers. The mutation analysis involved the detection of GAG deletion in the coding sequence of the DYT1 gene, based on either fragment-size analysis using capillary elecrophoresis or BSeRI restriction digestion, which creates three fragments of 161, 24, and 8 bp in the normal allele (Fig. 3.10), in contrast to only two fragments of 185 and 8 bp in the mutant gene.

Three closely linked markers, D9S62, D9S63, and ASS (intron 14), which were shown not to be involved in recombination with the DYT1 gene [23, 25–27], were used in the multiplex nested PCR system. To identify the paternal haplotypes, a single sperm testing was performed prior to PGD, which showed the linkage of the mutant allele in both couples to 121, 157, and 134 bp; the normal paternal allele in the first couple was linked to128, 140, and 124 bp, and in the second to 123, 155, and 130 bp repeat of D9S62, D9S63, and ASS markers, respectively. The maternal haplotypes were in the first couple 123/123, 149/155, and 128/124 bp, and in the second 123/123, 140/155, and 126/136 bp repeats of D9S62, D9S63, and ASS markers, respectively. Primer sequences and reaction conditions are presented in Table 3.7.

The embryos derived from the oocytes free of DYT1 3 bp deletion, in agreement with the information about the above polymorphic markers, were preselected for transfer back to patients, while those predicted to be mutant or with insufficient marker information were exposed to confirmatory analysis using genomic DNA from these embryos to evaluate the accuracy of single-cell-based PGD.

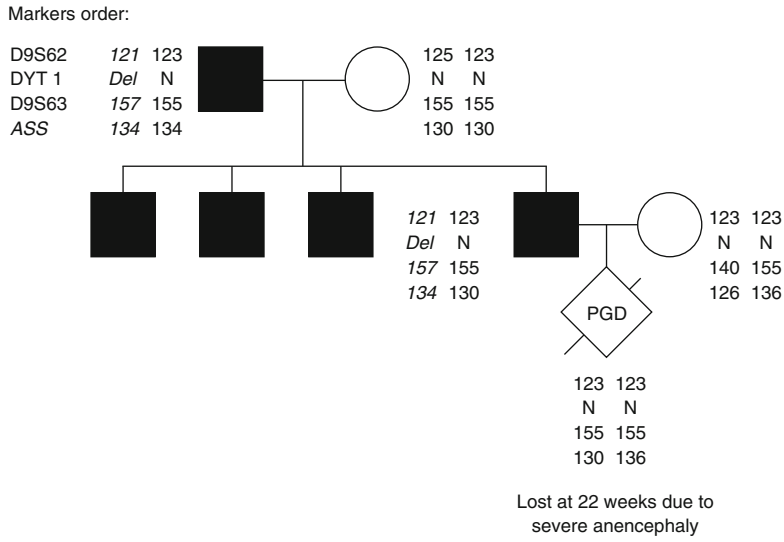



**Fig. 3.8** PGD for a couple at risk for torsion dystonia 1 resulting in miscarriage of an unaffected child with anencephaly. (*Upper panel*) Patient's parents, showing that he inherited 3 bp deletion of DYT1 gene from his father. (*Middle panel*) The father is a carrier of a 3 bp deletion of DYT1 gene, which is linked to 121, 157, and 134 bp repeats of D9S62, D9S63, and ASS markers, respectively, while the normal allele is linked to 123, 155, and 130 pb repeats of the same polymorphic markers, respectively. The mother is normal, with one normal DYT1 allele linked to 123, 140, and 126 and the other to 123, 155, and 136 pb repeats of D9S62, D9S63, and ASS markers, respectively. As seen from this panel, three paternal brothers are also affected obligate carriers of a 3 bp deletion of DYT1 gene, inherited from their father. (*Lower panel*) Reproductive outcomes of this couple, following PGD showing the 3 bp deletion-free fetus, which was terminated due to anencephaly

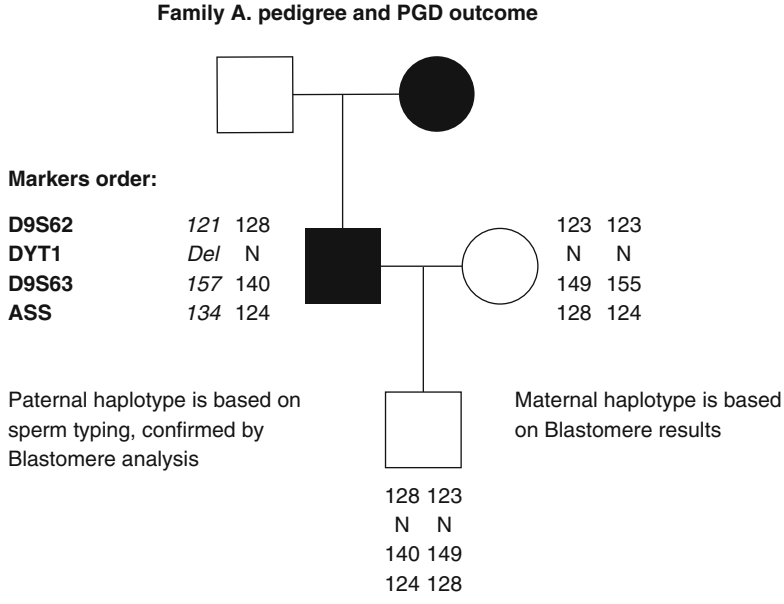



**Fig. 3.9** Pedigree of a couple whose PGD for DYT1 resulted in the birth of a mutation-free baby. (*Upper panel*) Patient's parents, showing that he inherited 3 bp deletion of DYT1 gene from his father. (*Middle panel*) The father is a carrier of a 3 bp deletion of DYT1 gene, which is linked to 121, 157, and 134 bp repeats of D9S62, D9S63, and ASS markers, respectively, while the normal allele is linked to 128, 140, and 124 bp repeats of the same polymorphic markers, respectively. The mother is normal, with one normal DYT1 allele linked to 123, 150, and 128 and the other to 123, 155, and 124 bp repeats of D9S62, D9S63, and ASS markers, respectively. As seen from the upper panel the mutation was inherited from the paternal mother, with no other family members available in the pedigree. (*Lower panel*) Reproductive outcomes following PGD, showing the 3 bp deletion-free baby, which is in agreement with polymorphic markers, also suggesting the presence of both paternal and maternal normal genes. This embryo originates from the transfer of embryo #6, as shown in Fig. 3.10

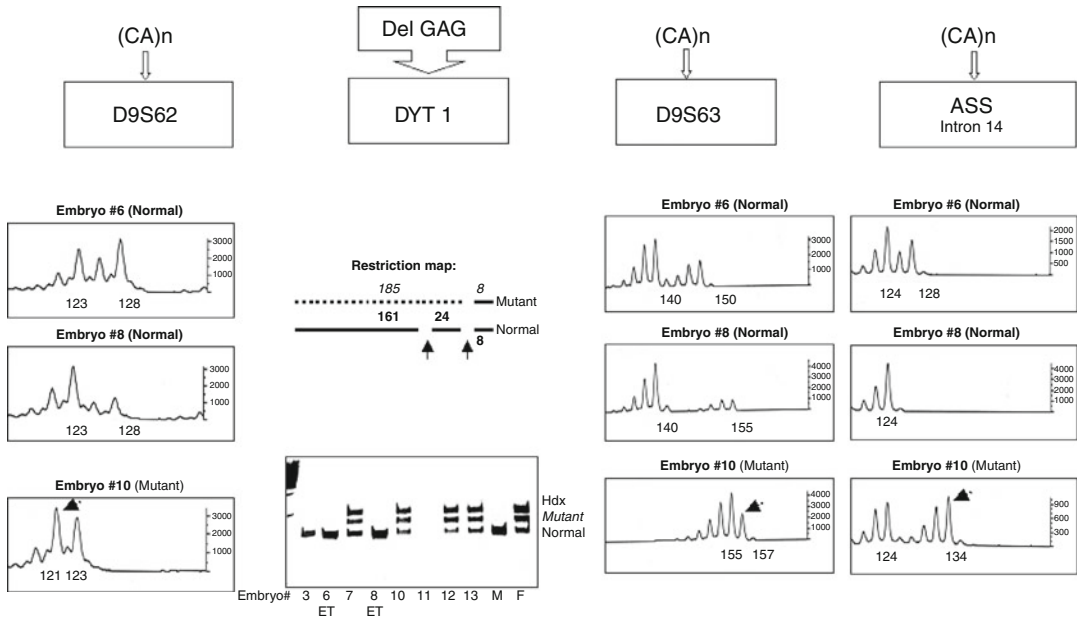



**Fig. 3.10** Preimplantation diagnosis for GAG deletion of DYT1 gene, resulting in the birth of mutation-free baby. Capillary electrophoregrams of fluorescently labeled PCR products of linked markers D9S62 (*first from the left*), D9S63 (*third from the left*), and ASS (*first from the right*), scored by Genotyper TM. The data of genotyping of only three embryos are shown as examples, including two transferred normal (embryos #6 and #8) and one affected (embryo #10) embryo. Paternally derived 128, 140, and 124 dinucleotides indicative of the DYT1 mutation are evident in blastomeres of embryos #6 and #8, together with the presence of maternal normal alleles. (*Second panel from the left*) The location (*top*) of the mutation in DYT1, restriction map for Dse RI digestion (*second panel from the top*), creating three fragments of 161, 24, and 8 bp in the normal allele, in contrast to only two fragments of 185 and 8 bp in the mutant gene. However, because this required a long incubation and high amount of enzymes, fluorescent genotyping was also performed (*bottom of this panel*). (*Middle section of the same panel*) The polyacrylamide gel electrophoregram of Dse RI-digested PCR products of 8 blastomeres from one of the cycles of PGD, paternal DNA from sperm (P), and maternal (normal) DNA. Hdx – the extra fragment in the heterozygous mutant embryos as a result of heteroduplex formation. (*Bottom section of this panel*) Capillary electrophoregrams of fluorescently labeled PCR products of some of the above blastomeres, including two normal (embryos #6 and #8) and one affected (embryo #10). Paternally derived GAG deletion shown by an arrow is evident in embryo #10, which is absent in embryos #6 and #8, in agreement with the linked marker analysis (*see relevant panels on the left and right*). These embryos inherited the paternal normal chromosome, but may be distinguished from each other by the inheritance of different maternal chromosomes, allowing the identification of the origin of the resulting mutation-free baby (see Fig. 3.9)

Three PGD cycles were performed including two for the first and one for the second couple, with a total of 19 embryos available for testing, of which 17 were with sufficient information on the mutation and marker analysis to predict the embryos' genotype. Of these 17 embryos, 9 were predicted to contain a 3 bp deletion, while the remaining 8 were free of the mutant gene, as also confirmed by the polymorphic markers. Six of these embryos, which reached the blastocyst stage, were transferred back to the patients, two in each of the three cycles, yielding a singleton DYT1 mutation-free pregnancy in two of them.

In one couple, only the second cycle resulted in a clinical pregnancy in which eight embryos were available for testing (Fig. 3.10). Although the biopsied blastomere of one of these embryos (embryo #3) was free of the paternal mutant gene, no other paternally derived alleles were present, suggesting that this cell contained only maternal alleles, probably due to monosomy 9. In the other embryo (embryo #11) one of the biopsied blastomeres showed no amplification, so the second

**Table 3.7** Primers for detection of GAG deletion in DYT1 gene and linked polymorphic markers

| Gene/polymorphism | Upper primer | Lower primer | Annealing $T_m$ (°C) |
|---|---|---|---|
| DYT 1 (del GAG) (heminested) | Outside | | 62–55 |
| | 5′ GCACAGCAGCTTAATTGACC 3′ | 5′ CGTAGTAATAATCTAACTTGGTGAA 3′ | |
| | Inside | | 56 |
| | 5′ GCACAGCAGCTTAATTGACC 3′ | 5′ Hex TTTATCTGAGAAAACTCTCTCCTCT 3′ | |
| D9S62 (heminested) | Outside | | 62–55 |
| | 5′ ACCTGTAATCCCAGTTGCT 3′ | 5′ TCACTTCTGACCCTCCTATCT 3′ | |
| | Inside | | 56 |
| | 5′ Hex GGCAACAGGGCAAGACT 3′ | 5′ TCACTTCTGACCCTCCTATCT 3′ | |
| D9S63 (heminested) | Outside | | 62–55 |
| | 5′ ATTCTGTGGGGGAATTATG 3′ | 5′ TTATAATGCCGGTCAACC 3′ | |
| | Inside | | 56 |
| | 5′ Fam CACAAAAGAAAGTCACAATCC 3′ | 5′ TTATAATGCCGGTCAACC 3′ | |
| ASS Intron 14 (CA)n (heminested) | Outside | | 62–55 |
| | 5′ GGGAGCTATAAAAATGACAAT 3′ | 5′ TTAACAGGCTGTCTGGCA 3′ | |
| | Inside | | 56 |
| | 5′ GGGAGCTATAAAAATGACAAT 3′ | 5′ Fam TAGGTCCGAAAACACAAAG 3′ | |

blastomere was removed, but did not show amplification of the DYT1 gene and ASS marker either, together with ADO of the D9S62 paternal allele. Despite this blastomere being informative for the D9S63 marker, which suggested the presence of both paternal and maternal normal alleles, the corresponding embryo was not transferred, to avoid the risk for misdiagnosis, associated with the use of a single linked marker. Four of the remaining six embryos were predicted to be mutant (embryos #7, #10, #12, and #13), evidenced by the presence of the DYT1 3 bp deletion and all three markers linked to the mutant gene (Fig. 3.10). The remaining two embryos (embryos #6 and #8) were free of 3 bp deletion, with all three markers not only excluding a possible ADO of the mutation, but also confirming the presence of both maternal and paternal normal alleles. These two embryos were transferred back to the patient resulting in the birth of a mutation-free boy. As can be seen from the inherited maternal normal chromosome, this baby originates from the transfer of embryo #6 (Figs. 3.9 and 3.10).

In the second patient, only four embryos were available for testing, two of which showed the presence of the DYT1 3 bp deletion confirmed by all three linked polymorphic markers. Of the remaining two embryos with no evidence for the presence of DYT1 3 bp deletion, the marker analysis also showed the presence of both paternal and maternal normal alleles, despite ADO of one of the paternal polymorphic markers in one of these embryos. The transfer of these embryos yielded a singleton pregnancy, which was terminated at 22 weeks of pregnancy due to a severe anencephaly (Fig. 3.8). The results of the mutation and marker analysis in the abortion material showed that the resulting fetus was free of mutation, with all three markers confirming the presence of both paternal and maternal normal alleles.

The presented results represent the first experience of PGD for TDY1, demonstrating the clinical usefulness of PGD in those couples that cannot accept prenatal diagnosis and termination of pregnancy. Because a single unique 3 bp deletion is involved in more than 70% cases of early-onset PTD, the presented PGD design for DYT1 mutation may probably be applied without extensive preparatory work in different couples, taking also into consideration the limited number of founder mutations [25]. As seen from the sperm haplotype analysis of our patients with GAG deletion of the DYT1 gene, the same haplotypes appeared to surround the DYT1 gene. The availability of a sufficient number of highly variable and closely linked markers also allows testing for the mutation simultaneously with at least three markers, to exclude misdiagnosis due to ADO, which may exceed 10% in blastomere analysis [28]. Because PTD is an autosomal-dominant disorder, to ensure a reliable preselection of mutation-free embryos for transfer, PGD should include the detection of both paternal and maternal normal alleles in addition to the exclusion GAG deletion, which may be masked by ADO. This also allows identification of individual embryos that was implanted, as usually two or embryos are transferred. As mentioned, the baby resulting from the transfer of two mutation-free embryos in one of the PGD cycles described has actually originated from the implantation of embryo #6 [29].

Our data also showed that the above microsatellite markers used in this study were also useful in detection of the chromosomal number containing the DYT1 gene in single blastomeres, without which the accuracy of the predicted embryo genotype might not be sufficient. For example, without such information the embryo #3 in one of the couples (Fig. 3.10) could have wrongly been predicted to be normal, which in fact contained no paternal linked markers either. This, therefore, may have suggested the presence of only maternal chromosome 9 due to mosaicism in this embryo, which in fact might have otherwise contained the paternal chromosome 9 with DYT1 mutation, so being affected.

Although prenatal diagnosis for PTD is also available, PGD may seem to be a more attractive option. The fact that approximately 70% of the offspring will not develop the disease in the obligate carriers of the mutation makes the decision of what to do in the case of a mutation carrier is very difficult for the parents.

As for the neonatal outcome, the presented case with anencephaly detected in the second trimester of pregnancy is probably not related to the procedure. As will be described in Chap. 6, the analysis of the outcome of many thousands of PGD cases showed that the prevalence of congenital malformations (5%) was not different from the population prevalence. It is too early to analyze the prevalence of specific types of congenital malformations, but it has been demonstrated that the prevalence of anencephaly and other neural-tube defects may be efficiently prevented by folic acid supplementation before pregnancy (see Chap. 1), so this should be recommended to all patients requesting PGD, as this is one of those rare occasions when the pregnancy is planned well ahead.

### 3.2.2 Charcot-Marie-Tooth Disease (CMT)

Charcot-Marie-Tooth disease (CMT) represents a clinically and genetically heterogeneous group of hereditary peripheral neuropathies, affecting approximately 1 in 2,500 in the United States. Although prenatal diagnosis is available, it may lead to termination of pregnancy, which is not acceptable for many couples. PGD has previously been applied in five couples with CMT1A, representing the most frequent autosomal-dominant type of CMT caused by 1.5 Mb tandem duplication including dosage-sensitive gene for peripheral myelin protein 22 (PMP22) on chromosome 17p11.2–12, presenting complexity of diagnosis requiring the application of multiple polymorphic markers [30]. PCR design for PGD of this condition involved the application of 13 highly polymorphic microsatellite markers, located within the duplicated area and closely linked to the PMP22 gene, some showing three alleles in patients with CMT1A duplication.

We performed PGD for CMT type 1A and 1B, with both paternally and maternally derived mutations. In those with paternally derived duplication, single-sperm analysis was performed to determine normal and mutant haplotypes. In the PGD cycles with maternal mutation, both PB1 and PB2 and blastomeres were analyzed, using markers D17S1357, D17S2229, D17S2226, D17S2225, D17S839, D17S2224, D17S2221, D17S2220, D17S291, D17S2219, D17S2218, D17S2217, and D17S2216, which were amplified in a multiplex heminested PCR system, followed by fragment analysis on an ABI 3100 analyzer. These cycles resulted in the transfer of the embryos free of PMP 22 duplication, yielding unaffected pregnancies and the birth of unaffected children.

In the other PGD cycle performed for maternally derived autosomal-dominant CMT-type 2E mutation in the light polypeptide neurofilament protein gene (NEFL), testing of oocytes by PB1 and PB2 and also blastomeres was performed for the presence of the mutation P8R, simultaneously with microsatellite marker D8S137, which resulted in the transfer of two unaffected embryos and the birth of a child with a normal NEFL gene. PGD was also performed for the X-linked forms of CMT, caused by mutations in connexin-32 gene (Cx32), for which 28 oocytes were tested by sequential PB1 and PB2 for the presence of the V95M mutation in the Cx32 gene, simultaneously with STRs (DXS453, DXS8052, DXS8030, DXS559, DXS441), and resulted in the transfer of four mutation-free embryos failing to yield a clinical pregnancy. The example of PGD for paternally derived CMT1A is presented in Fig. 3.11, showing the outcome of testing of ten embryos by blastomere analysis using ten linked markers (primers and reaction conditions are listed in Table 3.8). Five unaffected embryos were identified, two of which were transferred resulting in the birth of a healthy child, demonstrating the reliability and accuracy of PGD designs applied to PGD for CMT, although misdiagnosis in PGD for CMT was also reported, which was due to errors in linkage analysis in preparation to PGD (see below).

As can be seen in Table 3.3, 164 PGD cycles for dominant disorders were performed by the PB approach although the majority of these cases (118 cycles) still needed a follow-up sequential blastomere or blastocyst biopsy. As the mutation rate for dominant conditions is higher than for recessive and X-linked disorders,

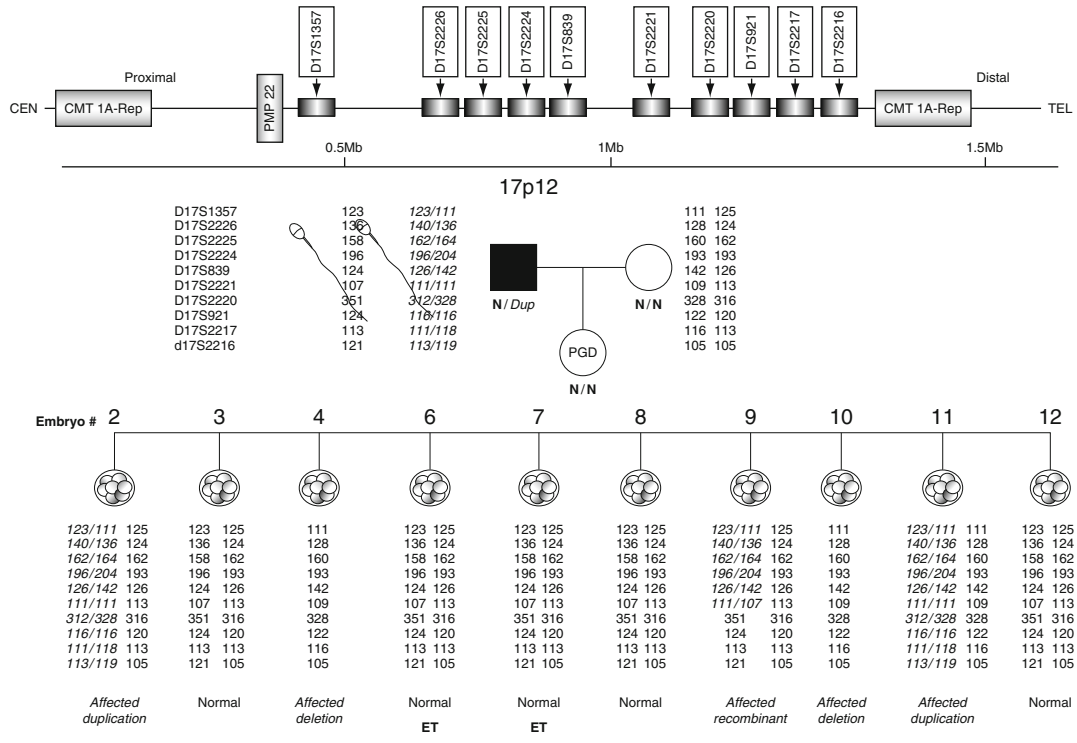



**Fig. 3.11** PGD for CMT1A resulted in the birth of a healthy baby. (*Upper panel*) Schematic representation of 1.5 Mb tandem duplication including dosage-sensitive gene for peripheral myelin protein 22 (PMP22) and linked markers, showing the complexity of the diagnosis. (*Middle panel*) Family pedigree, showing the affected father with duplication and the results of haplotype analysis established through sperm testing in the father and blastomere analysis in the mother. (*Lower panel*) Results of multiplex PCR analysis for ten markers, which identified five duplication-free unaffected embryos and five affected ones, including two with duplication, one recombinant containing partial duplication, and two with deletion. Two of the unaffected embryos were transferred resulting in the birth of an unaffected child

some of the PGD cycles for dominant disorders caused by *de novo* mutations will be presented in Sect. 3.6.

## 3.3 X-Linked Disorders

Almost half of the PGD cycles for single-gene disorders were done for X-linked conditions, the most straightforward indication from the very beginning of the introduction of PGD, either using PCR or FISH technique. Initially this was done by gender determination not only because the sequence information was not always available, but also because it was technically straightforward to identify female embryos by DNA analysis or FISH technique, despite the obvious cost of discarding 50% of healthy male embryos. So the couples at risk may choose either prenatal diagnosis at the cost of the risk for pregnancy termination, or PGD by gender determination at the cost of discarding 50% of male embryos. However, because X-linked disorders are maternally derived the most attractive option may be preselection of mutation-free oocytes through testing for specific causative mutations in PB1 and PB2. This allows avoiding any further testing of the resulting embryos, which may be transferred irrespective of gender or any contribution from the male partner.

PGD for the X-linked disorders was first attempted more than 20 years ago, and has been performed by gender determination [1]. Gender determination was initially done by single-cell

**Table 3.8** Primers and PCR conditions for PGD of CMT1 by linkage analysis

| Gene/polymorphism | Upper primer | Lower primer | Annealing $T_m$ (°C) |
|---|---|---|---|
| D17S2216 (Heminested) | Outside | | 62–45 |
| | 5′ TGGCAAAGAATAAGAAGGAAAGA 3′ | 5′ GTGTCTTGGACTCATTCGGAGACCCTT 3′ | |
| | Inside | | 53 |
| | 5′ TGGCAAAGAATAAGAAGGAAAGA 3′ | 5′ Hex GACAGGCACGGGATTAGGA 3′ | |
| D17S2217 (Heminested) | Outside | | 62–45 |
| | 5′ TCATATGCCCAGTGCCCACTC 3′ | 5′ GTGTCTTACACAGAGTTGATGAAAATCACTAA 3′ | |
| | Inside | | 55 |
| | 5′ TCATATGCCCAGTGCCCACTC 3′ | 5′ FamCTTGTCCTGTTTCTCTCCTGGTTTC 3′ | |
| D17S2218 (Heminested) | Outside | | 62–45 |
| | 5′ TTACAGAAGGACAAGGTAGCAAT 3′ | 5′ CTAAATGCTTGTGGATTAGTTGTA 3′ | |
| | Inside | | 55 |
| | 5′ TTACAGAAGGACAAGGTAGCAAT 3′ | 5′ HexGTTGTATATTGGATTGGACCCTT 3′ | |
| D17S2219 (Heminested) | Outside | | 62–45 |
| | 5′ GTGTCTTTGCTTTGCCAAGATTAC 3′ | 5′ CAACTGTAACACATAAAAGATGAGTTG 3′ | |
| | Inside | | 55 |
| | 5′ GTGTCTTTGCTTTGCCAAGATTAC 3′ | 5′ FamGGGGCTTAGTCTCCATCTGGT 3′ | |
| D17S2220 (Heminested) | Outside | | 62–45 |
| | 5′ GTGTCTTTGGGCAACAGAGCA 3′ | 5′ CTAATAATTTTCCATTTGTAACCTTG 3′ | |
| | Inside | | 48 |
| | 5′ GTGTCTTTGGGCAACAGAGCA 3′ | 5′ FamTTCTCTTGAAACCTCAGTCATCTTT 3′ | |
| D17S2221 (Heminested) | Outside | | 62–45 |
| | 5′ GTGTCTTGAAAATTTCCCACCCAAAG 3′ | 5′ GCCTCTCCCTGAGTGTCTGGT 3′ | |
| | Inside | | 55 |
| | 5′ GTGTCTTGAAAATTTCCCACCCAAAG 3′ | 5′ HexGCTGCTGTTCCTTCAAAATGTG3 ′ | |
| D17S839 (Heminested) | Outside | | 62–45 |
| | 5′ AACAACAGCGAAACTCTGTCTCA 3′ | 5′ CCAAACTTCCAAGTAAGAGAGGG 3′ | |
| | Inside | | 55 |
| | 5′ AACAACAGCGAAACTCTGTCTCA 3′ | 5′ FamAGAGAGACCCTGGAAGATCAACTAC 3′ | |
| D17S2224 (Heminested) | 5′ GTGTCTTGTTCATTCTATCGTCTCAAA 3′ | 5′ AGCAGAAATGAGAGAGGAGACATTA 3′ | 62–45 |
| | 5′ GTGTCTTGTTCATTCTATCGTCTCAAA 3′ | 5′ HexTACAAAGGATCATAAGGCTACCATA 3′ | 55 |
| D17S2225 (Heminested) | 5′ TGGTTAAATCTGTACTCATTATGGC 3′ | 5′ TGTCTTACAGTTGTAGAGGGCTTGTG 3′ | 62–45 |
| | 5′ Hex TTCATGTATCTGGGAGTATTCACG | 5′ TGTCTTACAGTTGTAGAGGGCTTGTG 3′ | 55 |

**Table 3.9** Results and outcomes of PGD for X-linked disorders

| Disease | Number of patients | Number of cycles | Cycle by PB | Cycle by PB &BL | Cycle by BL | Number of ET | Number of embryos | Pregnancy | Birth |
|---|---|---|---|---|---|---|---|---|---|
| DMD | 8 | 14 | 1 | 12 | 1 | 13 | 36 | 5 | 6 |
| CMT 1X | 2 | 2 | 1 | 1 | 0 | 2 | 4 | 0 | 0 |
| FRA X | 29 | 43 | 16 | 24 | 3 | 37 | 86 | 16 | 15 |
| Hemophilia A & B | 4 | 5 | 0 | 5 | 0 | 5 | 11 | 2 | 2 |
| Hyper IgM syndrome | 1 | 2 | 0 | 2 | 0 | 2 | 2 | 1 | 1 |
| Wiscott-Aldrich | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| Hydrocephalus | 3 | 8 | 2 | 6 | 0 | 8 | 18 | 0 | 0 |
| Hunter syndrome | 1 | 2 | 1 | 1 | 0 | 2 | 3 | 1 | 2 |
| Choroideremia | 1 | 1 | 1 | 0 | 0 | 1 | 2 | 1 | 1 |
| MTM | 1 | 2 | 2 | 0 | 0 | 1 | 3 | 0 | 0 |
| Norrie disease | 2 | 4 | 0 | 4 | 0 | 4 | 7 | 0 | 0 |
| X-ALD | 2 | 3 | 0 | 3 | 0 | 3 | 7 | 1 | 1 |
| OTC | 4 | 5 | 3 | 1 | 1 | 5 | 8 | 4 | 4 |
| Pelizaeus-Merzbacher disease | 2 | 2 | 0 | 2 | 0 | 2 | 5 | 1 | 1 |
| Incontinentia Pigmenti | 3 | 4 | 0 | 4 | 0 | 4 | 8 | 1 | 1 |
| Alport | 1 | 1 | 1 | 0 | 0 | 1 | 2 | 0 | 0 |
| *Total* | *65* | *99* | *28* | *66* | *5* | *90* | *202* | *33* | *34* |

PCR analysis, while then it was performed by the FISH technique in interphase cells. Although sufficiently accurate, as mentioned, this approach leads to a discard of 50% of unaffected male embryos, which cannot be accepted by many patients. Thus, there has been a need for specific genetic tests allowing the identification of the unaffected healthy male embryos for transfer as an option to preselect the X-linked mutation-free embryos, irrespective of gender.

In an attempt to preselect the X-linked mutation free embryos for transfer, a sequential PB1 and PB2 analysis was applied as an alternative to gender determination. One of such cases, involving PGD for ornithine transcarbamylase deficiency (OTC), was first reported more than 10 years ago, showing the feasibility of the approach [31].

The couples for whom PGD was applied now include those with previous male offspring affected by Duchenne muscular dystrophy (DMD), Charcot-Marie-Tooth disease X1 (CMTX1), fragile-X syndrome (FMR1), hemophilia A (F8) and B (F9), hyperimmunoglobulin syndrome (HIGM1), Wiscott-Aldrich syndrome (WAS), X-linked hydrocephalus (L1CAM), Hunter syndrome (IDS), choroideremia (CHM), myotubular myotonic dystrophy (MTMD), Norrie disease (NDP), X-linked adrenoleukodystrophy (ALD), ornithine transcarbamylase deficiency (OTC), Pelizaeus-Merzbacher disease (PMLD), incontinentia pigmenti (IP), and Alport disease (ATS). Our preferred approach for these conditions was testing PB1 and PB2, performed in 270 PGD cycles, which resulted in the transfer of 411 embryos originating from mutation-free oocytes, yielding 94 clinical pregnancies and the birth of 91 unaffected children (Table 3.3). The details of the first series of 100 cycles are presented in Table 3.9, showing that the application of all available methods allowed preselection of the embryos for transfer in over 90% of cycles, resulting in 37% pregnancy rate per transfer and the birth of 34 healthy children.

As described in Chap. 2, PB1s were removed at least 3 h after oocyte retrieval (38 h after chorionic gonadothropin (hCG) administration), using micromanipulation procedures described above in Chap. 2. Approximately a dozen oocytes, on an average, were fertilized by intracytoplasmic sperm insertion (ICSI), followed by PB2 removal, approximately 16–18 h after ICSI. Although PB1 and PB2 were removed in

sequence, they were amplified at the same time, using nested multiplex PCR, with the primer designs worked out for each of the conditions mentioned. Primers and PCR conditions for some of these conditions are presented in Table 3.10.

Based on sequential PB1 and PB2 testing, involving the mutation and/or linked marker analysis, results of which were available already on day 1 (approximately 30–32 h after oocyte retrieval), the embryos resulting from the oocytes predicted to be free from maternal mutation were transferred back to the patients on day 3, while those predicted as affected were further tested for confirmation of diagnosis, when available. Approximately 7.7 oocytes per cycle on an average were obtained with PB1 results, which appeared to be heterozygous in 66% and homozygous normal or mutant in 34% of oocytes. PB2 results were available in 79% of these oocytes (6.1 oocytes per cycle on an average), so in the remaining oocytes the genotype prediction was not possible, so additional testing by embryo biopsy was applied.

ADO still remains to be the major problem in avoiding misdiagnosis if undetected, as observed in one of the cases of PGD for FMR1, presented below. ADOs were detected either by the use of the linked markers or by sequential PB1 and PB2 analysis. For example, in the case of PGD for X-linked hydrocephalus, both PB1 and PB2 in one of the oocytes had identical homozygous mutant genotype, suggesting that PB1 was apparently heterozygous, with the normal allele not detected due to ADO. However, at least two embryos in each cycle for this condition were available for transfer, originating from the mutation-free oocytes preselected based on the heterozygous status of PB1 and homozygous mutant PB2, which not only predicted the mutation-free status of the resulting embryos, but also reliably excluded any probability of ADO.

In the PGD cycle for *MTMD* tested by both PB1 and PB2, three unaffected embryos were transferred, originating from the oocytes with heterozygous PB1 and homozygous mutant PB2, or with homozygous mutant PB1 and normal PB2, in which ADO in PB1 was excluded by two linked markers. In PGD cycles for *F8 and F9*, embryos originating from the mutation-free oocytes were also predicted based on heterozygous PB1 and mutant PB2, resulting in clinical pregnancies and the birth of unaffected children with no misdiagnosis.

The largest group of X-linked disorders for which PGD was offered was *FMR1*, which is tested indirectly using linkage analysis, as no test is available for direct analysis of the expanded allele [32]. As the mutant allele for this condition cannot be amplified, the preselection of the mutation-free oocytes was inferred from the presence of the linked markers for the normal allele. Only a few of these cycles were performed by blastomere biopsy alone, with the majority being done either by PB1 and PB2 only or PB1 and PB2 analysis followed by blastomere biopsy, if necessary. Only one misdiagnosis was observed, which was due to undetected ADO. Three embryos were transferred, two deriving from oocytes with heterozygous PB1 and mutant PB2, and one from the oocyte with presumably homozygous mutant PB1, which in fact turned out to be heterozygous because of an undetected ADO of both alleles linked to the normal gene (Fig. 3.12). As only two of four available markers were informative for linkage analysis in this cycle, in the absence of the direct test for the expanded allele, the chance of ADO of the normal allele in this case was predicted to be in the range of over 5%, based on our previous observations described in detail above. The second largest group, for which PGD cycles were performed, was *DMD* resulting in the preselection and transfer of mutation-free embryos in all but one cycle.

One of the first X-linked disorders for which the PB approach was utilized was *OTC* [31]. In this case, of five PGD cycles performed for four couples at risk for producing offspring with *OTC*, unaffected embryos for transfer were predicted in every cycle (1.6 embryos per cycles), resulting in four clinical pregnancies, and the birth of four healthy children free of mutation, following confirmation of PGD by prenatal diagnosis [31]. The preselection of mutation-free oocytes were based on heterozygous PB1 and mutant PB2, except only a few with the homozygous mutant PB1 and normal PB2, but with the linked marker

**Table 3.10** Primers to amplify mutation or linked markers for PGD of X-linked diseases

| Gene/polymorphism | Upper primer | Lower primer | Anneal $T_m$ (°C) |
|---|---|---|---|
| FMR1 AC1 | Outside | | 62–45 |
| | 5′ AGCTGCAAAGAGAAACAGACA 3′ | 5′ AATATCAGGCCAGGCACA 3′ | |
| | Inside | | 50 |
| | 5′ GTTGATGCTGAACATCCTTATCG 3′ | 5′ Fam GGCTGAGGCATGATGAGAGTC 3′ | |
| FMR1 AC2 | Outside | | 62–45 |
| | 5′ GCCCTAATCAGATTTCCACA 3′ | 5′ GATGCGGTGGCTCAAG 3′ | |
| | Inside | | 50 |
| | 5′ CAAAAAGAACCCAGATGTGA 3′ | 5′ Fam GGGAGGATAGCTCAAGCTC 3′ | |
| DXs548 | Outside | | 62–45 |
| | 5′ Fam CCTACATCAAAGTCCCAGCA 3′ | 5′ AAGCCTGCAACCAAACACTG 3′ | |
| | Inside | | 50 |
| | 5′ Fam CCTACATCAAAGTCCCAGCA 3′ | 5′ GTACATTAGAGTCACCTGTGGTGC 3′ | |
| DXs1193 | Outside | | 62–45 |
| | 5′ GCCAAGGCATAGAAGACAAC 3′ | 5′ TGTCAGCACAAAAGGGCTTA 3′ | |
| | Inside | | 50 |
| | 5′ ATTGTTCATGCAACTCTCCTC 3′ | 5′ Hex TCATCCAAGCTACTTATTTTAAG 3′ | |
| Factor IX RFLP *MseI* | Outside | | 62–45 |
| | 5′ AGAGGGATAAATACATCAATGGC 3′ | 5′ AATATATTGTCTCCAGCCTGTAGC 3′ | |
| | Inside | | 60 |
| | 5′ GATAGAGAAACTGGAAGTAGACCC 3′ | 5′ ATTAGGTCTTTCACAGAGTAGAATTT 3′ | |
| Factor IX RFLP *Mnl I* | Outside | | 62–45 |
| | 5′ TTGTAATACATGTTCCATTTGCC 3′ | 5′ GGGAATTGACCTGGTTTGG 3′ | |
| | Inside | | 60 |
| | 5′ TTCTAGTGCCATTTCCATGTG 3′ | 5′ ATCTTCTCCACCAACAACCC 3′ | |
| Factor IX VNTR(50 bp repeat) | Outside | | 62–45 |
| | 5′ GGGACCACTGTCGTATAATGTG 3′ | 5′ GAAGAGACACTCCTGAACTCTGG 3′ | |
| | Inside | | 60 |
| | 5′ CCAAAATGTCATTGTGCAGC 3′ | 5′ TCTGAATCATATTTCTCCTTCCC 3′ | |
| MTM1 R241C (loses artificial *HhaI site*) | Outside | | 62–45 |
| | 5′ TTGGATGTGGTGCTAATTAAG 3′ | 5′ CTCATCATCTTTATTTCGTTTC 3′ | |
| | Inside | | 55 |
| | 5′ CGCAGTGAGATTGCAAGTG 3′ | 5′ CAAGAGGCTGACTGCA *G* C 3′ | |

(continued)

**Table 3.10** (continued)

| Gene/polymorphism | Upper primer | Lower primer | Anneal $T_m$ (°C) |
|---|---|---|---|
| MTM1 DXS1684 | Outside | | 62–45 |
| | 5′ AGCACCCAGTAAGAGACTG 3′ | 5′ TGAATCAATCTATCCATCTCTC 3′ | |
| | Inside | | 55 |
| | 5′ CAGGCCACTACCACTTATG 3′ | 5′ HexTACTGTTTTCCACTCTAATGC 3′ | |
| X-Hydrocephalus Fr.168 (−C) (loses *Hpa II* site) | Outside | | 62–45 |
| | 5′ GAGTGTCAGCCCGTCTG 3′ | 5′ AGTAGAGGTTGCCGTTCTG 3′ | |
| | Inside | | 60 |
| | 5′ GTGGCCAAAGGAGACAGTGAA 3′ | 5′ GAAGACACCCCCGCTAACA 3′ | |
| PLP1 L86P (Msp I cuts mutant) | Outside | | 62–45 |
| | 5′ ATCTATGGAACTGCCTCTTTCTTCT 3′ | 5′ CGCTCCAAAGAATGAGCTTGAT 3′ | |
| | Inside | | 60 |
| | 5′ ATCTATGGAACTGCCTCTTTCTTCT 3′ | 5′ GGCCCCCTGTTACCGTTG 3′ | |
| 5′ PLP1 (CA)n (Heminested) | Outside | | 62–45 |
| | 5′ GGAAGGGACCTGAAGAAAAAGAT 3′ | 5′ GCTCTCATTTACCTGGCACACTA 3′ | |
| | Inside | | 55 |
| | 5′ FamGGGGTGATTCTAGTAACCAGGC 3′ | 5′ GCTCTCATTTACCTGGCACACTA 3′ | |
| DXS8020 (Heminested) | Outside | | 62–45 |
| | 5′ GATGGGTCGGTGATGAGAA 3′ | 5′ TCAGCATTACAATTCTGTATAGACT 3′ | |
| | Inside | | 60 |
| | 5′ FamGGGAGGTAGAAAAGGGTTGGAAAGG 3′ | 5′ TCAGCATTACAATTCTGTATAGACT 3′ | |
| DXS8096 (Heminested) | Outside | | 62–45 |
| | 5′ CCTAAGGTTTCCAGATTTAGCA 3′ | 5′ TGTGAGCCAGTTCTTGAAAAT 3′ | |
| | Inside | | 55 |
| | 5′ FamACATCCAGAGAAACAGAACCAA 3′ | 5′ TGTGAGCCAGTTCTTGAAAAT 3′ | |
| DXS8089 (Heminested) | Outside | | 62–45 |
| | 5′ ATGAATGGTGCTAGGATTTGG 3′ | 5′ ATTTTTACATTATGATGTGGTCAAA 3′ | |
| | Inside | | 60 |
| | 5′ ATGAATGGTGCTAGGATTTGG 3′ | 5′ Fam TTCTTGTATGGCTTCTGGGTC 3′ | |
| DXS1191 (Heminested) | Outside | | 62–45 |
| | 5′ TTGAAGGATGCACACTACAAA 3′ | 5′ GCCCCGTTTGATGCTTCTA 3′ | |
| | Inside | | 60 |
| | 5′ TTGAAGGATGCACACTACAAA 3′ | 5′ FamCAGCAGTAAACTGTTTCCCTTT 3′ | |

| Oocyte | Cell Type | DXs1193 | DXs548 | Predicted Genotype |
|---|---|---|---|---|
| 1 | PB1 | *154*/**156** | *245*/**247** | *Affected* |
| | PB2 | **156** | **247** | |
| 3 | PB1 | 154/**156** | *245*/**247** | *Affected* |
| | PB2 | **156** | **247** | |
| 5 | PB1 | 154/**156** | *245*/**247** | **Normal** |
| | PB2 | *154* | *245* | |
| 6 | PB1 | *245*/**247** | *245*/**247** | **Normal** |
| | PB2 | *154* | *247* | |
| 9 | PB1 | *154** | *245** | **Normal*** |
| | PB2 | **156** | **247** | |
| 10 | PB1 | *245*/**247** | *245*/**247** | *Affected* |
| | PB2 | **156** | **247** | |
| **Cord blood** | | *154* | *245* | *Affected* |

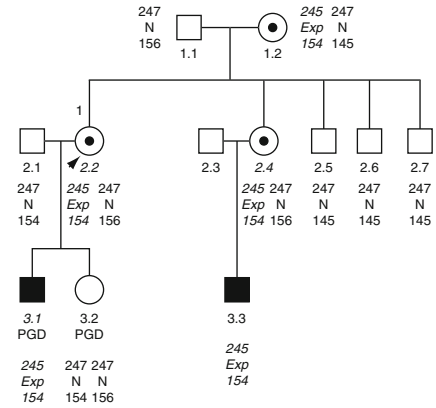



**Fig. 3.12** PGD for FRM1 with misdiagnosis due to undetected ADO in the first polar body. Family pedigree (*right*) and PGD results of haplotype analysis for CGG expansion in FMR1 gene using two linked markers (*left*). The mother is a carrier of an expanded allele linked to 154 and 245 markers. Her sister also has an expanded allele inherited from their mother. One of the sisters who presented for PGD is shown by *arrow*. The other sister has an affected son, who carries only an expanded allele (linked to 154 and 245 markers). On the left of the pedigree, an affected child carrying only an expanded allele (linked to 154 and 245 markers) was born following PGD, due to the predicted 5% risk of misdiagnosis, resulting from undetected ADO of the normal allele in PB1 of the corresponding oocyte (shown by * in the table on the *left*), from which the transferred embryo derived. As can be seen from the table, misdiagnosis originates from the undetected ADO of both markers linked to the expanded allele in PB1 of oocyte #9, which was erroneously considered homozygous mutant, while it was actually heterozygous; following the extrusion of PB2 with the normal allele, the maternal contribution to the resulting oocytes should have been considered mutant

information available to confirm the diagnosis. Despite transferring only a single embryo or two in each of these cycles, resulting from the mutation-free oocytes, an extremely positive clinical outcome of PGD for OTC was observed.

It should also be mentioned that, as in PGD for other conditions, an increasing number of patients presenting for PGD are of advance reproductive age, so a combined aneuploidy testing may be expected to be widely applied in the future, which in addition to avoidance of the birth of children with chromosomal disorders may also improve the PGD clinical outcome, as demonstrated in the example of PGD for PMLD, presented below.

*PMLD* is an X-linked recessive dysmyelinating disorder of the central nervous system that leads to deterioration of coordination, motor ability, and intellectual function, caused by mutation of the proteolipid protein 1 (PLP1) gene located on the long arm of the X (Xq22) chromosome. The gene consists of seven exons of about 15 Kbp, encoding two major proteins PLP1 and DM20, that results from alternative splicing, both serving an important structural component of compact myelin [16, 33].

PMD manifests in infancy or early childhood, with a span of continuum of neurological impairments from nistagmus, delayed motor and cognitive impairment to severe spasticity and ataxia with early mortality from respiratory complication during childhood. Mutations in PLD1 may result in misfolding of the coded proteins, failing to progress through the intracellular processes. This results in their accumulation in the endoplasmic reticulum, which triggers apoptosis, affecting oligodendrocyte survival and myelination. Although several nondisease-causing polymorphisms have

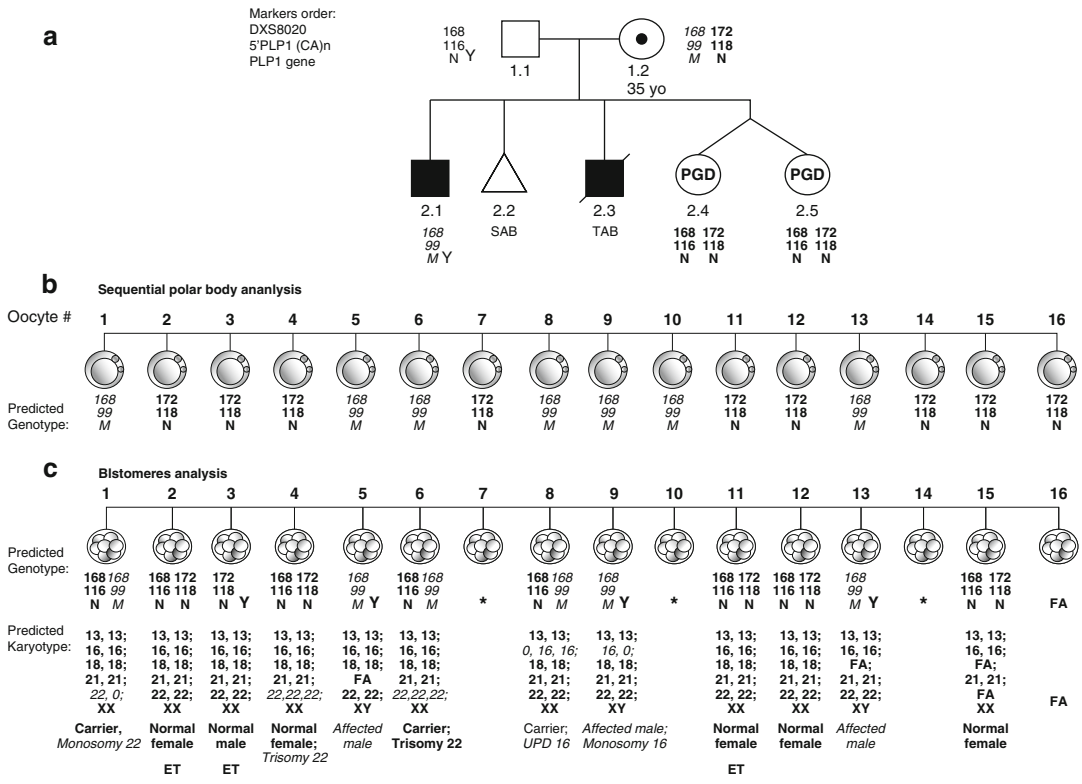



**Fig. 3.13** PGD for PMD with aneuploidy testing. (**a**) Pedigree showing that the mother (1.2) is a carrier of L86P mutation in exon 3 of the PLP1 gene, which is linked to 99 bp repeat of 5′ PLP1 (CA)n, and 168 bp repeat of DXS8020, while the normal allele is linked to 118 and 172 bp repeats of the same polymorphic markers, respectively. The father (1.1) is unaffected, carrying the normal PLP1 allele, linked to 116 and 168 bp repeats, respectively. (*Lower panel*) Reproductive outcomes of this couple, including one previous affected child with PMD (2.1), one spontaneous abortion (2.2), and one prenatal diagnosis, resulting in identification of the affected fetus and termination of pregnancy (2.3). *SAB* spontaneous abortion, *TAB* termination of pregnancy following chorionic villus sampling (*CVS*). (**b**) Sequential PB1 and PB2 analysis of 16 oocytes, of which 9 were predicted to be free of L86P mutation in exon 3 of the PLP1 gene, and 7 affected. (**c**) Blastomere analysis for causative gene (*upper panel*) and aneuploidy (*lower panel*), showing 3 mutant embryos (embryos #5, #9, and #13) and 9 unaffected, including 3 heterozygous female embryos. The remaining 4 embryos were inconclusive, including one with failed amplification. Aneuploidy testing revealed 2 embryos with trisomy 22 (embryos #1 and #6), 1 with monosomy 22, 1 with monosomy 16 (embryo #9), and 1 with uniparental disomy 16 (embryo #8). Three unaffected embryos (embryos #2, #3, and #11) were transferred, resulting in the birth of healthy twins. *Not available for testing (the IVF was performed in a different institution in another state and it is assumed that biopsy material was not sent for testing, because embryos did not develop further. For the same reason, the fate of the other two unaffected embryos (embryos #12 and #15) is also unknown, although it is presumed that they were frozen). *FA* failed amplification

been described, at least 100 different mutations in the PLP1 gene are known to be associated with PMD, including duplications, which are the most frequent ones, small insertions, deletions, and single-base substitutions, leading to missence, nonsense, or splicing mutations.

Because no specific treatment for PMD is presently available, prenatal diagnosis is offered as an option for couples at risk to avoid the birth of the affected child [34]. As mentioned above, PGD seems to be particularly attractive for at-risk couples who cannot accept prenatal diagnosis and termination of pregnancy. In addition, because of a wide individual phenotypic variability even within families, the decision about termination of the affected pregnancy may be extremely difficult even for those couples that accept prenatal diagnosis.

The couple presented for PGD had a history of a previous child diagnosed with PMD (Fig. 3.13). The phenotypically normal 35-year-old mother

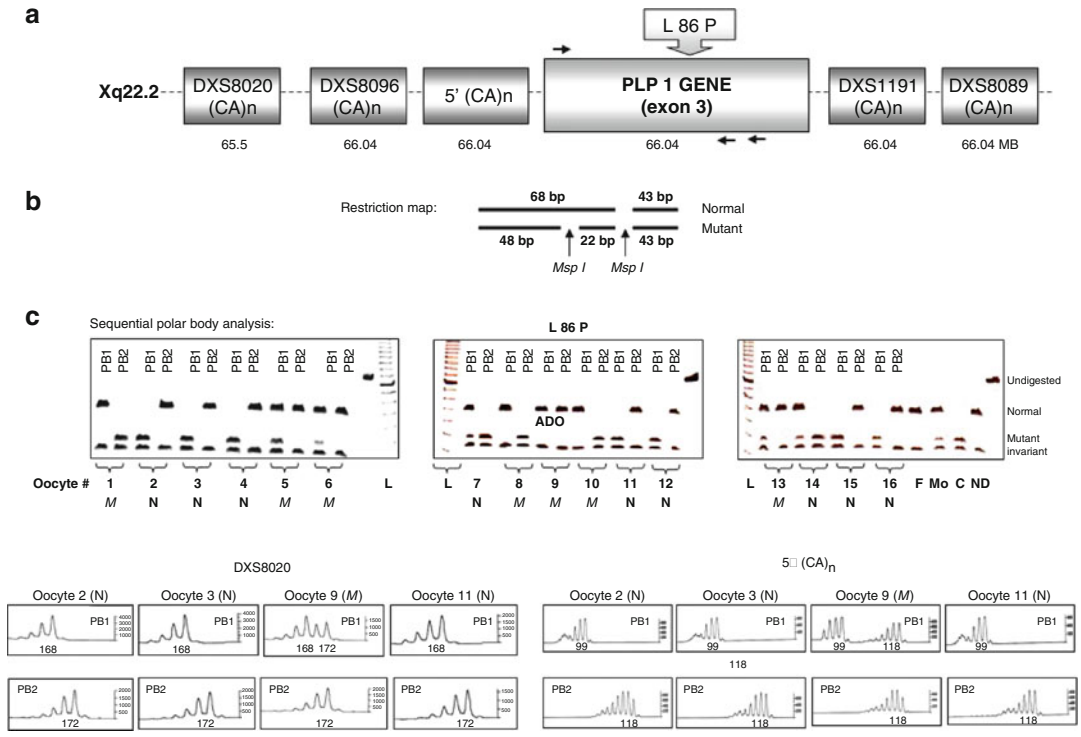



**Fig. 3.14** Preimplantation genetic diagnosis for major mutation and polymorphic markers in PMD. (**a**) Position of L86P mutation in PLP1 gene of exon 3 and polymorphic markers. (**b**) Restriction map. The mutation creates restriction site for *Msp*I enzyme. (**c**) PB analysis of normal and mutant sequences of PLP1 gene (*upper panel*). Of 16 oocytes tested, 9 were mutation-free based on heterozygous PB1 and affected PB2 in 2 of them (embryos #7 and #14) and mutant PB1 and normal PB2 in the remaining 7 oocytes. Six embryos were affected, based on heterozygous PB1 and normal PB2 in three of them (embryos #5, #6, and #13) and normal PB1 and mutant PB2 in the remaining three (embryos #1, #8, and #10). ADO of the mutant allele was observed in PB1 analysis of oocyte #9, which is confirmed by both linked polymorphic markers, suggesting the presence of both alleles in PB1 from oocyte #9 (*lower panel*). Linked polymorphic marker analysis confirmed the data of mutation analysis in all the oocytes, including the mutation-free status of oocytes #2, #3, and #11, shown on the *lower panel*; the embryos resulting from these three oocytes were transferred back to the patient resulting in unaffected twins (see Fig. 3.13). L ladder, *N* normal, *M* mutant, *Mo* maternal genotype, *F* paternal genotype, *C* affected child's genotype, *ND* normal (control) DNA

was a carrier of the T257C (L86P) mutation in exon 3 of the PLP1 gene, representing T to C substitution in 257 nucleotide position that results in lysine to proline substitution in 86 position of amino acid sequence of PLP1 protein. As seen from the pedigree (Fig. 3.14), the couples had two more pregnancies, one resulting in spontaneous abortion and the other terminated because the fetus was diagnosed with PMD by chorionic villus sampling (CVS).

The PGD cycle was performed using PB and blastomere analysis, as described above. PB1 and PB2 were removed sequentially following maturation and fertilization of the oocytes, and tested by the multiplex PCR analysis, involving the mutation testing simultaneously with two closely linked polymorphic markers, PLP-intragenic microsatellite PLP5' (CA)n and the nearest flanking extragenic microsatellite DXS8020. The mutation analysis involved the detection of T to C change in nucleotide position 257 of exon 3, based on Msp I restriction digestion, which does not cut the normal allele, while creating two fragments of 48 and 22 bp in the mutant allele (Fig. 3.14). The primers and reaction conditions are presented in Table 3.11.

Because the oocytes predicted as mutant may further form unaffected heterozygous female

**Table 3.11** Primers and reaction conditions for PGD of PMD

| Gene/ polymorphysm | Accession number | Heterozygosity index | Number of alleles | Upper primer | Lower primer | Annealing $T_m$ (°C) |
|---|---|---|---|---|---|---|
| PLP1 L 86 P (heminested) *Msp I* cuts mutant sequence | NT_011651 | NA | NA | Outside: 5′ ATCTATGGAACTGCCTCTTTCTTCT 3′<br>Inside: 5′ ATCTATGGAACTGCCTCTTTCTTCT 3′ | 5 CGCTCCAAAGAATGAGCTTGAT 3′<br>5′ GATGGTGGTCCTGTAGTCGCC 3′ | 62–45<br>60 |
| DXS8020 (heminested) | Z 52328 | 0.78 | 9 | Outside: 5′ GATGGGTCGGTGATGAGAA 3′<br>Inside: 5′ *Fam* GGGAGGTAGAAAAGGGTTGGAAAGG 3′ | 5′ TCAGCATTACAATTCTGTATAGACT 3′<br>5′ TCAGCATTACAATTCTGTATAGACT 3′ | 62–45<br>55 |
| DXS8096 (heminested) | Z 51652 | 0.8 | 12 | Outside: 5′ CCTAAGGTTTCCAGATTTAGCA 3′<br>Inside: 5′ *Fam* ACATCCAGAGAAACAGAACCAA 3′ | 5′ TGTGAGCCAGTTCTTGAAAAT 3′<br>5′ TGTGAGCCAGTTCTTGAAAAT 3′ | 62–45<br>55 |
| 5′ PLP1 $(CA)_n$ (heminested) | NT_011651 | 0.56 | 5 | Outside: 5′ GGAAGGGACCTGAAGAAAAAGAT 3′<br>Inside: 5′ *Fam* GGGGTGATTCTAGTAACCAGGC 3′ | 5′ GCTCTCATTTACCTGGCACACTA 3′<br>5′ GCTCTCATTTACCTGGCACACTA 3′ | 62–45<br>55 |
| DXS119 1 (heminested) | Z 23464 | 0.65 | 5 | Outside: 5′ TTGAAGGATGCACACTACAAA 3′<br>Inside: 5′ TTGAAGGATGCACACTACAAA 3′ | 5′ GCCCCGTTTGATGCTTCTA 3′<br>5′ *Fam* CAGCAGTAAACTGTTTCCCTTT 3′ | 62–45<br>55 |
| DXS8089 (heminested) | Z 51440 | 0.81 | 8 | Outside: 5′ ATGAATGGTGCTAGGATTTGG 3′<br>Inside: 5′ ATGAATGGTGCTAGGATTTGG 3′ | 5′ ATTTTTACATTATGATGTGGTCAAA 3′<br>5′ *Fam* TTCTTGTATGGCTTCTGGGTC 3′ | 62–45<br>55 |

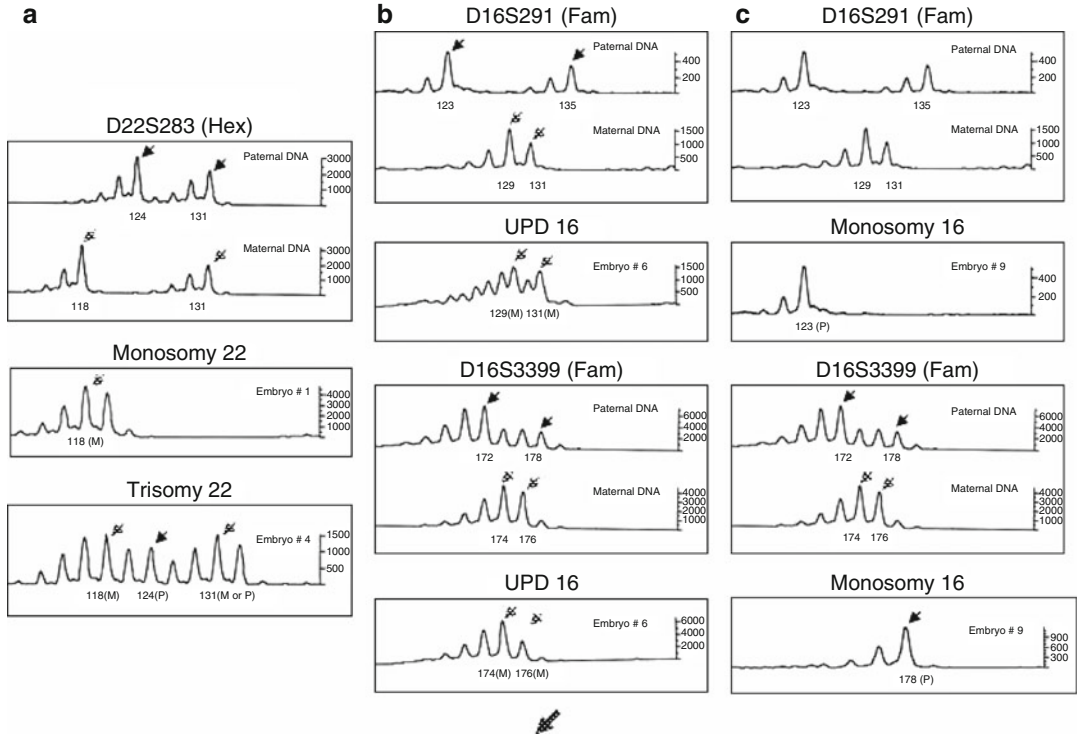



**Fig. 3.15** Aneuploidy testing as part of PGD for PMD. (**a**) *Upper panel* shows paternal and maternal haplotypes for D22S283, allowing identification of monosomy 22 in embryo #1, evidenced by only one maternal allele (*middle panel*) and trisomy 22 in embryo #4 (*lower panel*). (**b**) The *first* and *third panels* show paternal and maternal haplotypes for D16S291 and D16S3399, respectively, allowing identification of uniparental disomy 16 in embryo #8, evidenced by only maternal alleles (*second and fourth panels*). (**c**) The *first* and *third panels* show paternal and maternal haplotypes for D16S291 and D16S3399, respectively, allowing identification of monosomy16 in embryo #9, evidenced by only paternal alleles for each marker (*second and fourth panels*)

embryos, following fertilization by a sperm carrying the X chromosome, testing for paternal normal gene in the resulting embryos was also performed. Because the mother was 35 years old, the biopsied blastomeres were also tested for the age-related aneuploidies, for which purpose the copy number of chromosomes 13, 16, 18, 21, 22, X, and Y was identified (Fig. 3.15), using the patterns of alleles that uniquely identify the individual, relying on a multiplex fluorescent PCR of low-template DNA (primers are described in Chap. 2).

The maternal haplotypes, presented in Fig. 3.13, were established based on PB analysis: the mutant allele was linked to 168 bp repeat of DXS8020 and 99 bp repeat of PLP5' (CA) n marker; the normal allele was linked to 172 bp repeat of DXS8020 and 118 bp repeat of PLP5' (CA) n marker. The paternal haplotypes were established based on family blood sample DNA analysis: the normal paternal allele was linked to 168 bp repeat of DXS8020 and 116 bp repeat of PLP5' (CA)n marker.

Of 16 oocytes available for testing in one PGD cycle, 9 were mutation-free based on heterozygous PB1 and affected PB2 in 2 of them (embryos #7 and #14) and mutant PB1 and normal PB2 in the remaining 7 oocytes. Six embryos were affected, based on heterozygous PB1 and normal PB2 in 3 of them (embryos #5, #6, and #13) and normal PB1 and mutant PB2 in the remaining 3 (embryos #1, #8, and #10) (Fig. 3.13). This was in accordance with the two tightly linked polymorphic markers DXS 8020 and 5' (CA) n, as shown in three examples of three normal oocytes (oocytes #2, #3, and #11), in all but one (oocyte

#9), in which both PB1 and PB2 mutation analysis demonstrated the normal genotype, while the marker analysis revealed both normal and mutant alleles in PB1, suggested allele drop out (ADO) of the mutant allele.

Of 13 embryos available for blastomere analysis, 12 were with results, of which 3 were affected (embryos #5, #9, and #13) (Fig. 3.13), 5 chromosomally abnormal, including 2 trisomy 22 (1 of which, embryo #4, is shown in Fig. 3.15), 1 monosomy 22 (embryo #1), 1 monosomy 16 (embryo #9; also affected), and 1 uniparental disomy for chromosome 16 (embryo #8), and 5 unaffected. Three of these embryos, free of mutant gene and aneuploidy (embryos #2, #3, and #12) were transferred, resulting in a twin pregnancy and the birth of two unaffected children, confirmed to be euploid and free of T257C mutation in the PLP1 gene.

The presented case was the first PGD for PMD, demonstrating the realistic option available for couples at risk for producing offspring with the most severe type of PMD, caused by the T257C mutation. However, PGD may be useful also for milder but more frequent types of PMD caused by duplications, the wide variability of clinical manifestations of which may present difficulties in making decision of interruption of the affected pregnancies in prenatal diagnosis.

Because of an advanced reproductive age of the patient in the presented case, aneuploidy testing was also performed, using blastomere analysis to exclude both maternally and paternally derived aneuploidies. As for specific PLP1 mutation testing, which is maternally derived, although this may have been limited solely to oocyte analysis, the embryo testing allowed the identification of those unaffected female embryos which derived from the mutant oocytes detected by PB analysis. For example, embryos #1 and #6 were heterozygous unaffected, despite originating from mutant oocytes; however, embryo #1 appeared to be monosomic for chromosome 22, and embryo #6 trisomic for chromosome 22 (see Figs. 3.13 and 3.15). The fact that 5 of 12 tested embryos were detected to be chromosomally abnormal is in agreement with the expected risk for aneuploidies in patients of advanced reproductive age.

One of the incidental findings shown in Fig. 3.15 was a uniparental disomy of chromosome 16, which cannot be detected without PCR-based aneuploidy testing. Because both of these chromosomes 16 were of maternal origin, the extra chromosome 16 should have derived from the maternal meiosis I, followed by a subsequent trisomy rescue at the cleavage stage, which resulted in an incidental loss of the paternal chromosome 16 and the establishment of uniparental disomy 16 in the heterozygous status for PLP1 mutation in the female embryo. Unfortunately, this embryo could not be tested for the presence of the trisomic cell line, as the IVF was performed in a different institution in another state. It may be expected that the detection of uniparental disomies may in future help in avoiding them from transfer, which may contribute to the avoidance of at least some proportion of imprinting disorders described recently in association with assisted reproductive technologies [35].

With progress in obtaining the sequence information for X-liked disorders, PGD for an increasing number of X-linked disorders may soon be performed by specific diagnosis to avoid discarding 50% of healthy male embryos. On the other hand, as demonstrated above, testing for X-linked genetic disorders may be entirely limited to oocytes, because of the maternal origin of the mutations involved, making useless any further manipulation and testing of the resulting embryos, which may be transferred irrespective of gender or any contribution from the father. PGD using specific diagnosis for X-linked disorders has been reported also based on blastomere biopsy [36].

Presented results further demonstrate the clinical usefulness of PGD for X-linked disorders by a sequential PB1 and PB2 analysis, which resulted in the transfer of the embryos originating from the oocytes predicted to be free from X-linked mutation in 220 of 270 clinical cycles performed (Tables 3.3). As mentioned, these transfers yielded 94 clinical pregnancies, resulting in the birth of 91 healthy unaffected children in all but one case, in which the affected embryo was misdiagnosed to originate from the oocyte with the homozygous mutant PB1, predicted on the basis of only one additional polymorphic marker, which leaves an over 5% chance for misdiagnosis. With two

embryos having been reliably diagnosed for transfer, the couple may have had the reason for accepting such a misdiagnosis risk, so as to have three instead of two embryos for transfer, improving the chances to become pregnant. The results demonstrate once again that the embryos resulting from the oocytes with homozygous mutant PB1should not be transferred unless at least three linked markers are available to exclude the possible heterozygous status of PB1. This is especially relevant to testing for FMR1, which is entirely based on linkage analysis. To exclude completely the risk for misdiagnosis in the embryos resulting from homozygous affected PB1, testing for as many as four linked markers, overall, is required.

In conclusion, the data show that a specific genetic analysis by sequential PB1 and PB2 testing may be a practical option for PGD of X-linked disorders, providing an alternative to PGD by gender determination.

## 3.4 Homozygous or Double Heterozygous Recessive Conditions

PGD is of special value for dominant conditions and those couples with one homozygous and double heterozygous-affected partner, when only 50% chance of having an unaffected child may be expected. This was first applied for a couple with compound heterozygous male partner affected by *phenylketonuria* (*PKU*), and female partner carrying the third PKU mutation [37].

One in every 10,000 infants in the United States is born with PKU, an inherited metabolic disorder that causes mental retardation if untreated. When infants are fed a strict diet from birth, they have normal development and a normal lifespan. However, a strictly controlled diet must be maintained, especially during pregnancy in female patients, so as to avoid potential detrimental effects on the fetus. Because of considerable progress in screening newborns for PKU and dietary modification treatment, PKU has not often been included in prenatal diagnosis. With the introduction of PGD, which enables couples to have a healthy pregnancy, couples at risk may avoid giving birth to a child who needs a lifelong dietary treatment. PGD may be particularly useful for couples in whom one partner is homozygous or compound heterozygous-affected because there is a 50% chance of producing a child affected with PKU.

As mentioned, the first case of PGD for PKU was performed in a couple with compound heterozygous affected partner presenting for PGD in connection with their first offspring with PKU, and their 50% risk of producing another affected child. The 31-year-old mother was a carrier of the R408W mutation in exon 12 of the phenylalanine hydroxylase (PAH) gene, while the affected father was compound heterozygous for R408 and Y414C mutations in the same exon. Following maturation of oocytes in a standard IVF protocol, PB1s were removed following maturation of oocytes. Then following fertilization of oocytes by ICSI, PB2 were removed using the micromanipulation techniques described in Chap. 2. PB1 and PB2 were amplified by heminested multiplex PCR, using a primer design and primer-melting temperatures, presented in Table 3.12. Restriction digestion of the PCR product for mutation was performed overnight with Sty1 enzyme (Promega), followed by acrylamide gel electrophoresis. To detect possible ADO of mutant or normal allele, two linked markers were amplified simultaneously with the PAH gene, one representing an STR in intron 3, and the other a restriction fragment length polymorphism (RFLP) XmnI in intron 8. To detect potential contamination with extraneous DNA, an additional non-linked VNTR was also amplified.

The embryos resulting from oocytes predicted to contain only normal maternal alleles were cultured into the cleaving 6–8-cell embryos and transferred back to the patient on day 3 after aspiration. The resulting pregnancy was followed up by prenatal diagnosis to confirm the results of PGD. Accordingly, those embryos resulting from oocytes predicted to contain the mutant maternal gene were also analyzed further to investigate the accuracy of the results of the sequential PB1 and PB2 analysis.

Of 15 oocytes available for testing, the results for both PB1 and PB2 were obtained in 11 of

**Table 3.12** Primers and reaction conditions used in PGD for PKU

| Gene/polymorphism | Upper primer | Lower primer | Annealing $T_m$ (°C) |
|---|---|---|---|
| Y414C Nested PCR (*Pst I* cuts mutant allele) | Outside | | 62–45 |
| | 5′ ATGCCACTGAGAACTCTC 3′ | 5′ AGTCTTCGATTACTGAGAAA 3′ | |
| | Inside | | 54 |
| | 5′ TACCTTTCTCCAAATGGTG 3′ | 5′ CCTTTAGGTGTGTAGG*CT*G 3′ | |
| R408W Heminested PCR (*Sty I* cuts mutant allele) | Outside | | 62–45 |
| | 5′ ATGCCACTGAGAACTCTC 3′ | 5′ AGTCTTCGATTACTGAGAAA′ 3′ | |
| | Inside | | 54 |
| | 5′ TACCTTTCTCCAAATGGTG 3′ | 5′ AGTCTTCGATTACTGAGAAA 3′ | |
| VNTR Nested PCR | Outside | | 62–45 |
| | 5′ GCTTGAAACTTGAAAGTTGC 3′ | 5′ GGAAACTTAAGAATCCCATC 3′ | |
| | Inside | | 56 |
| | 5′ CTTGATTTAATCATTTTACAAT 3′ | 5′ CTCAGAGAAGCACATCTTTT 3′ | |
| SNP (*Xmn I*) Heminested PCR | Outside | | 62–45 |
| | 5′ CTGTACTTGTAAGATGCAGC 3′ | 5′ GCAGTAACCACACTTCTGAA 3′ | |
| | Inside | | 58 |
| | 5′ CTGTACTTGTAAGATGCAGC 3′ | 5′ ACTGTCCCAAGCAATCAAAG 3′ | |
| Exon 3 STR $(TCTA)^n$ Heminested PCR | Outside | | 62–45 |
| | 5′ CAAATTGCCAGAACACATA 3′ | 5′ TCATAAGTGTTCCCAGACA 3′ | |
| | Inside | | 52 |
| | 5′ TGTGGAAAGCAGAAAGAC 3′ | 5′ TCATAAGTGTTCCCAGACA 3′ | |

them, allowing a reliable diagnosis. Six oocytes with both PB1 and PB2 data were predicted normal, based on the heterozygous PB1, containing both the normal and mutant maternal alleles, and the mutant PB2, suggesting that no mutant allele was left in the resulting oocytes (Fig. 3.16). Another oocyte was predicted normal based on the presence of the mutant allele in PB1 and the normal allele in PB2. These results were in agreement with the linked marker analysis, showing that the homozygous status of PB1 was not due to ADO of the normal allele. Three oocytes were predicted affected, the diagnosis of two of which were based on the heterozygous status of PB1 and the normal PB2, and one on the homozygous normal status of PB1 and the mutant PB2. ADO of the mutant allele was observed in one oocyte, evidenced by the identical genotype of PB1 and PB2. This was confirmed by the presence of the linked polymorphic markers, suggesting that the resulting oocytes were mutant. The marker analysis also confirmed the mutant status of these three oocytes. ADO was observed in the STR analysis of two oocytes, evidenced by the identical patterns in PB1 and PB2. Three of four embryos resulting from these mutant oocytes were followed up by the mutation and marker analysis, confirming the presence of the mutant allele in all of them. Four of seven embryos resulting from the mutation-free oocytes were selected for transfer back to the patient, yielding a clinical twin pregnancy, confirmed by the chorionic villus sampling (CVS) to be unaffected. Healthy unaffected twins were born, presently of 7 years of age, with normal patterns of mental development.

The presented case was the first experience of PGD for PKU in the experience of PGD for more than 200 different single-gene disorders, which were mainly performed for the at-risk couples with both unaffected partners who were heterozygous carriers of an autosomal-recessive gene mutation, or one with a dominant mutation. Because, in the presented cases, it was a male partner who was compound heterozygous-affected, the PGD strategy was based on the preselection of the maternal mutation-free oocytes using a sequential PB1 and PB2 DNA analysis.

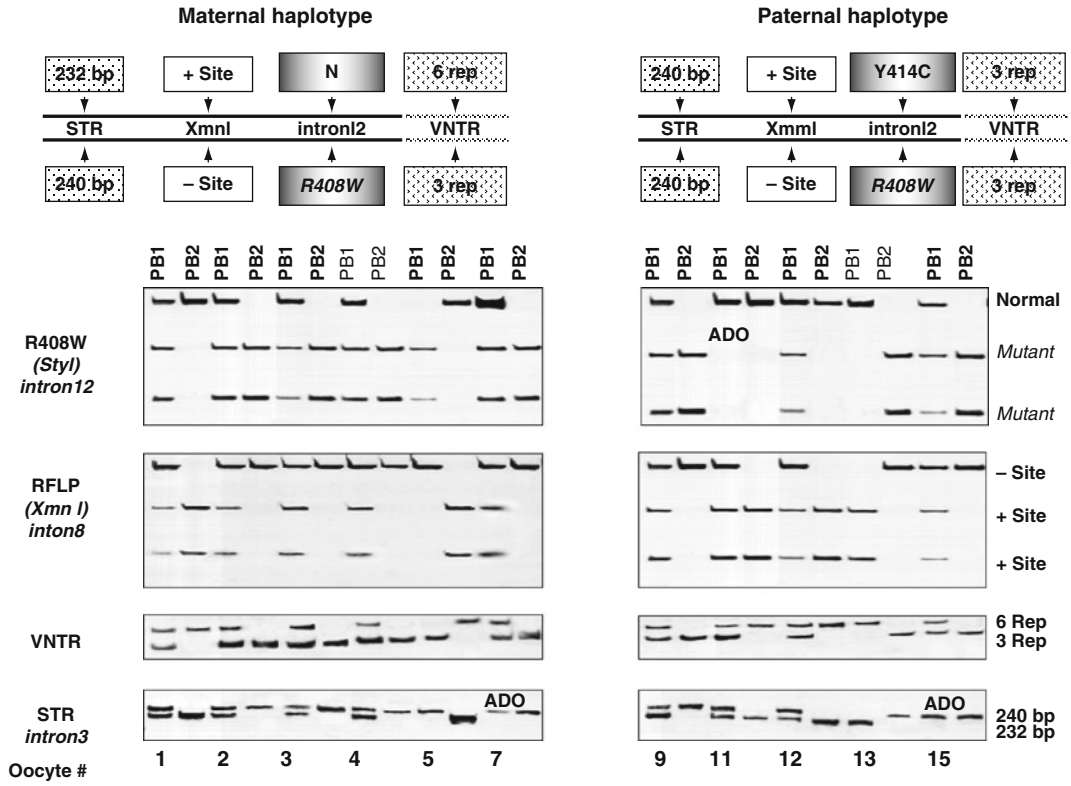



**Fig. 3.16** PGD for R408W mutation in phenylalanine hydroxylase gene in a couple with the male partner homozygous affected. (*Upper panel*) Schematic representation of maternal (*left*) and paternal (*right*) haplotypes. Heterozygous mother has R408W mutation linked to short tandem repeats (*STRs*) in intron 3, variable number of tandem repeats (VNTRs) close to 3′ of gene (3 rep), and restriction fragment-length polymorphism (*RFLP*) in intron 8 (−site). Homozygous affected father has two different mutations, one similar to maternal (R408W) mutation, and the other, Y414C mutation, with its own linkage pattern. (*Bottom panel*) Genotyping oocytes by sequential analysis of PB1and PB2 for R408W mutation and informative linked markers – STR, VNTR, and RFLP. All series include PB1 followed by PB2 in the lane to its immediate right, corresponding to 11 oocytes studied (oocytes are numbered at the bottom). As R408W mutation creates restriction site for StyI enzyme, oocytes #2, #3, #4, #7, #9, and #15 were predicted normal based on heterozygous PB1 and homozygous PB2. Oocyte #5 was also predicted normal, but based on homozygous mutant PB1 and normal PB2, which was in agreement with marker analysis, excluding the possibility for ADO in the corresponding PB1. ADO of mutant allele is evident from identical genotype of both PB1 and PB2 in oocyte #11 (confirmed by all three markers), suggesting affected status of this oocyte. Other three affected oocytes were predicted based on heterozygous PB1 and normal PB2 (oocytes #1 and #12), and homozygous normal PB1 and mutant PB2 (oocyte #13). ADO was also detected in oocytes #7 and #15 (identical genotype of PB1 and PB2 for intron 3 STR). These are not in conflict with unaffected genotype of resulting embryos, which were transferred together with two other unaffected embryos (#2 and #4), resulting in twin pregnancy and the birth of two healthy children, following confirmation of PGD by prenatal diagnosis

Based on the multiplex heminested PCR analysis, 6 of 11 oocytes with both PB1 and PB2 results were predicted to contain no mutant allele of the PHA gene. Four embryos resulting from these zygotes were transferred, yielding an unaffected twin pregnancy and the birth of healthy children, following the confirmation of PGD by CVS. The high accuracy of this strategy was obvious from the follow-up study of the embryos resulting from the mutant oocytes, confirming the results of the sequential PB1 and PB2 analysis.

With progress in the treatment of genetic disorders, PGD will have an increasing impact on the decision of the affected and well-treated

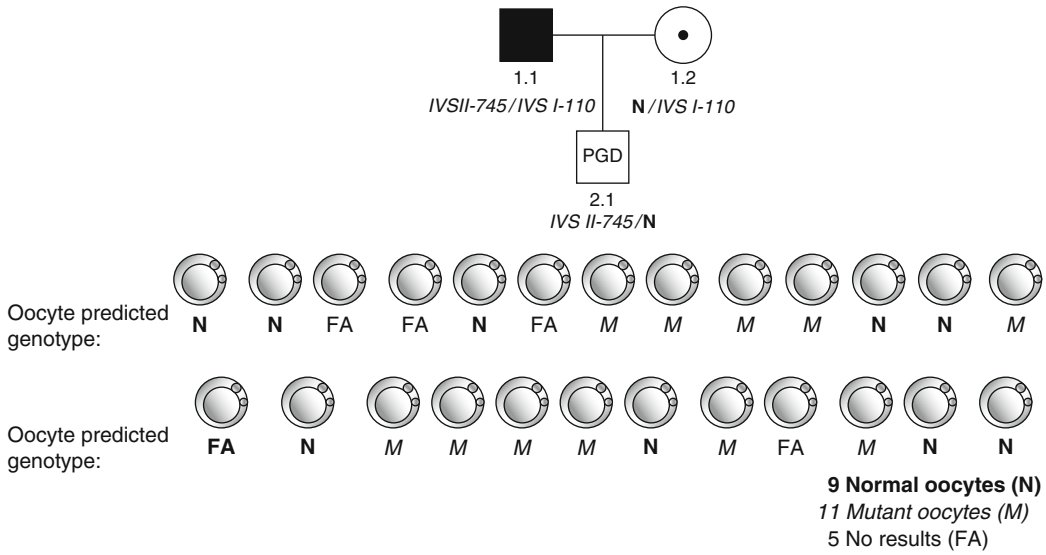



**Fig. 3.17** PGD for a couple with double heterozygous affected male partner with thalassemia. (*Top*) Family pedigree with father double-heterozygous IVSI-110 and IVSII-745, and mother carrier of IVSI-110. (*Bottom*) Genotyping of 20 embryos for maternal IVSI-110 mutation by sequential PB1 and PB2, resulting in prediction of 9 mutation-free oocytes

patients to reproduce. For example, the life expectancy has been significantly improved for such common conditions as *CFTR and thalassemias*, which presently may be treated radically by stem cell transplantation. In each of such cases, the strategy depends on whether the affected partner is male or female, because testing may be entirely based on oocytes if the male partner is affected, in contrast to embryo testing when the female partner if affected. We have performed PGD for couples with affected partners with both of these conditions presented below.

In *thalassemia* cases, there may be male factor infertility involved, so the male partners need appropriate treatments before PGD, or testicular biopsy has to be done. This was the case in one of our couples with *male thalassemic partner*, when a sufficient number of oocytes with homozygous mutant or heterozygous PB1 were detected, but no appropriated sperm can be found for fertilization, so matured oocytes were frozen for possible future use after treatment. In the other two cycles because the female partner was affected, blastomere biopsy was utilized, resulting in preselection of a single unaffected embryo for transfer in each cycle, yielding no clinical pregnancy. This was due to a limited number of oocytes obtained from thalassemic female partners. However, in the well-treated patients, the situation may be different as shown in the case with the male affected partner with two different mutations IVSI-110 and IVSII-745 and the female partner with IVSI-110, presented in Fig. 3.17. Of 25 embryos tested, 5 failed to amplify, 11 were mutant, and 9 heterozygous normal, providing a sufficient number of embryos for transfer (to be extended, if necessary).

We also performed PGD for couples, one with female and one with *affected male partner with CF*. In the couple with a maternal affected partner there were three different mutations in the CFTR gene. The mother was affected with *CF* and had two different mutations R117H and G542X. The father was a carrier of 1,717 mutation in the CFTR gene. Paternal haplotypes were established using single-sperm PCR, while maternal linkage was based on DNA amplification of PB1s. The couple had two previous pregnancies, the first one resulting in the spontaneous abortion of twins, and the second pregnancy being terminated following prenatal diagnosis which identified an affected fetus with CFTR. So PGD was performed for three different mutations in the CFTR gene, resulting in an unaffected

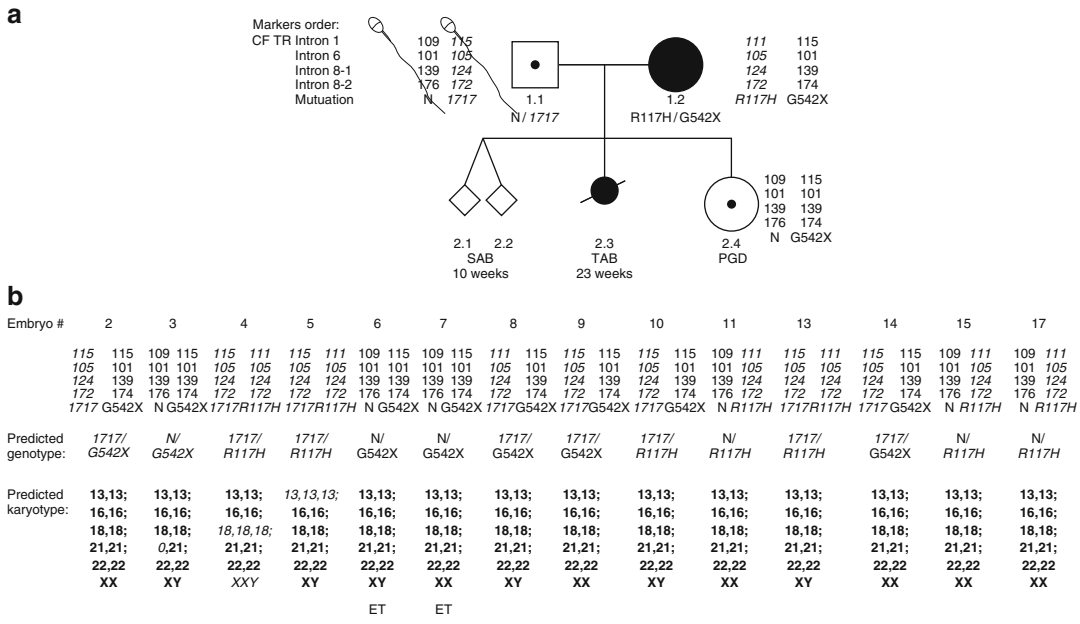



**Fig. 3.18** PGD for a couple with female affected double heterozygous partner with two different CFTR mutations combined with aneuploidy testing. (**a**) Family pedigree of a couple with three different mutations in CFTR gene. The mother is affected with cystic fibrosis (*CF*) and had two different mutations (R117H and G542X). The father is a carrier of 1717 mutation in CFTR gene. Paternal haplotype was established by multiplex heminested single-sperm PCR. Maternal linkage was based on DNA amplification of polar bodies. Marker order of the mutations and polymorphic markers in CFTR gene are shown on the *upper left*. The first pregnancy with twins resulted in spontaneous abortion and the second pregnancy in an affected fetus which was terminated; PGD was performed for three different mutations in CFTR gene, resulting in an unaffected pregnancy and the birth of a healthy girl predicted and confirmed to be the carrier of maternal mutation G542X. (**b**) Outcome of PGD cycle for three mutations in CFTR gene combined with age-related aneuploidy testing. Multiplex heminested PCR performed on blastomeres from 14 embryos allowing simultaneous detection of the paternal and maternal CFTR haplotypes and non-syntenic short tandem repeats (STRs) located on chromosomes 13,16,18,21,22, and XY. Six embryos (#3, #6, #7, #11, #15, and #17) were predicted to be carriers based on the presence of the maternal mutation and normal paternal CFTR gene. Monosomy 21 was found in the blastomere from embryo #3, which was excluded from the transfer and freezing. Trisomy 18 and XXY was observed in the blastomere from embryo #4, and trisomy 13 was detected in the blastomere from embryo #5. Embryos #6 and #7 were transferred, resulting in pregnancy and the birth of a healthy girl. DNA analysis of the newborn baby revealed a genetic profile identical to that of embryo #7

pregnancy and the birth of a healthy girl predicted and confirmed to be the carrier of maternal mutation G542X. It is of interest that multiplex PCR performed for three mutations in the CFTR gene in this case was combined with age-related aneuploidy (see below), because of the advanced reproductive age of the mother. Multiplex heminested PCR was performed on blastomeres from 14 embryos allowing simultaneous detection of the paternal and maternal CFTR haplotypes and non-syntenic short tandem repeats (STRs) located on chromosomes 13, 16, 18, 21, 22, and XY. Six embryos were predicted to be carriers based on the presence of the maternal mutation and normal paternal CFTR gene. In addition to avoiding the transfer of the affected double heterozygous embryos, three aneuploid embryos were identified, involving aneuploidy for chromosomes 13, 18, 21, and X and excluded from the transfer and freezing. Two unaffected embryos were transferred resulting in pregnancy and the birth of a healthy girl. DNA analysis of the newborn baby revealed a genetic profile identical to that of embryo #7, evidencing the usefulness and the accuracy of the combined mutations analysis, linkage, and aneuploidy testing in PGD for single-gene disorders in patients with advanced reproductive age (Fig. 3.18).

PGD for another couple with three different mutations in the CFTR gene was performed in which the paternal partner was affected carrying both delta F508 and R117H mutations in the CFTR gene, which was described above (Fig. 3.4).

## 3.5 Conditions with no Available Direct Mutation Testing

Although the availability of the sequence information is one of the major conditions in undertaking PGD for Mendelian diseases, it is also possible to perform PGD when no exact mutation is known. One of the examples demonstrating feasibility of the approach may be the report of PGD for *autosomal-dominant polycystic kidney disease* (*ADPKD*), caused by either PKD1 or PKD2 genes, for which no direct testing was available, making the linkage analysis the only method of choice.

ADPKD is a common genetic disorder present in 1/1,000 individuals worldwide, which causes progressive cyst formation and may eventually lead to renal failure by late middle age, requiring renal transplantation or dialysis [16]. The overall health implications of ADPKD are obvious from the fact that approximately 10% of all patients in need for renal transplantation or dialysis have this disease. ADPKD is caused by either PKD1 or PKD2 genes, for which no direct testing was available, making the linkage analysis a method of choice [38–40]. Because of an extremely variable expression and the age of onset, much higher in PKD2, only half of the patients carrying these genes may present with severe clinical manifestation and renal failure, making prenatal diagnosis and pregnancy termination highly controversial. PGD may therefore be more attractive for at-risk couples, because an ADPKD-free pregnancy may be established from the onset, without risk for pregnancy termination after prenatal diagnosis.

The majority (85%) of ADPKD is caused by PKD1, assigned to chromosome 16p13.3, the rest being attributed to PKD2, located on chromosome 4q21-q23 [16]. Because both PKD1 and PKD2 are characterized by the enlargement of kidneys due to the formation of bilateral or multiple unilateral fluid-filled cysts, presymptomatic diagnosis is available by abdominal ultrasound examination of young adults at risk, which also allows improving hypertension management in these patients, appearing long before the actual manifestation of renal disease. Although mutation rate is believed to be high, especially in PKD1, approximately half of the cases are still ancestry-related, allowing the diagnosis by linkage analysis. The PKD1 gene contains 46 exons, encoding the membrane protein polycystin 1 involved in cell-to-cell interaction, while PKD2 has at least 15 exons and encodes polycystin 2, which is a channel protein. Both of these proteins interact to produce new calcium-permeable nonselective cation currents, contributing to fluid flow sensation by the primary cilium in renal epithelium. Therefore, a loss or dysfunction of any of these proteins leads to polycystic kidney disease (PKD), due to the inability of cells to sense mechanical cues that normally regulate renal tubular morphology and function.

Because direct mutation testing was not available either for PGD1 or PKD2 in the case presented below, and their clinical manifestations are almost identical, except for the severity and earlier onset of PKD1, the linkage analysis was applied for both of these genes to trace the disease gene from the affected person through the family to the patient. DNA sequencing may be applied for PKD2, but this may not be expected to be useful for the PKD1 gene, because a large part of this gene is a duplication of multiple pseudogenes, which produce mRNA but are not translated.

PGD was performed for a woman with a family history of ADPKD, who inherited the disease from her father with a severe ADPKD and also had a brother with clinical symptoms of ADPKD (Figs. 3.19 and 3.20). Initial linkage analysis could not exclude either PKD1 or PKD2 as a cause of ADPKD in this family, so a set of linked markers have been designed to trace both PKD1 and PKD2 in the same reaction.

A PGD cycle was performed using PB sampling, and blastomere biopsy, described above. PB1 and PB2 were removed in sequence and

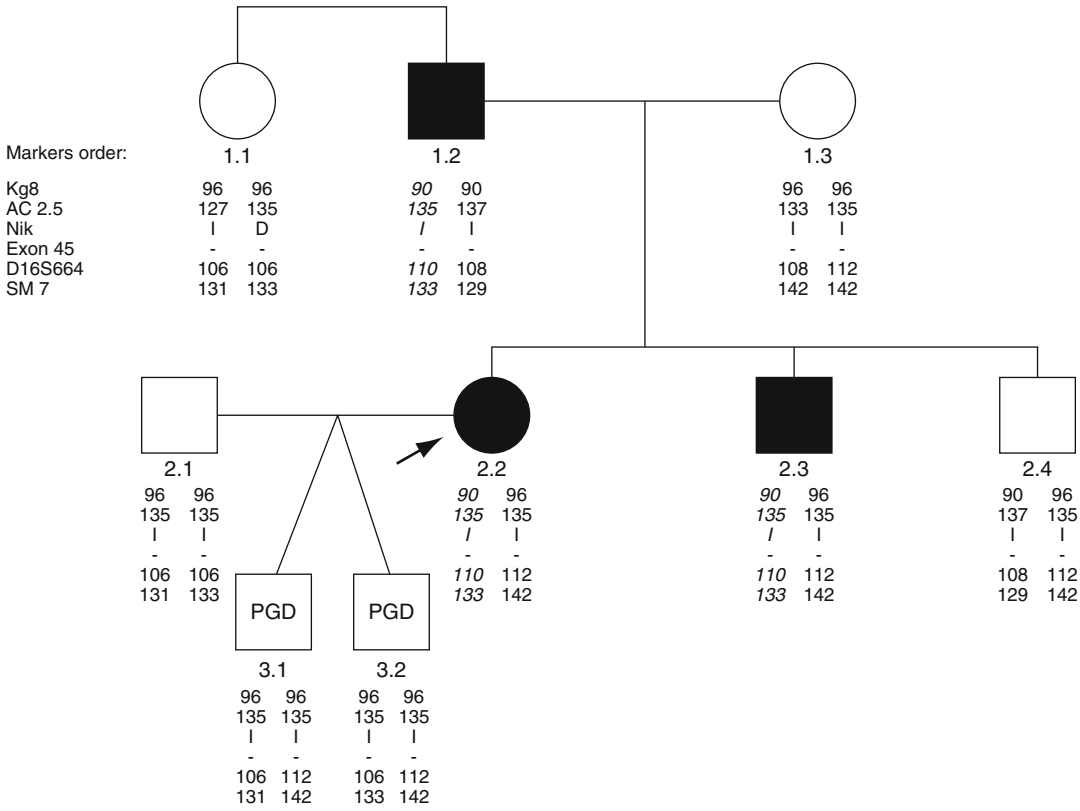



**Fig. 3.19** Preimplantation linked marker analysis for PKD-1 resulting in the birth of unaffected twins. (*Upper panel*) ADPKD mutation originated from the affected maternal father (1.2), from whom the patient (2.2) inherited the mutant chromosome 16 (*bold*). Mutation-free chromosomes of maternal mother (1.3) are shown in *non-bold face*. (*Middle panel*) The affected patient (2.2) had one affected brother (2.3), who also inherited the mutant chromosome from his father, and the same normal chromosomes 16 from the mother. Her unaffected husband's (2.1) normal chromosomes 16 differ by one of the markers (131 vs. 133 bp), which makes it possible to identify twins, resulting from PGD (3.1 and 3.2) (*lower panel*)

tested by the multiplex nested PCR analysis, involving the above markers simultaneously in a multiplex heminested system. The preselection of mutation-free oocytes was performed based on the linked marker analysis, involving three closely linked markers to PKD1, Kg8, D16S664, and SM7, and four closely linked markers to PKD2, D4S2922, D4S2458, D4S423, and D4S1557 (primers and reaction conditions are presented in Tables 3.13 and 3.14). The maternal haplotypes were 90, 110, and 133 bp repeats for Kg8, D16S664, and SM7 markers, respectively, to trace PKD1 (Fig. 3.19), and 123, 91, 133, and 100 bp repeat for D4S2922, D4S2458, D4S23, and D4S1557 markers, respectively, to trace the PKD2 gene (Fig. 3.20).

The embryos derived from the oocytes free of PKD1 and PKD2, based on the information about polymorphic markers, were preselected for transfer back to the patient, while those predicted to be mutant or with inconclusive marker information were further tested by blastomere analysis removed from the resulting embryos either to confirm the diagnosis or identify additional mutation-free embryos for transfer.

Of 14 oocytes available for testing, only 11 were with the information for both PB1 and PB2. Two of these oocytes were predicted to contain PKD1 (oocytes #1 and #4), 3 to contain PKD2 (oocytes #2, #8, and #9), 3 neither PKD1 nor PKD2 (oocytes #5, #7, and #10), and 3 inconclusive for one or both genes, due to failed

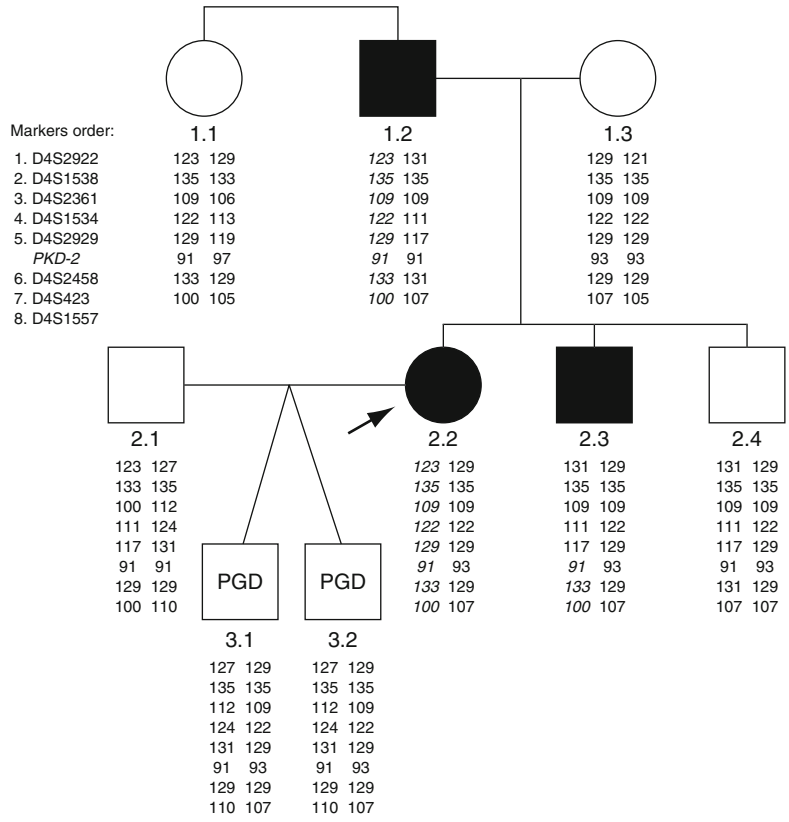



**Fig. 3.20** Preimplantation linked marker analysis for PKD-2 (same pedigree as in Fig. 3.19). (*Upper panel*) ADPKD mutation (mutant chromosome 4 is shown in *non-bold face*) originated from the affected maternal father (1.2), from whom the patient (2.2) inherited the mutant chromosome 4 (*non-bold face*). Mutation-free chromosomes of maternal mother (1.3) are shown in *bold face*. (*Middle panel*) The affected patient (2.2) had one affected brother (2.3), who also inherited the mutant chromosome from his father, but following recombination between D4S2929 and D42458, which resulted in a recombinant chromosome 4 (*bold* and *non-bold*), and the same normal chromosomes 16 from his mother. Her unaffected husband's (2.1) normal chromosomes 16 cannot be distinguished due to sharing the same markers, so the fact that the resulting PGD twins are dizygotic cannot be demonstrated (3.1 and 3.2) (*lower panel*)

amplification of PB2 (oocytes #3 and #6) or ADO in PB1 (oocyte #11) (Table 3.15). The latter oocytes, however, were shown to be normal following blastomere analysis, although there was still a probability of recombination between Kg8 marker and PKD1 gene. The other two embryos resulting from oocytes #3 and #6 with inconclusive results were left with insufficient information because the amplification failure of embryo #6, or shared parental markers for PKD2 in embryo #3. Blastomere biopsy from the embryos resulting from the oocytes predicted mutant have been confirmed to be affected, including one (embryo #9), in which according to linkage analysis, neither PKD1 nor PKD2 could be excluded.

Three embryos resulting from oocytes #5, #7, and #10 were transferred, yielding a twin pregnancy and the birth of two children confirmed to be free of both PKD1 and PKD2. As seen from the pattern of markers used to exclude PKD-1, these mutation-free children are dizygotic twins, as they inherited different normal chromosomes 16 from the father. The follow-up analysis of the embryos, resulting from the mutant oocytes, also confirmed the accuracy of sequential PB1 and PB2 by linked marker analysis, which is in accordance with the previously reported data

**Table 3.13** Primers for the detection of linked markers to PKD 1 (16p13.3)

| Gene/ polymorphism | Upper primer | Lower primer | Annealing $T_m$ (°C) |
|---|---|---|---|
| D16S3252 (Kg 8) nested | Outside | | 62–45 |
| | 5′ CGGCCATGCCCACAGAAG 3′ | 5′ CCTGGGGGCTGGCTCC 3′ | |
| | Outside | | 58 |
| | 5′ TGCAGCCTCAGTTGTGTTTC 3′ | 5′ Fam CAGGGTGGAGGAAGGTGAC 3′ | |
| D16S291 (AC2.5) heminested | Outside | | 62–45 |
| | 5′ TGCAGCCTCAGTTGTGTTTC 3′ | 5′ TGCTGGGATTACAGGCATG 3′ | |
| | Inside | | 58 |
| | 5′ TGCAGCCTCAGTTGTGTTTC 3′ | 5′ Fam AAGGCTGGCAGAGGAGGTGA 3′ | |
| Nik 2.9 Nested | Outside | | 62–45 |
| | 5′ GGCCCCAGGTCTCTTTCC 3′ | 5′ TCCGTGAGTTCCACTTGTC 3′ | |
| | Inside | | 58 |
| | 5′ GTGGACGGGCATACATCAGC 3′ | 5′ CCAGGCCGATGATGTGCAGC 3′ | |
| EXON 45 (*Ava II* & *Bsp1286 I* ) Nested | Outside | | 62–45 |
| | 5′ CATCCTGGTAGGTGACTGC 3′ | 5′ GAACAACTCCACCATCTCG 3′ | |
| | Inside | | 58 |
| | 5′ TACGCCCTCACTGGTGTC 3′ | 5′ ACAGCTCTCCACGCAAGG 3′ | |
| SM7 Heminested | Outside | | 62–45 |
| | 5′ CTCCGTCTCAAACAAACAAC 3′ | 5′ TTGTGGCCCAAATATATCA 3′ | |
| | Inside | | 58 |
| | 5′ CTCCGTCTCAAACAAACAAC 3′ | 5′ Hex TAGTCCTGGTCCCTTCCA 3′ | |
| D16S664 (CW3 ) Heminested | Outside | | 62–45 |
| | 5′ TCATCGTTAGTGGGAGTCTG 3′ | 5′ TGCCCGGTCATAAATTG 3′ | |
| | Inside | | 58 |
| | 5′ Fam TCAATGAGATTTCGGGTAA | 5′ TGCCCGGTCATAAATTG 3′ | |

(see Chap. 2). In addition, in cases of inconclusive results by PB analysis, the blastomere biopsy of the resulting embryo may still allow preselecting additional embryos for transfer, as in embryo #11, mentioned above. However, as for avoiding a potential recombination between PKD1 and Kg8 alleles closer linked markers were required, this embryo was frozen for possible future use by the couple.

The presented case was the first PGD for ADPKD [41]. The clinical relevance of PGD for ADPKD is evident from the fact that prenatal diagnosis and termination of pregnancy may not be acceptable for a considerable proportion of ADPKD cases, which might be benign with no clinical manifestation during the whole lifetime.

The practical implication of PGD for ADPKD is also obvious from the high worldwide prevalence of this disease. So the information about the availability of PGD for ADPKD will be useful for practicing physicians, to provide the at-risk couples with appropriate advice regarding the available options for avoiding the risk of producing a child with ADPKD. Because of a straightforward clinical manifestation of the disease and the availability of a simple ultrasound screening, with no need for the mutation identification, the PGD strategy described may be applied without extensive preparatory work, usually required for PGD for many other conditions.

The presented case also demonstrates the feasibility of PGD in cases without exact information on the causative gene involved, and, therefore, may have practical implications for PGD of other conditions, in which the mutation might not be known, but tracing of the mutant chromosome is possible using highly variable linked markers, which presently has much wider clinical implications [42]. Also, because it was not known

**Table 3.14** Primers for the detection of linked markers to PKD 2

| Gene/polymorphism | Upper primer | Lower primer | Annealing $T_m$ (°C) |
|---|---|---|---|
| D4S2922 (heminested) | Outside | | 62–45 |
| | 5′ AATAATAGAAAACAATCTCCTCA 3′ | 5′ TACACATCTTCTTTACTCAAACTAC 3′ | |
| | Inside | | 55 |
| | 5′ Hex AGTTCTTCCTCTCCTGCTTAT 3′ | 5′ TACACATCTTCTTTACTCAAACTAC 3′ | |
| D4S1538 (heminested) | Outside | | 62–45 |
| | 5′ AGCCTGGGTGACAGAGACTC 3′ | 5′ AACAATAAGTACATAAAAGAATGAAAC 3′ | |
| | Inside | | 55 |
| | 5′ AGCCTGGGTGACAGAGACTC 3′ | 5′ Hex ATAGAAGCCTCAGTGCCTCTT 3′ | |
| D4S2361 (heminested) | Outside | | 62–45 |
| | 5′ TCCTCCTCCCCACTGAAGT 3′ | 5′ GCACATGTACCCTAGAACTTAAAG 3′ | |
| | Inside | | 55 |
| | 5′ Fam AAAGCCACGTGACTTTCATTA 3′ | 5′ GCACATGTACCCTAGAACTTAAAG 3′ | |
| D4S1534 (heminested) | Outside | | 62–45 |
| | 5′ GTATTCAGTTTCAGCCCCATC 3′ | 5′ CCAAGGTAGAGGAGGGAAGG 3′ | |
| | Inside | | 55 |
| | 5′ HexGTGAATATGCAGGTTACTCCCA 3′ | 5′ CCAAGGTAGAGGAGGGAAGG 3′ | |
| D4S2929 (heminested) | Outside | | 62–45 |
| | 5′ TGTATTCAGTTTCAGCCCCATC 3′ | 5′ AGACCAGCCCAAGGTAGAGG 3′ | |
| | Inside | | 55 |
| | 5′ Fam GAATATGCAGGTTACTCCCAGGA 3′ | 5′ AGACCAGCCCAAGGTAGAGG 3′ | |
| D4S2458 (heminested) | Outside | | 62–45 |
| | 5′ CGAATGTTTCTATGTCATTGGAC 3′ | 5′ TGTCTTTCATTACAGAGATTCCTTC 3′ | |
| | Inside | | 55 |
| | 5′ CGAATGTTTCTATGTCATTGGAC 3′ | 5′ Hex CCCCTGTAGCTGCCCTGTA 3′ | |
| D4s423 (heminested) | Outside | | 62–45 |
| | 5′ TATTTGTTTCTCTTGAGTAGTTCCT 3′ | 5′ TGATCTCTTCTTTGGAAATATAAAT 3′ | |
| | Inside | | 55 |
| | 5′ TATTTGTTTCTCTTGAGTAGTTCCT 3′ | 5′ Hex GTACATTGCAAAGTCCTCCATC 3′ | |
| D4S1557 (heminested) | 5′ TTAATTTCAGACTTTTCTATTTTCA 3′ | 5′ GGAGATATTGGAAAGGCTGT 3′ | 62–45 |
| | 5′ FamTATATTCTCGATATGAGATTGTGG 3′ | 5′ GGAGATATTGGAAAGGCTGT 3′ | 55 |

**Table 3.15** Results of polar body and blastomere testing for PKD 1 and PKD 2 by linked marker analysis

| | | PKD 1 | | | PKD 2 | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Oocyte # | Cell type | Kg8 | D16S664 | SM7 | D4S2922 | D4S2458 | D4S423 | D4S1557 | Predicted genotype | ET |
| **1** | **PB1** | 96 | 112 | 142 | **123**/129 | **ADO**/93 | **133**/129 | **100**/107 | **Oocyte : PKD1 affected** | **NO** |
| | **PB2** | **90** | **110** | **133** | **123** | **91** | **133** | **100** | **PKD2 normal** | |
| **2** | **PB1** | 90/**96** | 110/**112** | 133/**ADO** | **123**/129 | **91**/93 | 129 | **100** | **Oocyte : PKD1 normal** | **NO** |
| | **PB2** | 90 | 110 | 133 | **129** | 93 | 129 | 107 | **PKD2 affected** | |
| 3 | **PB1** | **90**/96 | **110**/112 | **133**/ADO | 129 | **91**/93 | **133**/129 | **100**/107 | Oocyte : PKD1 normal | **NO** |
| | **PB2** | FA | **110** | **133** | **123** | **91** | **133** | **100** | PKD2 inconclusive | |
| | **Blast** | 96/96 | 106/112 | 133/142 | 127/**123** | 91/93 | 129/129 | 110/107 | **Embryo** : PKD1 normal | |
| | | | | | | | | | **PKD2 inconclusive** | |
| 4 | **PB1** | **ADO**/96 | **110**/112 | **ADO**/142 | **123**/129 | **91**/93 | **133**/129 | **100**/107 | Oocyte : PKD1 affected | **NO** |
| | **PB2** | 96 | 112 | 142 | **123** | **91** | **133** | **100** | PKD2 normal | |
| | **Blast** | 96/**90** | ADO/**110** | 133/**133** | 123/129 | 91/93 | 129/129 | 100/107 | **Embryo** : PKD1 affected | |
| | | | | | | | | | **PKD2 normal** | |
| 5 | **PB1** | **90**/96 | **110**/112 | **133**/ADO | **123** | **91** | **133** | **100** | Oocyte : PKD1 normal | **YES** |
| | **PB2** | **90** | **110** | **133** | 129 | 93 | 129 | 107 | **PKD2 normal** | |
| 6 | **PB1** | 96 | **110**/112 | **133**/142 | **123** | **91**/93 | **133**/129 | **100**/107 | Oocyte : PKD1 inconclusive | **NO** |
| | **PB2** | FA | **110** | **133** | 129 | **91** | **133** | **100** | PKD2 inconclusive | |
| | **Blast** | FA | FA | FA | FA | FA | FA | FA | **Embryo : inconclusive** | |
| 7 | **PB1** | **90**/96 | **110**/112 | **133**/142 | **123**/129 | **91**/93 | **133**/129 | **100**/107 | **Oocyte : PKD1 normal** | **YES** |
| | **PB2** | **90** | **110** | **133** | **123** | **91** | **133** | **100** | **PKD2 normal** | |

(continued)

**Table 3.15** (continued)

| | | PKD 1 | | | PKD 2 | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Oocyte # | Cell type | Kg8 | D16S664 | SM7 | D4S2922 | D4S2458 | D4S423 | D4S1557 | Predicted genotype | ET |
| 8 | **PB1** | 96 | **110**/112 | 142 | **123**/129 | **91**/93 | **133**/129 | **100**/107 | Oocyte : PKD1 inconclusive | **NO** |
| | **PB2** | FA | **110** | **FA** | 129 | 93 | 129 | 107 | PKD2 affected | |
| | **Blast** | 96/[a] | 106/112 | 131/142 | 127/**123** | 91/**91** | 129/**133** | 110/**100** | Embryo: PKD1 inconclusive<br>**PKD2 affected** | |
| 9 | **PB1** | 96 | **ADO**/112 | 142 | **123**/129 | **91**/93 | **133**/129 | **100**/107 | Oocyte : PKD1 inconclusive | **NO** |
| | **PB2** | FA | 112 | **FA** | 129 | 93 | 129 | 107 | PKD2 affected | |
| | **Blast** | 96/**90** | 106/**110** | 133/**133** | 123/**123** | 91/**91** | ADO/**133** | 100/**100** | **Embryo : PKD1 affected**<br>**PKD2 affected** | |
| 10 | **PB1** | **90/ADO** | **110**/112 | **133**/142 | **123**/129 | **91**/93 | **133**/129 | **100**/107 | Oocyte : PKD1 normal | **YES** |
| | **PB2** | **90** | **110** | **133** | **123** | **91** | **133** | **100** | PKD2 normal | |
| 11 | **PB1** | 96/[a] | **110**/112 | **ADO**/142 | **123** | **91** | **133** | **100** | **Oocyte : PKD1 inconclusive** | **YES** |
| | **PB2** | **90** | **110** | **133** | 129 | 93 | 129 | 107 | PKD2 normal | |
| | **Blast** | 96/[a] | 106/112 | 133/142 | 123/129 | 91/93 | 129/129 | 100/107 | **Embryo**: PKD1 normal<br>**PKD2 normal** | |
| **Affected mother** | | **90**/96 | **110**/112 | **133**/142 | **123**/129 | **91**/93 | **133**/129 | **100**/107 | | |
| **Affected maternal brother** | | **90**/96 | **110**/112 | **133**/142 | **131**/129 | **91**/93 | **133**/129 | **100**/107 | | |
| **Unaffected father** | | 96/96 | 106/106 | 131/133 | 123/127 | 91/91 | 129/129 | 100/110 | | |

*PB1* first polar body, *PB2* second polar body, *Blast* blastomere, *FA* failed amplification, *ADO* allele dropout, *ET* embryo transfer
Bolded number of repeats corresponds to the expected mutant alleles
[a]Means inconclusive results

whether the disease was caused by PKD1 or PKD2, the couple was treated as if both parents were carriers of both PKD1 and PKD2 genes. So the PGD strategy was to exclude the possibility of inheritance of both conditions by tracing markers for PKD1 and PKD2.

The other case, in which we performed concomitant testing for more than one genetic condition, was simultaneous PGD for Charcot-Marie-Tooth (CMT) and Fabry diseases in a couple, with both partners carrying mutations for both of these diseases. Testing was performed by sequential PB1 and PB2 analysis of Fabry disease, followed by embryo biopsy and testing for CMT, which allowed the identification and transfer of an unaffected embryo, which resulted in a triplet pregnancy and the birth of three healthy children, which appeared to be monozygotic triplets. Although the mechanism for the formation of monozygotic triplets is not understood, the data show that concomitant PGD for more than one condition is feasible and may be performed using the combination of different biopsy techniques, allowing the accurate detection of a healthy child free from both of conditions.

A similar approach has previously been described in a childless Ashkenazi Jewish couple, at risk for producing a child with Tay Sacs (TS) and Gaucher disease (GD), with both parents carrying two different mutations in both the β hexosaminidase A (HEX A) and the β glucocerebrosidase (GBD) genes [43]. The authors were able to diagnose six embryos, of which one was wild type for both TS and GD, and three were wild type for GD and carriers of TS, with the remaining two being compound heterozygote for TS. Two of the four transferable embryos which developed into blastocysts were transferred, resulting in a singleton pregnancy and the birth of a healthy child free from both of conditions.

These cases demonstrate the feasibility and advantages of analyzing a large number of markers in a single multiplex reaction allowing the analysis of multiple diseases in cases where couples are carriers of mutations in several genes. This is particularly common in the Ashkenazi Jewish population, in which there are a large number of prevalent autosomal-recessive diseases.

## 3.6 De Novo Mutations

As presented above, with an increasing number of different genetic disorders for which PGD is being applied each year, PGD may presently be applicable for any inherited disorder for which sequence information or relevant haplotypes are available for the detection by direct mutation analysis or haplotyping in oocytes or embryos [3, 4]. According to the current guidelines, performing PGD requires knowledge of sequence information for Mendelian diseases, but may also be performed when the exact mutation is unknown, through the application of the linkage analysis [3]. As described above, with expanding use of polymorphic markers, the linkage analysis may allow PGD for any genetic disease irrespective of the availability of specific sequence information (see also 42). This approach is more universal, making it possible to track the inheritance of the mutation without actual testing for the gene itself.

However, the above approaches cannot be applied in cases of de novo mutations (DNM) in parent(s) or affected children, as neither origin nor relevant haplotypes are available for tracing the inheritance of this DNM in single cells biopsied from embryos or in oocytes. On the other hand, with the improved awareness of PGD, an increasing number of couples request PGD, without any family history of the genetic disease that has been first diagnosed in one of the parents or in their affected children. So the developments in PGD strategies for the genetic conditions determined by DNM are presented below, which represents the first systematic clinical experience of PGD for over 100 cases with DNM [44].

The PGD strategy was developed for a total of 80 families with 38 different genetic disorders, determined by 33 dominant, 3 recessive, and 2 X-linked DNM (the list of DNM for which this PGD strategy was performed is presented in Table 3.16). The majority of these families (71 of 80) were with dominant mutations, of which 40 were of paternal origin, including 2 cases of gonadal mosaicism, 46 of maternal origin, including 1 with gonadal mosaicism, and 9 detected for the first time only in the affected children. All

**Table 3.16** Outcome of PGD for DNM

| Disease | Origin of de novo mutation | | | Number of patients with PGD | Number of cycles | Number of transfers | Number of embryos transferred | Pregnancy | Birth |
|---|---|---|---|---|---|---|---|---|---|
| | Paternal | Maternal | Detected in child | | | | | | |
| Autosomal-dominant [33] | | | | | | | | | |
| Adenomatous polyposis of the colon (FAP) | 1 | 3 | | 2 | 7 | 7 | 13 | 1 | 1 |
| Basal cell nevus syndrome (Gorlin syndrome) | 3 | 1 | | 4 | 5 | 4 | 7 | 2 | 2 |
| Brachydactyly (BDB1) | 1 | | | 1 | 3 | 3 | 4 | 2 | 2 |
| Corneal dystrophy | | 1 | | 0 | 0 | 0 | 0 | 0 | 0 |
| Crouson syndrome (CFD1) | 1 | 2 | | 3 | 5 | 4 | 8 | 3 | 2 |
| Darier disease (DAR) | | 1 | | 1 | 1 | 1 | 2 | 1 | 1 |
| Diamond–Blackfan anemia (DBA) | | 2 | 3 | 4 | 9 | 6 | 8 | 3 | 3 |
| Emery–Dreifuss muscular dystrophy (EDMD2) | 2 | | | 2 | 3 | 2 | 4 | 1 | 1 |
| Epileptic encephalopathy, early infantile | | | 1 | 1 | 1 | 1 | 2 | 1 | 1 |
| Exostoses, multiple (EXT1) | 2 | | | 2 | 2 | 2 | 5 | 1 | 1 |
| Facioscapulohumeral muscular dystrophy (FSHD) | 4 | | | 3 | 9 | 6 | 9 | 3 | 5 |
| Kallmann syndrome (KAL2) | | 1 | | 1 | 1 | 1 | 2 | 1 | 1 |
| Loyes–Dietz syndrome (LDS1A) | | 1 | | 1 | 1 | 1 | 2 | 1 | 1 |
| Malignant rhabdoid tumor (SMARCB1) | | | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| Marfan syndrome (MFS) | 5 | 3 | | 6 | 13 | 12 | 21 | 6 | 4 |
| Metaphyseal dysplasia | 1 | | | 1 | 1 | 1 | 2 | 1 | 1 |
| Multiple endocrine neoplasia, type I (MENI) | 1 | | | 1 | 2 | 2 | 4 | 1 | 1 |
| Multiple endocrine neoplasia, type II (MEN2B) | 1 | | | 1 | 1 | 1 | 2 | 0 | 0 |
| Neurofibromatosis, type I (NF1) | 3 | 13 | 1 | 13 | 24 | 22 | 38 | 11 | 10 |
| Neurofibromatosis, type II (NF2) | 1 | | | 1 | 2 | 2 | 4 | 2 | 3 |
| Optic atrophy 1 (OPA1) | 1 | 1 | | 0 | 0 | 0 | 0 | 0 | 0 |
| Osteogenesis imperfecta I (OI I) | 5 | 5 | | 7 | 19 | 11 | 22 | 5 | 6 |
| Peutz–Jeghers syndrome (PJS) | | 1 | | 1 | 2 | 2 | 3 | 2 | 1 |
| Pfeiffer syndrome | | 1 | | 1 | 1 | 1 | 2 | 1 | 1 |
| Retinoblastoma (RB1) | 3 | 3 | | 3 | 7 | 7 | 12 | 1 | 1 |
| Rett syndrome (RTT) | | | 1 | 1 | 1 | 1 | 1 | 0 | 0 |
| Sotos syndrome | | 1 | | 1 | 1 | 1 | 1 | 1 | 1 |
| Spinocerebellar ataxia 6 (SCA6) | | 1 | | 1 | 2 | 1 | 2 | 0 | 0 |
| Stickler syndrome (STL1) | | 1 | | 1 | 1 | 1 | 3 | 0 | 0 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Treacher–Collins syndrome (TCOF) | 1 | 1 | | 1 | 1 | 1 | 2 | 1 | 2 |
| Tuberous sclerosis 1 (TSC1) | 1 | 2 | | 3 | 7 | 7 | 10 | 3 | 3 |
| Tuberous sclerosis 2 (TSC2) | | 1 | | 1 | 1 | 1 | 2 | 0 | 0 |
| Von Hippel–Lindau syndrome (VHL) | 3 | | | 2 | 3 | 3 | 4 | 2 | 2 |
| Subtotal | 40 | 46 | 7 | 71 | 136 | 115 | 201 | 57 | 57 |
| X-linked [2] | | | | | | | | | |
| Granulomatous disease, chronic (CGD) | | 1 | | 1 | 3 | 3 | 4 | 1 | 0 |
| Incontinentia pigmenti (IP) | | 5 | | 5 | 8 | 5 | 8 | 3 | 4 |
| Subtotal | | 6 | | 6 | 11 | 8 | 12 | 4 | 4 |
| Autosomal-recessive [3] | | | | | | | | | |
| Cystic fibrosis (CF) | | | 1 | 1 | 1 | 1 | 2 | 1 | 2 |
| Fanconi anemia I (FA) | | | 1 | 1 | 2 | 2 | 2 | 0 | 0 |
| Spinal muscular atrophy, type I (SMA) | 1 | | | 1 | 1 | 1 | 2 | 1 | 1 |
| Subtotal | 1 | 0 | 2 | 3 | 4 | 4 | 6 | 2 | 3 |
| Total [38] | 41 | 52 | 9 | 80 | 151 | 127 (84%) | 219 1.72 | 63 (49.6%) | 64 |

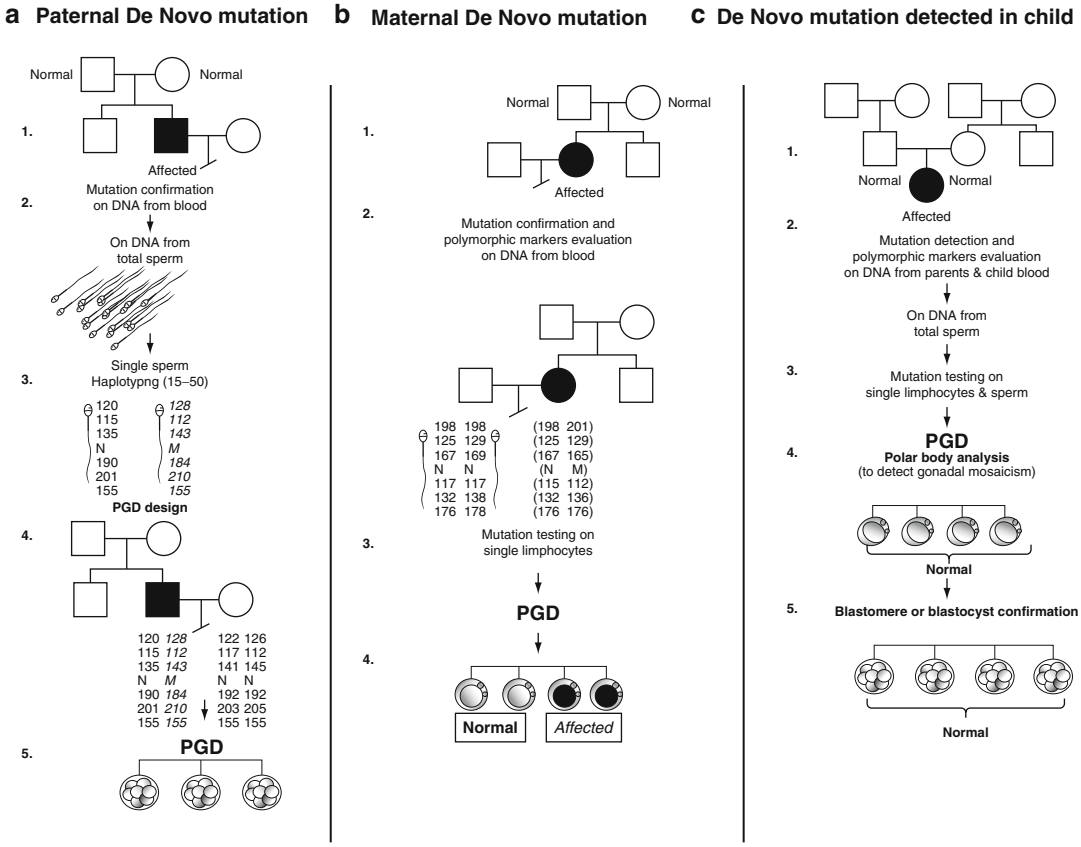



**Fig. 3.21** PGD strategies for DNM of different origin. (**a**) Case workout for DNM detected in male partner: (1) Pedigree in two generations. (2) Mutation verification in DNA extracted from blood and total sperm. (3) Amplification of partner's single sperm to establish normal and affected haplotypes required for PGD cycle preparation. (4) Amplification of patient's DNA to identify the most informative markers for PGD. (5) PGD by blastomere or blastocyst biopsy for combined mutation and linkage analysis. (**b**) Case workout for DNM detected in mother (patient): (1) Pedigree in two generations. (2) Mutation verification in DNA extracted from whole blood or cheek swabs and single lymphocytes. Paternal haplotypes are analyzed on a single sperm for more accurate embryo genotype prediction. (3) PGD by PB1 and PB2 analysis to identify DNM-free oocytes and establish maternal haplotypes, followed by blastomere or blastocyst analysis to confirm the diagnosis. (**c**) Case workout for DNM detected first in affected offspring: (1) Pedigree in three generations. (2) Verification of DNM in child's DNA extracted from blood or cheek swabs, and mutation testing on DNA extracted from parents' whole blood and total sperm. (3) Mutation evaluation on single lymphocytes and single-sperm testing to rule out paternal gonadal mosaicism. (4) PGD by polar body analysis to detect potential maternal gonadal mosaicism. (5) Blastomere or blastocyst analysis to confirm the absence of the mutation

three couples with DNM of autosomal-recessive inheritance were of paternal origin, including cystic fibrosis (CFTR), spinal muscular atrophy (SMA) and Fanconi anemia (FA). PGD for 2 X-linked DNM included PGD for chronic granulomatosys and incontinentia pigmenti.

As seen from Fig. 3.21, PGD strategies for these families were different depending on the origin of DNM, and included an extensive DNA analysis of the parents and affected children prior to PGD, with the mutation verification, polymorphic marker evaluation, whole- and single-sperm testing, and PB analysis in order to establish the normal and mutant haplotypes, without which PGD cannot always be performed. In cases of DNM of paternal origin, the DNM was first confirmed on the paternal DNA from blood and total sperm, followed by single-sperm typing to

**Table 3.17** Summary of testing and clinical outcome of PGD for DNM

| Type of inheritance | Number of patients | Number of cycles | Number of embryo transfers | Number of embryos | Number of pregnancies | Number of births |
|---|---|---|---|---|---|---|
| Autosomal-dominant | 71 | 136 | 115 | 198 | 57 | 57 |
| X-linked | 6 | 11 | 8 | 14 | 4 | 4 |
| Autosomal-recessive | 3 | 4 | 4 | 7 | 2 | 3 |
| Total | 80 | 151 | 127 (84%) | 219 (1.72) | 63 (49.6%) | 64 |

determine the proportion of sperm with DNM and relevant normal and mutant haplotypes, as described earlier. For a higher reliability of testing, the relevant maternal linked markers were also detected, to be able to trace for possible shared maternal and paternal markers. To exclude misdiagnosis, PGD involved simultaneous detection of the causative gene, and at least three highly polymorphic markers, closely linked to the gene tested, to ensure detecting preferential amplification and ADO, the main potential causes of PGD misdiagnoses. This involved a multiplex nested PCR analysis, with the initial first round PCR reaction containing all the pairs of outside primers, followed by amplification of separate aliquots of the resulting PCR product with the inside primers specific for each site. Following the nested amplification, PCR products were analyzed either by restriction digesting or direct fragment-size analysis.

In cases of DNM of maternal origin, DNM was first confirmed in maternal blood, and PGD was performed, when possible, by PB analysis, to identify the normal and mutant maternal haplotypes. Also, in order to trace the relevant paternal haplotypes, single-sperm typing was performed, whenever possible, for avoiding misdiagnosis caused by possible shared paternal and maternal markers.

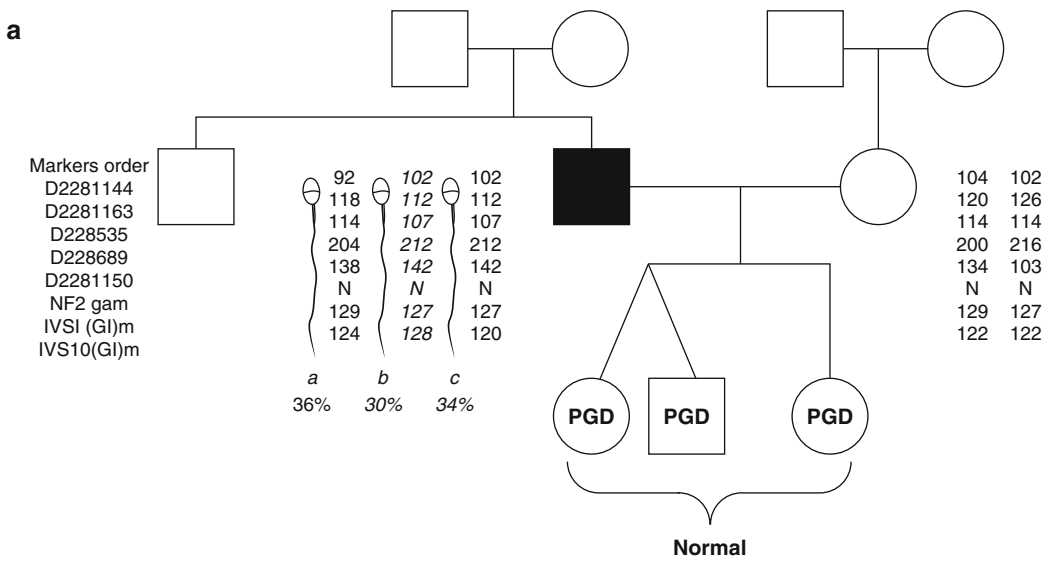
In cases of DNM-detected first in children, the mutation was verified in their whole blood DNA, followed by testing for the mutation in paternal DNA from blood, total and single sperm, if the DNM appears of paternal origin. In DMD of maternal origin, PGD was performed by the PB approach, with confirmation of the diagnosis by embryo biopsy, if necessary.

So, in contrast to previous PGD practice, performing PGD for DNM required extensive preparatory DNA work before performing the actual PGD, with the additional tests including single-sperm analysis and the requirement of performing sequential PB1 and PB2, followed by blastomere or blastocyst analysis, described in detail in Chap. 2. As in previous PGD protocols, the embryos without DNM were transferred in the same cycles, while the affected ones were used for confirmation of diagnosis, at least for in-house cases. Predicted diagnoses were followed up after delivery, while the spare unaffected embryos were frozen for future use by the families.

Overall, 151 PGD cycles for DNM were performed for 80 families under study, resulting in preselection and transfer of 219 (1.72 per cycle) DNM-free embryos in 127 (84%) PGD cycles, yielding 63 (49.6%) unaffected pregnancies and the birth of 64 healthy children, confirmed to be free of DNM tested (Table 3.17).

The largest group was PGD for DNM of autosomal-dominant type, including 136 cycles from 71 patients, which resulted in transfer of 201 DNM-free embryos in 115 cycles, yielding 57 pregnancies and the birth of 53 unaffected children, with 4 ongoing pregnancies by the present time. The most frequent conditions in this group were neurofibromatosis type 1 (NF1) (24 cycles), osteogenesis imperfecta (19 cycles), Marfan syndrome (13 cycles), facioscapularhumoral muscular dystrophy (FSHD) and Blackfan Diamond anemia (BDA) (9 cycles each), familial adenomatous polyposis (FAP), tuberous sclerosis type 1 (TSC1) and retinoblastoma (RB) (7 cycles each), and Gorlin and Crouson syndromes (5 cycles each). From 1 to 3 cycles were performed for the remaining conditions, listed in Table 3.16.

The example of PGD design for DNM of dominant inheritance is presented below for a



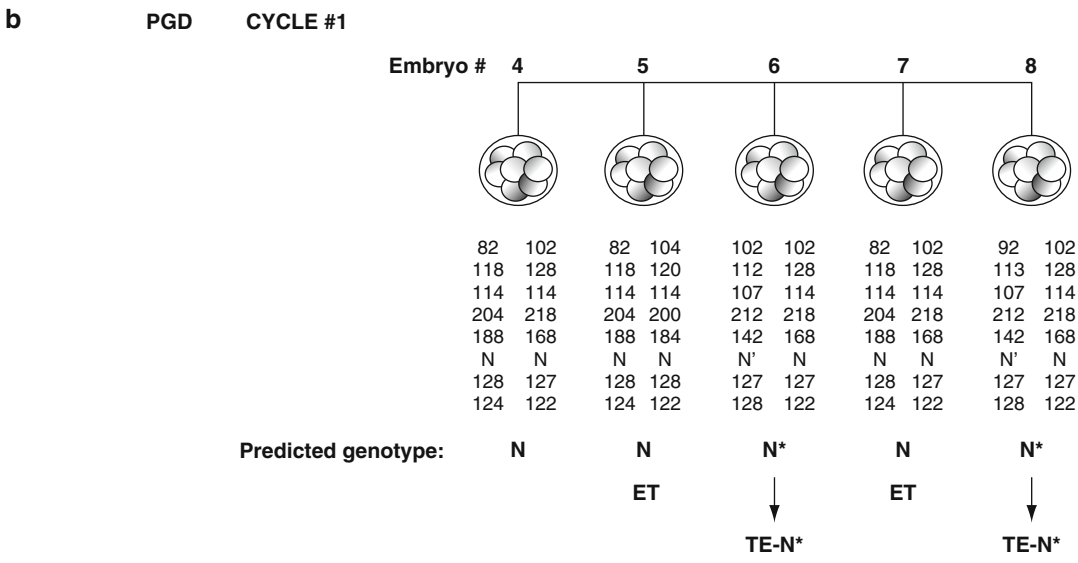




**Fig. 3.22** PGD for DNM in NF2 gene (c114+ 2T-C splicing mutation) of paternal origin. (**a**) Family pedigree showing that that DNM in NF2 gene was first detected in the father. Single-sperm analysis via multiplex heminested PCR revealed gonadal mosaicism with three different haplotypes: *a* – normal; *b* – mutant containing c.114+2 T-C allele; and *c* – mutant without c.114+2 T-C allele in NF2. Maternal linkage was based on DNA amplification of blastomeres in PGD cycle. Mutation and marker order is printed in *upper left corner*. (a) Outcome of the first PGD cycle. Blastomeres from five embryos were subjected to combined mutation and linkage analysis by multiplex heminested PCR. Three embryos (#4, #5, and #7) were predicted to be free from the paternal mutation based on the presence of normal sequence (*N*) in NF2 gene and confirmed by linked markers (haplotype *a*). Two embryos (#6 and #8) were predicted to have normal sequence (*N**) on haplotype *c*. The accuracy of this prediction was decreased due to a potential allele dropout (ADO) of the dominant mutation in single blastomere. Trophectoderm (*TE*) biopsy from these embryos confirmed the presence of the normal sequence of NF2 gene. Embryos #5 and #7 were transferred, resulting in an unaffected pregnancy and the birth of a healthy boy and girl confirmed by postnatal testing. (**b**) Outcome of the second PGD cycle. Combined mutation and linkage analysis by multiplex heminested PCR was performed on blastomeres from ten embryos. Mutant haplotype b was detected only in embryo #13. Embryo #6 was missing all the maternal markers, suggesting monosomy of chromosome 22, in which the gene is localized. Although all the remaining embryos were predicted to be normal and free of mutation, only four of them (embryos #3, #4, #9, and #12) were with normal (*N*) paternal haplotype *a*, while embryos #2, #5, #7, and #11 were predicted to have normal sequence (*N**) on the mutant haplotype *c*. Blastocyst biopsy confirmed normal genotypes predicted on blastomeres. Two normal embryos (embryos #3 and #4) were transferred, resulting in clinical pregnancy and the delivery of a healthy girl confirmed by postnatal analysis

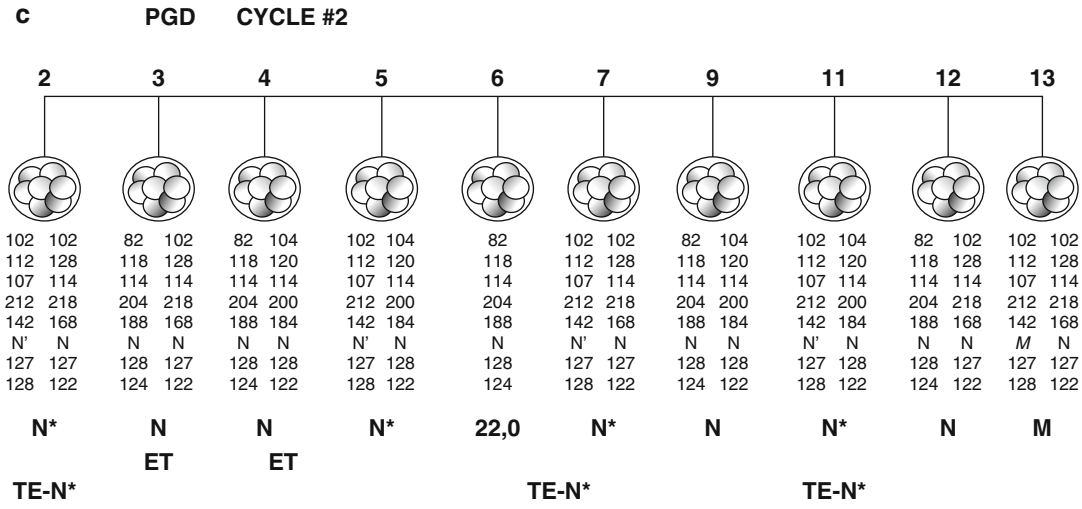



**Fig. 3.22** (continued)

couple with NF2 splicing mutation (c114+2T-C) detected in the husband with no previous family history of the disease (Fig. 3.22). DNA analysis in paternal blood confirmed the presence of NF2 splicing mutation (c114+2T-C), while testing of single sperms showed a gonadal mosaicism, represented by three types of sperms corresponding to three different haplotypes. Only 30% of sperms were represented by actual mutant haplotype, while 36% were normal, characterized by normal haplotype, and 34% contained a normal allele in the mutant haplotype.

PGD was based on detecting and avoiding the transfer of embryos with mutant haplotype with or without a mutant gene, while the embryos with normal haplotypes of paternal and maternal origin were transferred. As can be seen from Fig. 3.22, all the five tested embryos from the first PGD cycle may appear unaffected, despite the finding of the mutant haplotype in two of them (embryos #6 and #8), which, however, were missing the mutant gene. The remaining three embryos were with normal paternal and maternal haplotypes, of which two (embryos #5 and #7) were transferred, resulting in a twin pregnancy and the birth of two unaffected children. In the second PGD cycle for this couple, ten embryos were examined, of which only one contained the actual mutant haplotype, while three were with mutant haplotypes without the mutant gene, and the remaining six were with normal haplotypes. Two of these embryos (embryos #3 and #4) were transferred, resulting in a singleton pregnancy and the birth of an unaffected child.

The example of dominant DNM of maternal origin is presented in Fig. 3.23, in which gonadal mosaicism was also detected. DNM in the NF1 gene (intron 17–38 deletion) was first presented in the affected child, and appeared to be originated from the mother, who had three cell populations, represented by three haplotypes, including the normal, mutant with intron 17–38 deletion, and mutant without deletion. So PGD was based on preselection and transfer of the embryos with either normal maternal haplotype or with mutant maternal haplotype lacking intron 17–38 deletion. As seen from Fig. 3.23, of 11 embryos examined, despite the presence of 6 embryos with mutant maternal haplotypes, actually only 2 were affected (embryos #6 and #8), the other 4 (embryos #1, #2, #10, and #12) being unaffected as they contained no inton 17–38 deletion. Of the remaining 5 embryos, 2 were monosomic for the maternal chromosome (embryos #7 and #11), and 3 (embryos #3–5) contained only the normal parental haplotypes. One of these embryos (embryo #3) and the other demonstrating mutant maternal haplotype without deletion (embryo #2) were transferred, resulting in chemical pregnancy (Fig. 3.23).

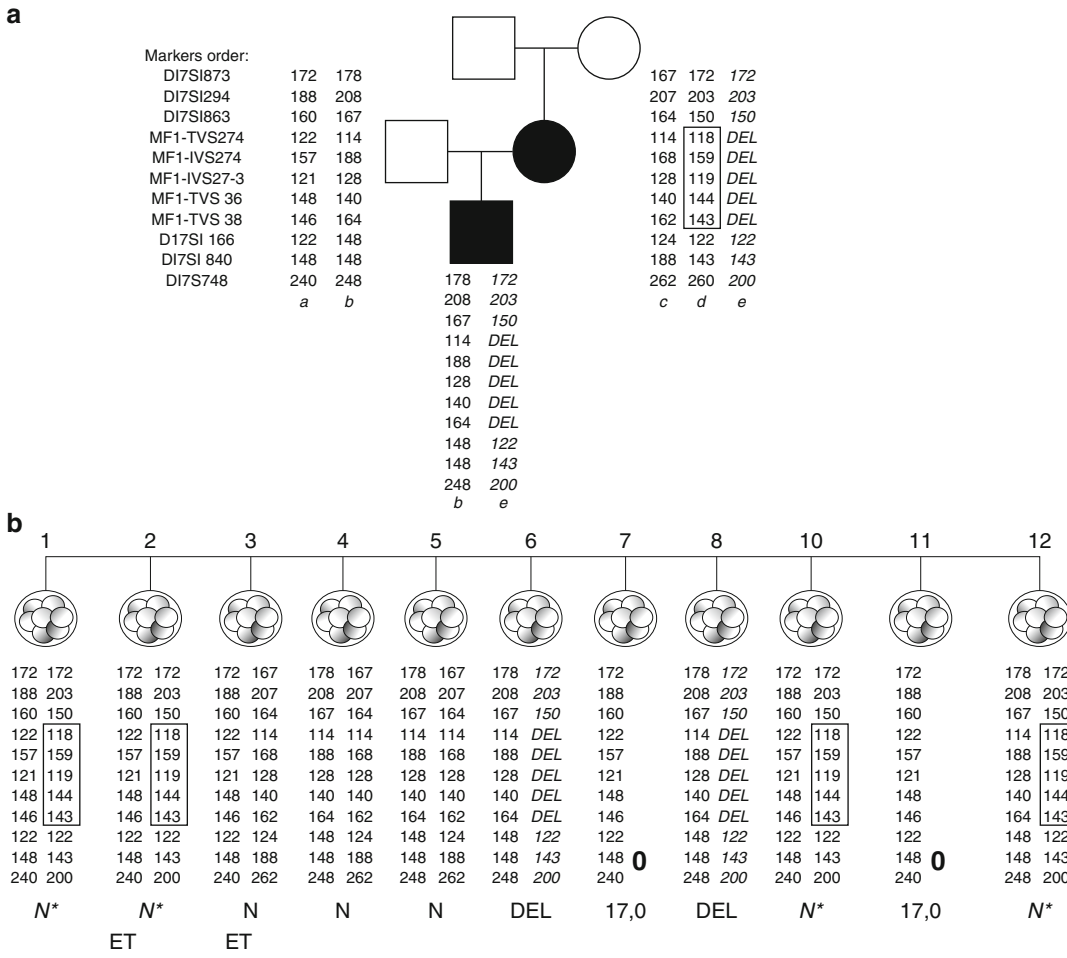



**Fig. 3.23** PGD for DMD in NF1 gene (intron 27–38 deletion) of maternal origin. (**a**) Family pedigree of a couple with an affected son carrying deletion of intron 27–38 in the NF1 gene. This deletion was not detected in maternal DNA from whole blood, although two haplotypes (*c*) and (*d*) were present, the latter corresponding to mutant haplotype corresponding to affected son's haplotype, but with no deletion. The expected deleted area on this "benign" chromosome (same haplotype as affected son received from the mother but without deletion) is framed. The actual mutant haplotype (*e*) with deletion was detected on maternal single lymphocytes. Paternal normal haplotypes (*a*) and (*b*) were established based on markers detected on the son's normal chromosome. Position and order of the markers and deletion in NF1 gene are shown on the *upper left*. (**b**) Outcome of PGD cycle, performed by multiplex heminested PCR on blastomeres from 11 embryos. Three embryos (embryos #3, #4, and #5) were predicted normal (*N*) based on the presence of maternal normal haplotype (*c*) and suitable for embryo transfer (*ET*). Four embryos (embryos #1, #2, #10, and #12) inherited the "benign" mutant maternal haplotype (*d*) and were also predicted normal (*N**) and suitable for embryo transfer (*ET*). Of the remaining four embryos, embryos #7 and #11 were predicted to have monosomy of chromosome 17, based on the absence of maternal alleles, while the other two (embryos #6 and #8) were predicted to be affected, based on the absence of maternal markers in deleted area (*DEL*). Two embryos (embryos #2 and #3) were transferred and resulted in a biochemical pregnancy

Only four PGD cycles were performed for DNM of autosomal-recessive type mentioned, including two cycles for *CF*, one for SMA, and one for Fanconi anemia (FA), presented in Fig. 3.24, which resulted in the transfer of seven embryos, yielding two pregnancies and the birth of two unaffected children (Table 3.17).

As presented in Fig. 3.24, DNM for FANC I was first detected in a child who was compound heterozygous for the C750G/E837X mutation.

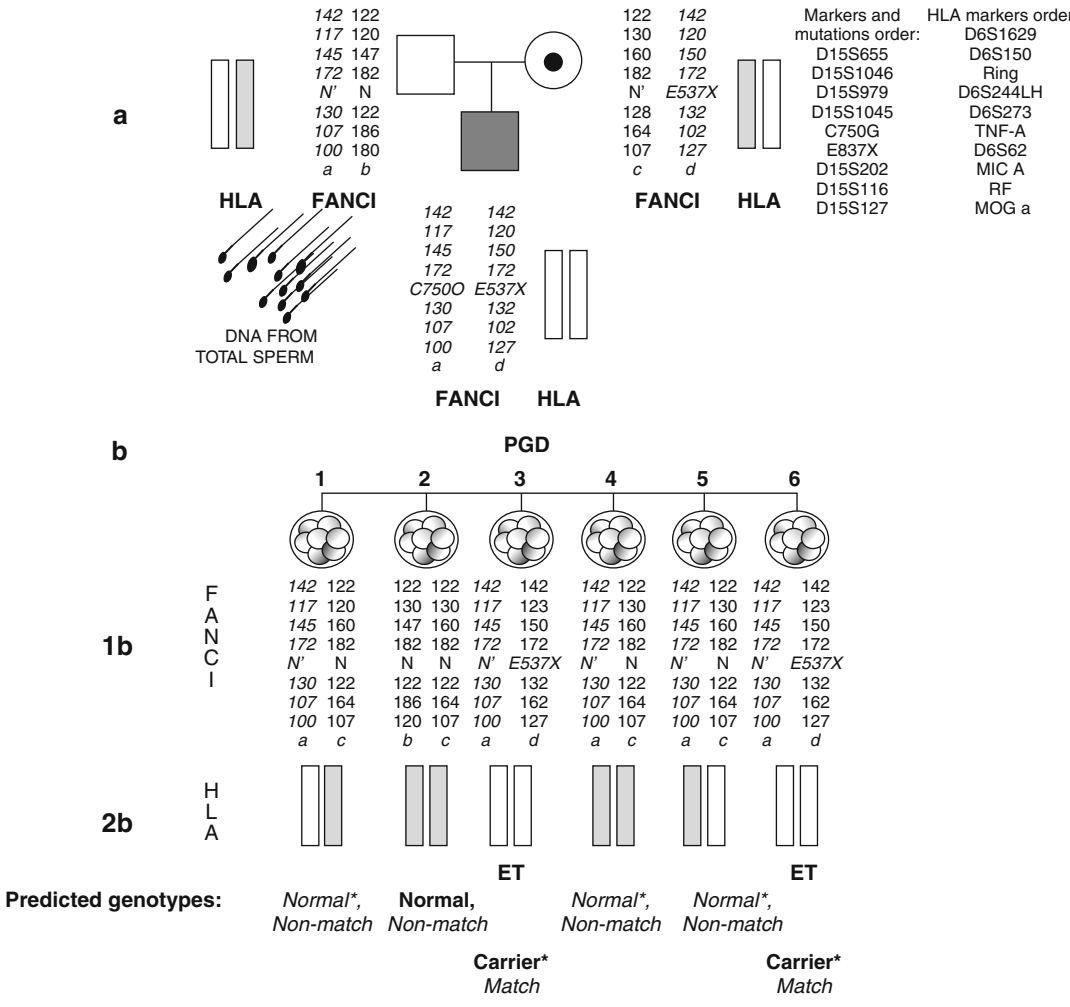



**Fig. 3.24** PGD for autosomal-recessive DNM detected first in an affected child who was compound heterozygous for C750G E837X mutations in the FANC I gene, combined with HLA genotyping. (**a**) Family pedigree showing HLA and mutation haplotypes, based on parental and affected child's genomic DNA testing. E837X mutation was detected in the carrier mother, but C750G mutation was absent in DNA extracted from paternal blood or whole-sperm samples. However, both normal and mutant haplotypes were detected in testing of single sperm. White bars represent HLA-matched genotypes. *Gray bars* depict non-matched haplotypes. Mutation and marker orders are printed in the *upper right corner*. (**b**) PGD cycle combined with HLA testing. *1b* – Multiplex heminested and fully nested amplification performed on blastomeres from six embryos did not reveal the paternal mutation (*1b*). Four embryos (embryos #1, #2, #4, and #5) were predicted normal (*N*) based on the absence of both mutations, of which embryos #1, #4, and #5 inherited "benign" paternal haplotype *a*, similar to one of the mutant haplotypes in the affected child, and embryo #2 inherited the normal haplotype *b*. The remaining embryos (#3 and #6) were predicted to be carriers of the maternal mutation E837X, but inherited the paternal haplotype *a*. *2b* – HLA marker analysis demonstrated the presence of two HLA-matched embryos (#3 and #6), which were transferred, but no pregnancy was achieved. *N** – shows benign paternal haplotype *a* similar to the mutant haplotype of the affected child

Testing of both parents for the presence of these mutations showed that the mother was a carrier of the E837X mutation, and characterized by two relevant haplotypes, while no mutation was found either in the paternal blood or whole sperm, despite the presence of both normal and mutant haplotypes in single sperms, which however was lacking the C750G mutation. Because the couple also requested HLA typing for possible stem cell transplantation required for the affected sibling,

the embryos were also tested for HLA haplotypes. As can be seen from Fig. 3.24, of the six embryos tested, only one embryo (embryo #2) inherited both maternal and paternal normal haplotypes, while two were with both paternal and maternal mutant haplotypes (embryos #3 and #6), but were unaffected heterozygous carriers, because the paternal mutant haplotype was missing the expected paternal FANC750G mutation. The remaining three embryos had normal maternal and mutant paternal haplotype, without a mutant gene involved. So all the embryos were actually unaffected, of which two heterozygous carriers appeared to be also an HLA match to the affected child (embryos #3 and #6) and transferred, resulting in no pregnancy.

Eleven PGD cycles were performed for X-linked DNM, including eight for incontinentia pigmenti and three for chronic granulomatosys, resulting in four pregnancies and the birth of four unaffected children (Table 3.17). The results of PGD for chronic granulomatosys, determined by DNM IVS 9+5G-A in the CYBB gene is presented in Fig. 3.25. DNM in this case was first detected in an affected child, who also required HLA-matched stem cell transplantation. So in addition to mutation analysis, HLA typing was performed, together with aneuploidy testing, because the mother was 36 years old.

DNA analysis in maternal blood failed to detect the mutant gene, while both normal and mutant haplotypes were present, despite the latter missing the mutant gene. PGD was performed by sequential PB1 and PB2 analysis in nine oocytes, showing that all the oocytes were normal, although four of them (oocytes #2, #3, #7, and #11) contained the maternal mutant haplotype, without the mutant gene. The testing of the embryos resulting from each of these oocytes confirmed the PB haplotype analysis, showing the lack of the mutant gene. All the embryos were found to be also aneuploidy-free, of which four (embryos #7, #8, #9, and #11) appeared to be also the exact HLA match to the affected sibling. Two of these embryos (embryos #8 and #9) were transferred, resulting in clinical pregnancy, which was spontaneously aborted in the first trimester.

The overall clinical outcome of PGD for DNM showed a success rate as high as 84% of identifying unaffected embryos for transfer (127 of 151 initiated cycles), with the average 1.72 embryos per transfer (219 embryos transferred in 127 cycles), resulting in a 49.6% pregnancy rate (63 clinical pregnancies) and the birth of 59 unaffected children (five pregnancies still ongoing at the time of submission of the paper), with no misdiagnosis observed in the follow-up analysis.

The presented experience is the first cumulative report of PGD for DNM, which could not be performed previously, due to unavailability of family history and lack of any affected family member to identify the origin of mutation and trace the inheritance of the mutant and normal alleles in oocytes and embryos. However, the presented data show that the strategies may be developed in search for the possible origin of DNM and relevant haplotypes as the basis for developing a PGD design for each particular couple with DNM, allowing a highly accurate preselection of oocytes and embryos free from DNM in question.

Although the strategies may differ depending on the type of DNM inheritance, the general approach involves the identification of DNM origin and search for a possible gonadal mosaicism and relevant parental haplotypes. As demonstrated in Fig. 3.21, one of the important steps is single-sperm typing, which was performed in 37 of 80 patients (46.3%). Overall 964 single sperms were tested, with the requirement for testing of at least 15 single sperms per patient, and as many as 50 per patient to exclude a possible gonadal mosaicism. Even if DNM is not identified, single-sperm typing may identify a “benign” mutant haplotype, represented by a mutant haplotype without DNM. The other important requirement is to identify the relevant linked markers in both parents even if only one is a DNM carrier. Although not always possible, PGD by PB approach to detect or confirm the maternal normal and mutant haplotypes is always the method of choice, performed in our material in 54 (35.8%) of 151 cycles.

The implications of gonadal mosaicism for genetic counseling of dominant disorders, such as NF1, TSC1, TSC2, lethal osteogenesis imperfecta, familial adenomatous polyposis, retinoblastoma, and X-linked dominant trait incontinentia

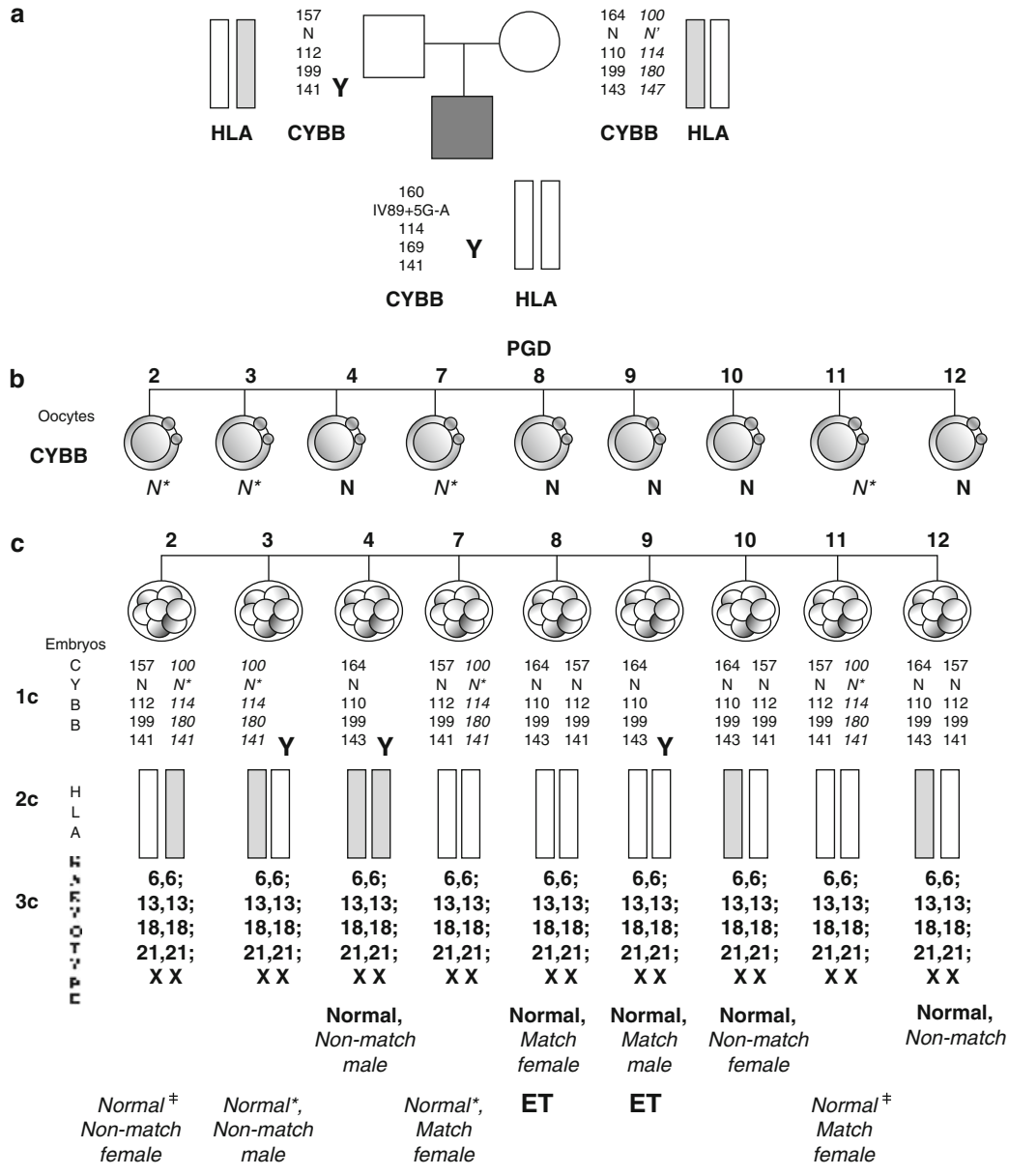



**Fig. 3.25** PGD for chronic granulomatous disease, determined by X-linked DNM IVS9+ 5G-A, combined with HLA genotyping and aneuploidy testing. (**a**) Family pedigree showing the mutation and closely linked to CYBB gene markers, and HLA-matched haplotypes depicted as white and non-matched colored in gray. (**b**) Sequential PB1 and PB2 analysis, showing that all the tested oocytes are normal, despite 4 of them containing the "benign" mutant haplotype without IVS9+ 5 mutation (*N**). Multiplex heminested PCR for combined mutation analysis (*1c*), HLA genotyping (*2c*) and karyotyping (*3c*) for six chromosomes by PCR on blastomeres. Two of four embryos (embryos #8 and #9), predicted to be HLA-matched and free of mutation and aneuploidy, were transferred resulting in a singleton pregnancy and the birth of an unaffected child (as indicated in the family pedigree by PGD). *ET* embryo transfer

pigmenti, have been recognized previously [45–50]. Although germinal mosaicism is thought to be common, its presence in families is usually difficult to detect and depends on the gene penetrance. A majority of the newly mutant genes will have mutated during the development of the

affected individual and not during the development of one of the parents' gonads, so testing of the affected child will often reveal mosaicism for the gene in question, but in many cases will remain undetected.

For example, all the oocytes and embryos tested from such cases in our experience appeared to be unaffected, irrespective of the origin of DMN (29 oocytes and 87 embryos). However, this does not mean that PGD is not justified in these cases, because the possibility of a low level of mosaicism in parents' gonads cannot be completely excluded. In our cases of DNM detected first in children, although no mutation was identified in either parent, the presence of mutant haplotypes without the mutant gene was evident, suggesting that there may still be a proportion of germ cells with the mutation remaining undetected. So PGD is indeed indicated in such cases to exclude any possibility of the mutant oocyte and embryo production due to undetected germinal mosaicism.

As expected, the majority of cases involved DNM of dominant inheritance, in agreement with the high mutation rate of dominant disorders. However, almost a similar proportion of DNM of dominant type was either of paternal (45%) or maternal (55%) origin, requiring testing for the presence of DNM in both parents. On the other hand, all the cases of DNM of recessing inheritance were of paternal origin, but the number of cases is not sufficient for conclusions.

It should be mentioned that, despite the complexity of PGD for DNM, the applied strategies appeared to be highly accurate. Based on testing of 631 oocytes by PB analysis and 1,145 embryos by blastomere biopsy, 219 mutation-free embryos were transferred, resulting in extremely high pregnancy rate (49.6%) and the birth of 59 healthy children with no misdiagnosis detected, suggesting 100% accuracy of the applied technique of PGD.

The presented data show that PGD for DNM is an important addition to the practice of PGD for Mendelian diseases, as it makes it now possible to offer PGD to any couple at risk for producing offspring with genetic disease, despite the traditional requirement of family data, which is not always available even in cases with known family history for the disease. So the data demonstrate feasibility of PGD for DNM, which may now be routinely performed with the accuracy of over 99%, using the established PGD strategy.

## 3.7 Late-Onset Disorders with Genetic Predisposition

The diseases with genetic predisposition have not traditionally been considered an indication for prenatal diagnosis, as this would lead to pregnancy termination, which may hardly be justified on the basis of genetic predisposition alone. On the other hand, the possibility of choosing the embryos free of genetic predisposition for transfer would obviate the need for considering pregnancy termination, as only potentially normal pregnancies are established. PGD for such conditions appeared to be acceptable on ethical grounds because only a limited number of the embryos available from hyperstimulation are selected for transfer.

### 3.7.1 Inherited Predisposition to Cancer

Cancers are the largest group of conditions with genetic predisposition for which PGD was performed. Our experience (Table 3.18) is currently the world's largest series, involving PGD for 197 PGD cycles performed for 103 couples at risk for producing 20 different inherited cancers, including BRCA 1 and 2, Li-Fraumeni disease, familial adenomatosis polyposis (FAP), familial colorectal cancer, hereditary nonpolyposis coli (HNPCC) (type 1 and 2), Von Hippel-Lindau syndrome (VHL), familial posterior fossa brain tumor (hSNF5), retinoblastoma (RB), neurofibromatosis 1 and 2 (NF1 and NF2), Nevoid basal cell carcinoma (NBCCS) or Gorlin syndrome, tuberous sclerosis (TSC type 1 and type 2), ataxia telangiectasia, and Fanconi anemia (FA). This resulted in the transfer of 284 (1.7 embryos on an average) unaffected embryos in 163 transfer cycles, yielding 66 (40.5%) unaffected pregnancies and

**Table 3.18** PGD experience for cancer predisposition

| DISEASE | Number of patients | Number of cycles | Number of transfers | Number of embryo transferred | Pregnancies | Births |
|---|---|---|---|---|---|---|
| Ataxia telangiectasia | 1 | 3 | 2 | 3 | 1 | 1 |
| BCNS (Gorlin) | 4 | 5 | 4 | 7 | 2 | 2 |
| Brain tumor | 1 | 1 | 1 | 1 | 0 | 0 |
| BRCA1 &2 | 19 | 31 | 23 | 39 | 10 | 14 |
| Fanconi anemia | 17 | 51 | 32 | 52 | 7 | 6 |
| FAP | 7 | 20 | 19 | 33 | 4 | 3 |
| HNPCC 1&2 | 3 | 8 | 8 | 16 | 4 | 5 |
| LFS | 4 | 6 | 5 | 9 | 2 | 2 |
| MEN 1&2 | 2 | 2 | 2 | 4 | 1 | 2 |
| NF1 & 2 | 22 | 40 | 38 | 69 | 18 | 21 |
| RB1 | 3 | 4 | 4 | 9 | 4 | 3 |
| TSC 1&2 | 9 | 13 | 13 | 18 | 5 | 8 |
| VHL | 5 | 5 | 4 | 9 | 3 | 4 |
| Peutz–Jegher | 1 | 2 | 2 | 3 | 2 | 1 |
| Exostosis mult. | 5 | 6 | 6 | 12 | 3 | 2 |
| Total | 103 | 197 | 163 | 284 | 66 | 74 |

the birth of 74 healthy children free from predisposition to those cancers. Some of these data were reported previously [51–53]. All these disorders are relatively rare autosomal-dominant conditions, with prevalence of 1 in 5,000 in the American populations for FAP, 1 in 15,000 for RB, 1 in 36,000 for VHL, and even rarer for others.

The first PGD for inherited predisposition has been performed for couples carrying p53 tumor suppressor gene mutations [53], known to determine a strong predisposition to the majority of cancers, which is described below.

#### 3.7.1.1 p53 Tumor Suppressor Gene Mutations

Two couples presented for PGD, one with the maternally and one with the paternally derived p53 tumor suppressor mutations. The paternal one was a missence mutation due to a transversion of a G to A in exon 5 of the p53 tumor suppressor gene, resulting in a change from Arginine to Histidine at the 175 amino acid residue of the protein [16, 54]. The carrier was a 38-year-old proband with Li-Fraumeni syndrome (LFS), diagnosed with rhabdomyosarcoma of the right shoulder at the age of 2 followed by right upper extremity amputation. At the age of 31, he was also diagnosed with a high-grade leimyosarcoma of the bladder and underwent a radical cystoprostatectomy. His mother was diagnosed with leimyosarcoma at the age of 37 (Fig. 3.26).

In the other couple, the 39-year-old mother with LFS was a carrier of 902insC mutation of the p53 tumor-suppressor gene, representing an insertion of C in exon 8. She was diagnosed with breast cancer at the age of 30, followed by bilateral mastectomy. She also had thyroid cystocarcenoma, which was also removed. Her mother died from a stomach cancer at age 51. One of her sisters diagnosed with breast cancer at 48 followed by mastectomy also died at age 51. Two of her four brothers were diagnosed with bone or brain tumour in their teens (Fig. 3.27).

Prior to PGD cycles, linkage analysis has been performed for each couple in order to establish the maternal and paternal haplotypes, needed for performing a linked marker analysis in addition to mutation testing. Thus, in both cases, a multiplex nested PCR was performed by the testing of the mutations simultaneously with the linked polymorphic markers, representing the short tandem repeats (STR) in intron 1. To establish the paternal haplotypes, a single-sperm analysis was

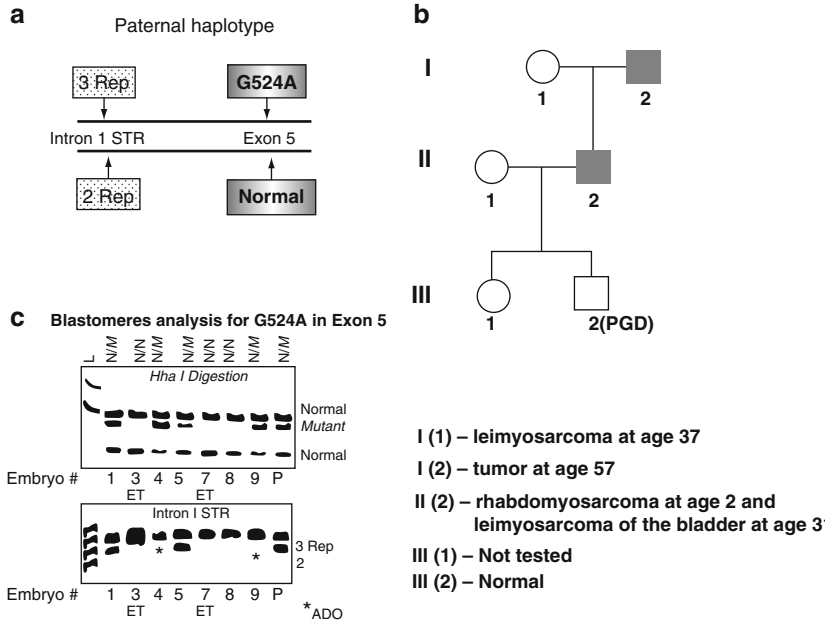



**Fig. 3.26** PGD for Paternally Derived Mutation in p53 Tumor Suppressor Gene Mutation. (**a**) Paternal haplotypes are based on single sperm analysis, showing linkage of affected allele to three repeats and normal allele to two repeats in intron 1 STR. (**b**) Family pedigree, also showing a medical history on the bottom. (**c**) Duplex blastomere analysis for G524A mutation in exon 5 and liked STR in intron 1, showing that embryos #1, #4, #5 & #9 are affected, while embryo #3, #7 and #8 are mutation free and suitable for transfer. ADO of three repeats in intron 1 STR was detected in blastomere from embryo#4 and #9

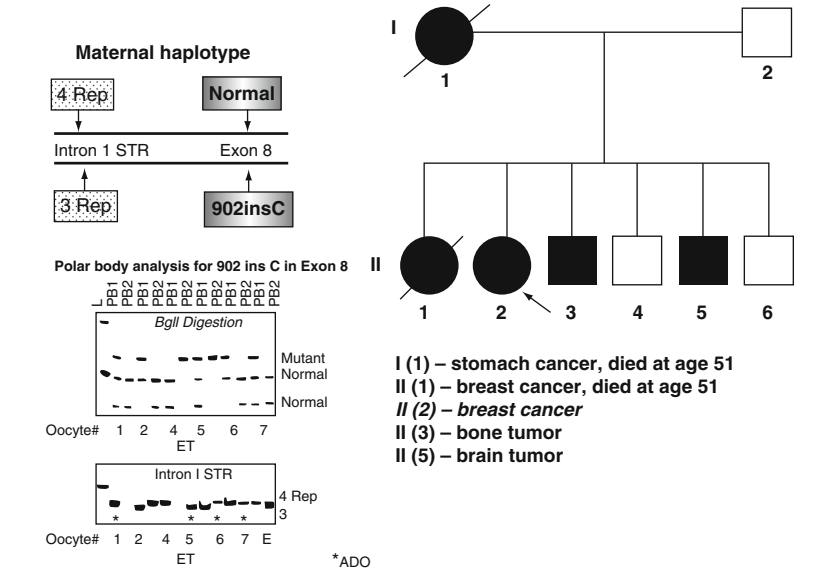



**Fig. 3.27** PGD for maternally derived mutation in p53 tumor suppressor gene mutation. (**a**) Maternal haplotypes are based on PB1 and PB2 analysis, showing linkage of affected allele to three repeats and normal allele to four repeats in intron 1 STR. (**b**) Family pedigree, also showing a medical history at the bottom. (**c**) Duplex PB1 and PB2 analysis for 902insC mutation in exon 8 and linked to STR in intron 1, showing that all but one oocyte (oocyte #5) are affected, based on heterozygous PB1 and homozygous normal PB2. Thus, only embryos deriving from oocyte #5 were predicted normal and suitable for transfer, as shown by heterozygous PB1 and affected PB2. ADO of 3 repeats in intron 1 STR linked to 902insC mutation was detected in 4 of 7PB1 studied, all of which were heterozygous in mutation analysis

**Table 3.19** Primers and reaction conditions for PGD of p53 tumor suppressor gene mutations

| Gene/polymorphism | Upper primer | Lower primer | Annealing $T_m$ (°C) |
|---|---|---|---|
| G524A (−Hha I) | Outside | | 62–45 |
| | 5′ GTGCAGCTGTGGGTTGA 3′ | 5′ GGAATCAGAGGCCTGGG 3′ | |
| | Inside | | 56 |
| | 5′ CCATGGCCATCTACAAGCA 3′ | 5′ GGGACCCTGGGCAACC 3′ | |
| 902insC (−Bgl I) | Outside | | 62–5 |
| | 5′ AAGAGAATCTCCGCAAGAA 3′ | 5′ GAGGCAAGGAAAGGTGATAA 3′ | |
| | Inside | | 60 |
| | 5′ GCCTGTCCTGGGAGAGAC 3′ | 5′ GCTTACCTCGCTTAGTGCG 3′ | |
| (TAAA)n Heminested | Outside | | 62–45 |
| | 5′ CATTTGAATCCGGGAGGAG 3′ | 5′ ACAAAACATCCCCTACCAAACA 3′ | |
| | Inside | | 60 |
| | 5′ GCCTGGGCAATAAGAGCTG 3′ | 5′ ACAAAACATCCCCTACCAAACA 3′ | |

performed, showing a linkage of the affected allele to the three repeats and the normal one to the two repeats. The maternal haplotypes were established by PB1 and PB2 analysis, demonstrating the linkage of the affected allele to the three repeats, and the normal one to the four repeats. The outside and inside primer sequences, the restriction sites, and the primer melting temperature for DNA analysis of both mutations are shown in Table 3.19. The PCR products were identified by restriction digestion, using HhaI for G524A and BglI for 902insC mutation.

Two PGD cycles were performed for each couple as described above. Testing for the maternal 902insC mutation was done by DNA analysis of PB1 and PB2, removed sequentially following maturation and fertilization of oocytes. The paternal G524A mutation was tested by DNA analysis of single blastomeres, removed from the eight-cell embryos. Based on both mutation and STR analysis, unaffected embryos were preselected for transfer back to the patients, while those predicted mutant were exposed to the confirmatory analysis using the genomic DNA from these embryos to evaluate the accuracy of the single-cell-based PGD.

A total of 18 day-3 embryos were tested in the couple with the paternally derived G524A mutation. Eleven embryos were heterozygous, with both alleles of the exon 5 present, while seven contained only the normal allele, in agreement with the STR analysis in intron 1. Figure 3.26 demonstrates the results of the testing of eight embryos in one of the cycles performed for this couple, showing that the embryos #1, #4, #5, and #9 were affected, and the embryos #3, #7, and #8 were unaffected. These results were in agreement with the marker analysis in all but two embryos (embryos #4 and #9), in which the three-repeat STR marker linked to the mutant gene was missing, probably due to ADO, because these embryos were clearly heterozygous according to the mutation analysis.

A total of ten oocytes were tested in two PGD cycles from the couple with the maternally derived 902insC mutation in exon 8. Mutation analysis was performed simultaneously with the linked STR in intron 1, using a sequential PB1 and PB2 analysis. Six oocytes with the available PCR results in both PB1 and PB2 were predicted affected, based on the heterozygous PB1 and normal PB2, and four unaffected, based on the heterozygous PB1 and the mutant PB2. As in the previous case, ADO of the three-repeat marker linked to the 902insC mutation was observed in four of seven heterozygous PB1 (Fig. 3.27).

The unaffected embryos were preselected and transferred back to the patients in each of the four PGD cycles performed for these two couples. Neither transfer yielded clinical pregnancies in the two cycles performed for the maternal mutation 902insC, while one of the transfers resulted in a singleton pregnancy and the birth of a

mutation-free child in a couple with the paternally derived G525A mutation, following the confirmation of PGD by prenatal diagnosis. The follow-up PCR analysis of the embryos predicted affected was possible for the embryos resulting from the six affected oocytes for the maternal mutation 902insC, and showed a concordance with the results of the PB analysis.

In one of our couples, the maternal carrier of the p53 tumor suppressor mutation was 39 years old, thus her elevated age-related risk for aneuploidy may have contributed to the failure of establishing pregnancy in the two subsequent clinical cycles. Although the testing for chromosomal abnormalities could be done for this couple, only one or two embryos, respectively, were available for the transfer in each of the two cycles, preventing further embryo preselection for the age-related aneuploidies. On the contrary, the other patient with the established pregnancy was neither a carrier of the p53 tumor-suppressor mutation, nor had the risk for the age-related aneuploidy.

As seen from the presented cases, indications for PGD are being extended steadily compared to the practice of prenatal diagnosis. This is due to the potential of PGD for the preselection of the mutation-free embryos and the establishment of an unaffected pregnancy, instead of the testing and termination of the pregnancies already in process. In fact, many at-risk couples have had such unfortunate experiences of repeated prenatal diagnoses and termination of affected pregnancies, that they regard PGD as their only hope for having children of their own, despite having to undergo IVF. This has made PGD also attractive for the couples at risk for late-onset disorders with the genetic predisposition, although such conditions have never been an indication even for prenatal diagnosis.

The presented cases demonstrate practical feasibility of PGD for the late-onset disorders, providing a principally new option for a large group of couples who wish to avoid the risk of having children with a strong inherited predisposition to common disorders. The application of PGD for avoiding the establishment of pregnancy with neurofibromatosis is presented below.

#### 3.7.1.2 Neurofibromatosis

Neurofibromatosis (NF) is a common autosomal-dominant neurological disorder, with at least two distinct major forms, including NF type I (NF1), which is more common (1:4,000) and characterized by fibromatous skin tumors with café-au-lait spots, known also as Von Recklinghausen disease, and NF type II (NF2), which is less common (1:100,000) and characterized by bilateral acoustic neuromas, meningeomas, schwannomas, and neurofibromas [16].

The NF1 gene is located on chromosome 17ql 1.2, while the NF2 gene is mapped on chromosome 22q12.2. Alterations in the sequence of these genes affect the tumor suppressor function of their products (neurofibromin and merlin, respectively), leading to a strong predisposition to malignancies. Different mutations in these genes have been described, resulting in a variety of clinical manifestations. Approximately half of these mutations are sporadic [55, 56], with the rest representing germ-line mutations, which may be detected before the establishment of pregnancy to ensure unaffected pregnancy and the birth of a healthy child without an inherited predisposition to malignancy.

Although preimplantation genetic diagnosis (PGD) for inherited cancer predisposition is still a controversial issue [57, 58], the possibility of establishing only mutation-free pregnancies makes PGD an attractive option for the late-onset disorders with genetic predisposition, because of the possibility for preselection of the genetic predisposition-free embryos for transfer, avoiding the risk for pregnancy termination, as only potentially normal pregnancies could be established.

Three couples presented for PGD, two at risk for producing a child with NFI and one with the NF2 mutation. In one of the couples at risk for producing a child with maternally derived NF1, presented in Fig. 3.28, the 23-year-old mother with café-au-lait spots, axillary freckling, and Lisch iris nodules had a nonsense mutation (Trp->Ter) resulting from TGG->TGA substitution in exon 29 of the NF1 gene coding sequence. This was a de novo mutation, as no clinical symptoms of NF1 were known in the extended pedigree of the patient.

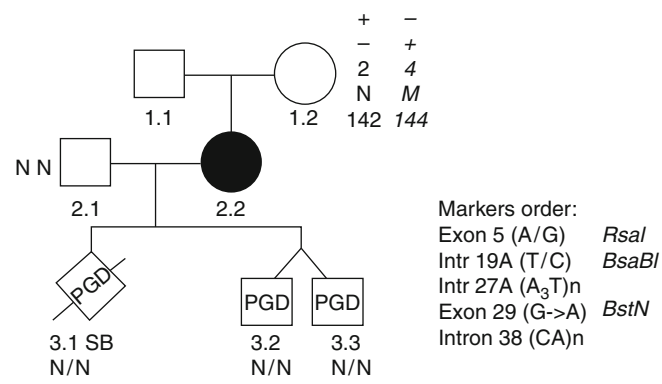



**Fig. 3.28** PGD for neurofibromatosis type 1 (NF1): family pedigree. Haplotype analysis in a PGD couple with maternal de novo nonsense NF1 mutation Trp->Ter (TGG->TGA) in exon 29 of NF1 gene (based on sequential polar body analysis) Rsa – in exon 5, Bsa+in intron 19A, and AAATup in intron 27A (*Top*) Patient's parents (1.1 & 1.2) who are normal (*Middle*) markers (2.1 and 2.2) (*Bottom*) Sibling (SB) (3.1) representing a still-born baby born after the first PGD cycle, with confirmed mutation-free status in agreement with linked presented markers, while 3.2 and 3.3 represent unaffected twins resulting from next PGD cycle

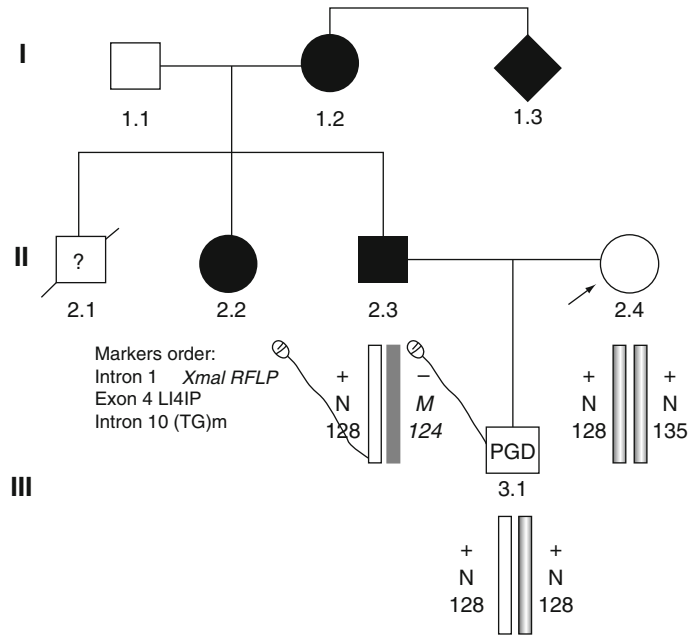



**Fig. 3.29** PGD for neurofibromatosis type 2 (*NF2*): Family Pedigree Haplotype Analysis in Couple with Paternal L141P Mutation in Exon 4 of NF2 Gene. Paternal haplotype (2.3) was established by multiplex heminested PCR analysis of single sperm. Maternal haplotype was based on embryo genotypes. PGD cycle resulted in delivery of the normal boy (3.1)

In the couple at risk for producing an offspring with NF2, the 32-year-old father had an NF2 mutation, represented by the DNA sequence alteration at codon 141 of the merlin gene, due to a T–C base substitution in the nucleotide position 422, leading to the change from leucine (L) to proline (P) (Fig 3.29).

For testing of the maternal NF1 mutation, PB1 and PB2 were removed sequentially following maturation and fertilization of oocytes, while single blastomeres were removed from the eight-cell embryos for testing the paternal NF2 mutation. Linkage analysis had been performed for each couple, in order to establish the maternal and paternal haplotypes and to amplify these markers simultaneously with the mutation testing. In the NF1 case, three markers were informative, including Bsa B1 restriction site in intron 19A [59], short tandem repeat (STR) (AAAT) in intron 27A [60], and Rca I restriction site in exon 5 [61]. In the NF2 case, two markers were informative, one representing single nucleotide polymorphism (SNP) in intron 1, following XmaI restriction digestion, and the dinucleotide repeat (TG) in intron 10 [62, 63]. Thus, in both cases, a multiplex nested PCR was performed, amplifying the mutations simultaneously with the linked polymorphic

**Table 3.20** Primers and reaction conditions for PGD of NF1 & NF2

| Gene/ polymorphism | Upper primer | Lower primer | Annealing $T_m$ (°C) |
|---|---|---|---|
| NF1exon 29 (TGG-TGA) *Bst NI* cuts normal | Outside<br>5′ GTTTAATTCTTCTCCACTTCACC 3′ | 5′ CAACACTGCATACCTTCCA 3′ | 62–45 |
| | Inside<br>5′ TTCATATCCGGACCCCC 3′ | 5′ CTTTTGGCCGAATCTTGG 3′ | 53 |
| NF1 (AAAT)n | Outside<br>5′ TGGTGGCACATACCTGTAG 3′ | 5′ TTACAGATTAAGGCAATTCTGA 3′ | 62–45 |
| | Inside<br>5′ TGCATTCTAGCCTGAGTGA 3′ | 5′ AAACAAGCAAGAATAGAAAAAG 3′ | 48 |
| NF1 SNP *Bsa BI* | Outside<br>5′ ATTAGTGGGTTTTACTGTG 3′ | 5′ CTGAGGCTTTATGTATCTTA 3′ | 62–45 |
| | Inside<br>5′ TGTGTATTTAAACTTTTGAG 3′ | 5′ TTCCCAATAACTGTAGAC 3′ | 53 |
| NF1 SNP Rsa I | Outside<br>5′ CATGTGGTTCTTTATTTATAGG 3′ | 5′ TTGACACCAGTTGACAATAG 3′ | 62–45 |
| | Inside<br>5′ GGTAGAAAATTATCCAGATGA 3′ | 5′ AACTTGGAAAACGATGATAG 3′ | 55 |
| NF2 *Msp I* cuts mutant | Outside: 5′ GGCAGCCCTCATTAGAAC 3′ | 5′ AGAATACAGAAAACCCAAAG 3′ | 62–45 |
| | Inside: 5′ AAGATCTACTGCCCTCCTG 3′ | 5′ TGATCCCATGACCCAAATTA 3′ | 54 |
| NF2 SNP Xma I | Outside: 5′ AAGAATATTCGCCGTGTGTC 3′ | 5′ GACTTTCCCGCTCCGTC 3′ | 62–45 |
| | Inside: 5′ CAACGAAGGACCCAATTCC 3′ | 5′ AAGCAGGCCTAGGCTCG 3′ | 54 |
| NF2 Intron 10 (TG)n | Outside: 5′ GGAGAAAATTGGAGAAGAACT 3′ | 5′ CCACTCTGGTCATACAACG 3′ | 62–45 |
| | Inside: 5′ TTCACTGTTTTATTGCTTGTC 3′ | 5′ FamGACTGCTGCTTTTTCTAAATC 3′ | |

markers [64, 65]. The primers designed for amplification of NF1 and NF2 genes and their linked markers mentioned are listed in Table 3.20.

Testing for NF1 was performed using BstNI restriction digestion, which produces two fragments of 20 and 52 bp in the second-round PCR product of the normal allele, in contrast to a single undigested fragment of 72 bp in the mutant gene. The maternal haplotypes were established by PB1 and PB2 analysis, demonstrating the linkage of the affected allele to Rsa –, Bsa+, and AAATup markers (Fig. 3.30).

Testing for NF2 mutation was done by blastomere analysis, using MspI restriction digestion, which produces two fragments of 36 and 86 bp in the second-round PCR product of the mutant allele, in contrast to a single 122 bp fragment in the normal gene. The single-sperm haplotype analysis demonstrated the linkage of the affected allele to the XmaI undigested fragment, and to the 124 bp dinucleotide repeat in intron 10, the latter being tested using fluorescent PCR (Fig. 3.31).

A total of 57 oocytes were tested in 5 PGD cycles from the patient carrying the NF1 mutation. Based on simultaneous mutation and marker analysis in PB1 and PB2, 20 oocytes with the available PCR results in both PB1 and PB2 were predicted as affected. Of 26 oocytes containing no mutant gene, 8 (two in each cycle) with sufficient marker information available resulted in the embryos of acceptable quality for embryo transfer, and 3 reached blastocyst stage and were frozen. Due to the close location of the gene to the centromere, which might explain the presence of only 2 oocytes with heterozygous PB1, the information on the linked markers was of particular importance to avoid misdiagnosis due to a potential ADO of one of the alleles in PB1.

Two of these transfers resulted in clinical pregnancies. In one of these cycles, of 17 tested oocytes, 9 contained no mutant allele, from which only 2 with sufficient linked marker information to exclude the risk for misdiagnosis resulted in the embryos of acceptable quality for transfer, yielding a singleton clinical pregnancy. Prenatal diagnosis by chorionic villus sampling (CVS)

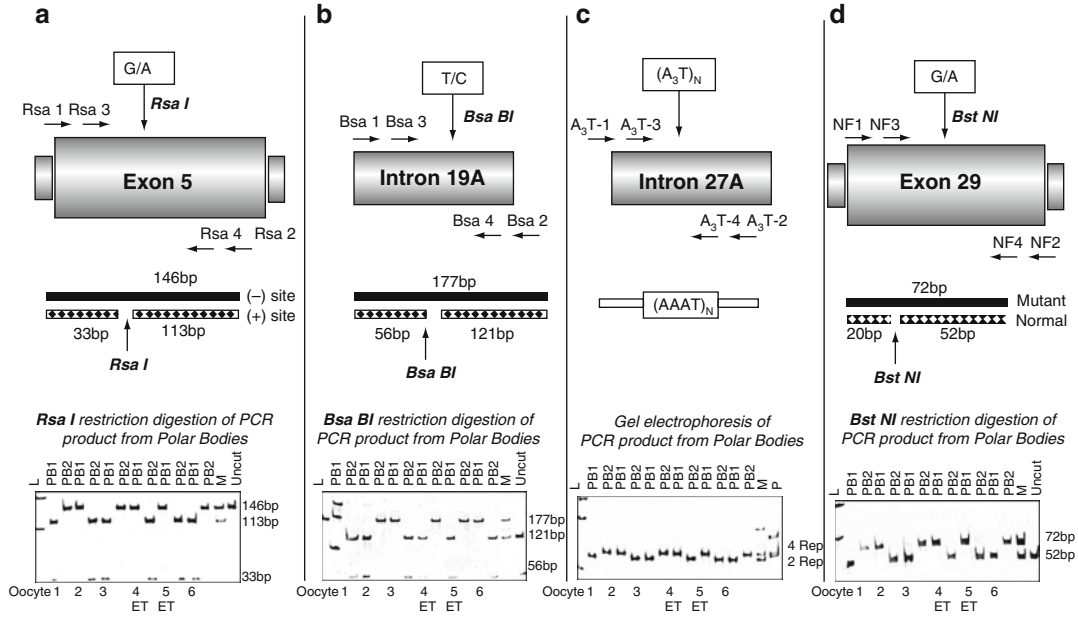



**Fig. 3.30** PGD for NF1 nonsense mutation Trp->Ter (TGG->TGA) in exon 29: mutation and polymorphic marker analysis. (*Top*) Schematic diagram of the mutation (**d**) and linked markers (**a**–**c**) on chromosome 17q11.2. (*Middle*) Restriction maps for BstN I (**d**), Rsa I (**a**), and Bsa BI (**b**) restriction fragment-length polymorphism; and short tandem repeat in intron 27A (**c**). (*Bottom*) Polyacrylamide gel electrophoresis of the restriction-digested PCR products of PB1 and PB2 from six oocytes in one of the cycles of PGD for NF1, showing mutation-free oocytes #2, #4, and #5, evidenced by 72 bp undigested fragment in PB1 and a 52 bp digested fragment in PB2 (**d**), in agreement with all three marker analyses (**a**–**c**) (for linkages to mutant and normal alleles)

confirmed the mutation-free status of the fetus, but the pregnancy ended in a stillbirth at 24 weeks. Similar results were obtained in the next cycle, resulting in preselection and transfer of two mutation-free oocytes, yielding a twin pregnancy and the birth of healthy twins (Figs. 3.28 and 3.30). As seen from polyacrylamide gel electrophoresis of the restriction-digested PCR products of PB1 and PB2 from six oocytes from this cycle, mutation-free status of oocytes #2, #4, and #5 is evidenced by 72 bp undigested fragment in PB1 and a 52 bp digested fragment in PB2, in agreement with all three marker analyses. One of the cycles performed for this couple, resulting in the establishment of embryonic stem cell line from the affected NF1 embryos, will be described in Chap. 7.

Of 18 day-3 embryos tested in one cycle from the couple with the paternally derived NF2 mutation, 8 were predicted to be mutation-free including four with no or only one linked marker information available to exclude the risk for misdiagnosis. Three of four normal embryos with both linked markers in agreement with mutation analysis (Fig. 3.31) were transferred back to the patient, resulting in a clinical pregnancy and the birth of a healthy child free from NF2 mutation, following confirmation of PGD by CVS. Three of five remaining mutation-free embryos that continued development were frozen for future possible use by the couple.

The presented data demonstrate the acceptable diagnostic accuracy of both the PB and blastomere analysis for PGD of NF1 and NF2 [66]. As shown by the follow-up analysis of the mutant embryos or those with insufficient marker information, the PGD results were confirmed in all resulting embryos available for the study. As mentioned, in one of the cycles performed for maternally derived NF1 mutation, the pregnancy resulted in a stillbirth, following confirmation of the mutation-free status of the fetus by CVS. Mutation-free status was also confirmed in a baby born following PGD for NF2 (Fig. 3.31).

As seen from the presented cases, together with the other application of PGD to cancer

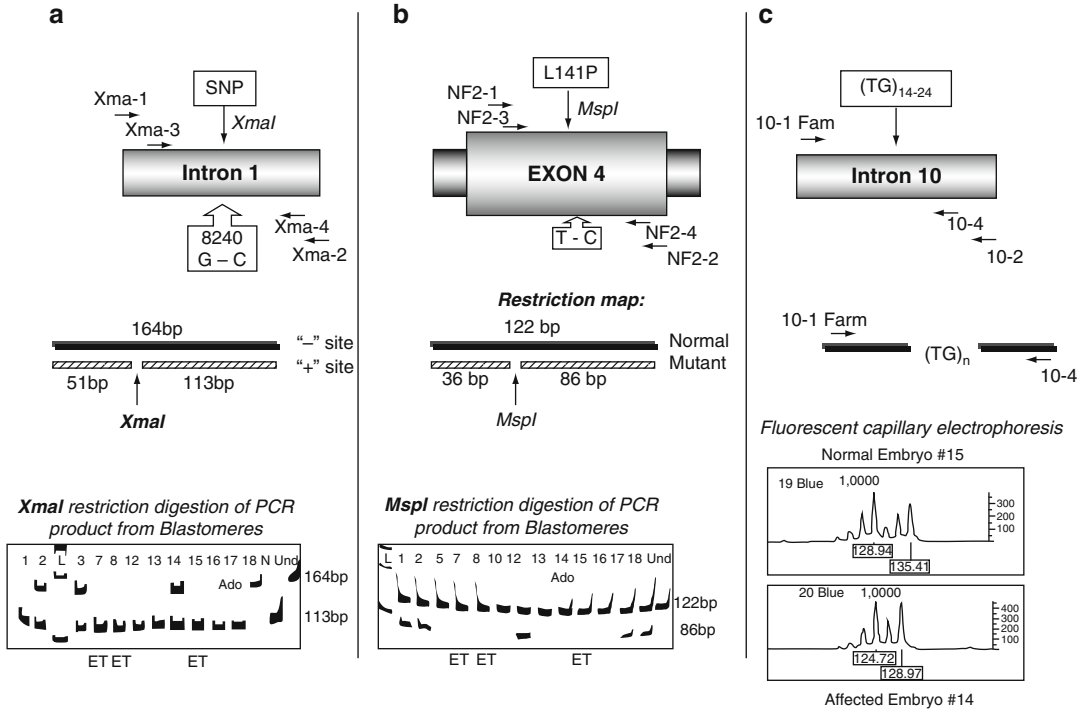



**Fig. 3.31** PGD for L141P mutation in exon 4 of NF2 gene: mutation and polymorphic marker analysis. (*Top*) Schematic diagram of the mutation (**b**) and linked markers (**a**, **c**) on chromosome 22q12.2. (*Middle*) Restriction maps for Msp I (**b**) and Xma I (**a**) restriction digestion in exon 4 and intron 1 of NF2 gene, and design for the dinucleotide TG repeat testing in intron 10 (**c**). (*Bottom*) Polyacrylamide gel electrophoresis of the restriction digested PCR products of blastomeres, showing seven mutation-free embryos (#5, #7, #8, #10, #13, #15, and #16), evidenced by the presence of a 122 bp undigested fragment, as compared to the presence of a digested 86 bp fragment in addition to a 122 fragment in the rest of the embryos (**b**), in agreement with XmaI restriction length polymorphism in intron 1 (**a**), and dinucleotide TG repeats in intron 10 (the latter being detected by fluorescent capillary electrophoresis (**c**)) shown for normal embryo #14, as an example (for the linkages to normal and mutant genes, see Fig. 3.29)

predisposition, the approach seems to be acceptable to the couples at risk, despite important ethical implications. So current genetic counseling services may consider informing patients at risk of having children with a strong genetic predisposition to cancers and late-onset disorders about the availability of PGD, without which these couples may remain childless because of their fear to opt for prenatal diagnosis and possible pregnancy termination.

### 3.7.1.3 Other Cancers

At the present time the most common cancer for which PGD has been performed is inherited breast cancer [52, 67–71]. Almost half of inherited breast cancers are caused by BRCA1 and BRCA2, which were indications for 31 PGD cycles in our experience. A total of 39 embryos free from these mutations were preselected for transfer in 23 cycles, resulting in 10 clinical pregnancies and the birth of 14 children without predisposition to breast cancer. Because of the high prevalence of these conditions, some of the couples were at risk not only of producing offspring with genetic predisposition to breast cancer, but also with other genetic disorders at the same time.

The examples of combined PGD for BRCA1 and SMA, and BRCA2 and MEN1, are presented in Fig. 3.32, the latter also involving aneuploidy testing by FISH analysis. Despite testing for both mutations in each of these cycles, two embryos were identified for transfer in both cases.

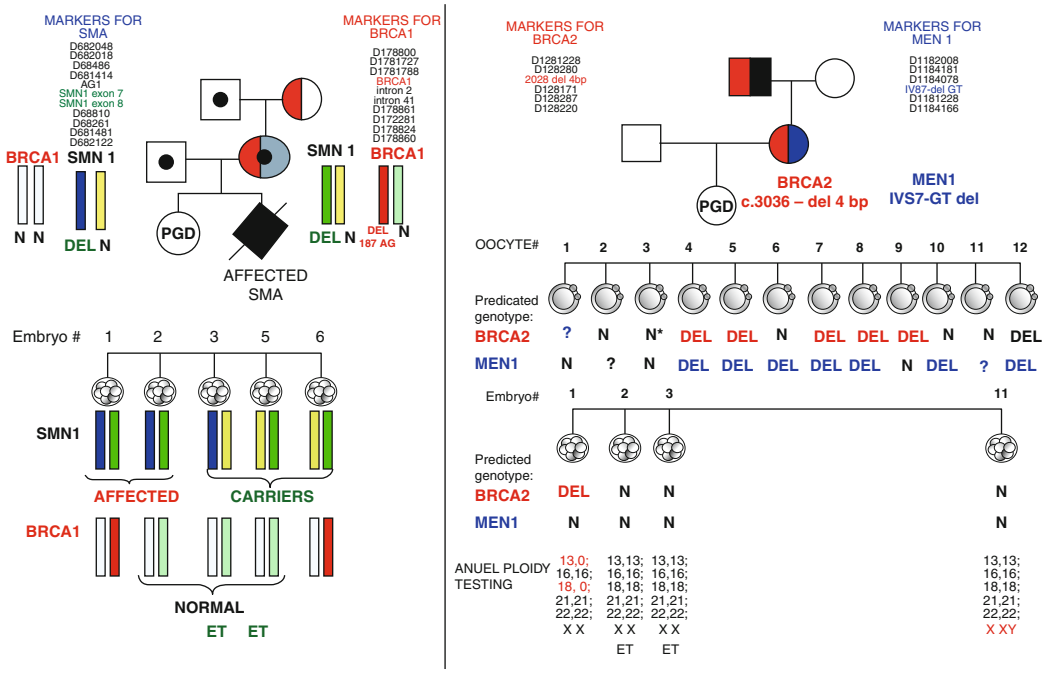



**Fig. 3.32** Concomitant PGD for breast cancer, BRCA1 and BRSA2, and SMA and MEN1. (**a**) PGD for BRCA1 and SMA in the same couple. (*Upper panel*) Pedigree showing that the patient and her mother are carriers of BRCA1 mutation (Del 187 AG; shown in *red*) – linked markers are listed on the *right*. The patient is also a carrier of SMN1 mutation (deletion, shown in *green*), inherited from her father. The male partner (the father) is also an unaffected carrier of the same deletion in SMN gene (shown in *blue*). The couple had one previous pregnancy resulting in the birth of an affected child with SMA who died (linked polymorphic markers for SMN1 mutation are listed on the *left*). (*Lower panel*) Five embryos were tested for both SMN1 and BRCA1 in the same reaction, showing that embryos #1 and #2 contained deletion in SMNA from both parents (*blue and green*), and the remaining three embryos were carriers of either maternal (embryos #4 and #5) or paternal (embryo #3) deletion. Two of these embryos (embryos #1 and #5) were also carriers of BRCA1 mutation, so three embryos (embryos #2, #3, and #4) were predicted to be free of BRCA 1 mutation and unaffected by SMA. Two of these embryos (embryos #3 and #4) were transferred, resulting in the birth of an unaffected child, shown in pedigree as PGD (*ET* embryo transfer). (**b**) Combined PGD for BRCA2, MEN1, and aneuploidy. (*Upper panel*) Pedigree showing that the patient and her father are carriers of BRCA2 (c.3036-del4 bp, shown in *red*) and MEN1 (IVS7-GT del, shown in *blue*) mutations. Polymorphic markers for testing of BRCA2 mutation are shown on the *left*, and for MEN1 deletion on the *right*. (*Middle panel 1*) Twelve oocytes were tested by sequential PB1 and PB2 analysis simultaneously for both mutations, which detected only one oocyte (oocyte #3) to be free of both mutations (in addition, oocyte #2 had insufficient marker information to confirm a normal allele for MEN1), so the resulting four embryos (embryos #1, #2, #3, and #11) were further tested by blastomere biopsy, presented in the *middle panel 2*. (*Middle panel 2*) Blastomere analysis of embryos #1, #2, #3, and #11, showing that all but one (embryo #1) are free of both mutations. (*Lower panel*) Two of these embryos were chromosomally abnormal (embryos #1 monosomic for chromosomes 13 and 18; and embryo #11 with extra chromosome X), while the other two (embryos #2 and #3) were euploid. These two embryos were transferred, resulting in an unaffected singleton pregnancy and the birth of a healthy child (shown in pedigree as PGD) free of both BRCA2 and MEN1 mutations

As seen from Table 3.18, the second most frequent indication was *FAP*. Patients with FAP usually present with colorectal cancer in early adult life, secondary to extensive adenomatous polyps of the colon, determined by mutation of the adenomatous polyposis coli (APC) gene located on chromosome 5 (5q21-q22). Over 826 germ-line mutations have been found in families with FAP, causing a premature truncation of the APC protein, through single amino acid substitutions or frameshifts, with the most common mutation being a 5 bp deletion resulting in a frameshift

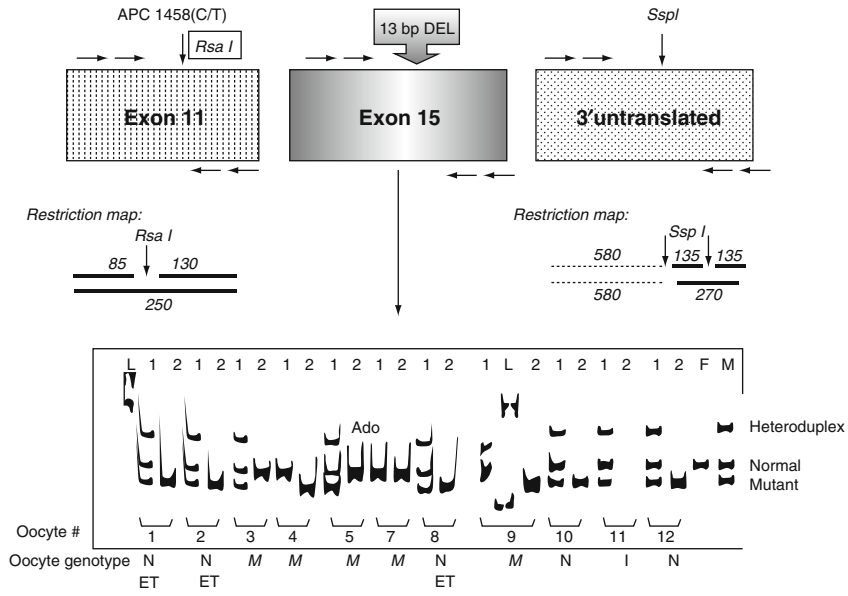



**Fig. 3.33** PGD for 13 bp deletion in APC gene resulted in the birth of a normal child. (*Top*) Schematic diagram of the mutation, and linked markers on chromosome 5q21. (*Middle*) Restriction map for Rsa I and Ssp I restriction digestion in exon 1 and untranslated area of APC gene. (*Bottom*) Polyacrylamide gel electrophoresis of the PCR products of PB1 and PB2 from 11 oocytes in one of the cycles of PGD for FAP, showing mutation-free oocytes #1, #2, #8, #10, and #12, evidenced by normal and mutant fragments in PB1 and a mutant fragment in PB2, in agreement with both marker analyses

mutation at codon 1309. The APC mutations lead to a premalignant disease with one or more polyps progressing through dysplasia to malignancy with a median age at diagnosis of 40 years. Because the mutations in the APC gene are almost completely penetrant, although with striking variation in expression, even presymptomatic diagnosis and treatment of carriers cannot exclude the progression of polyps to malignancy, making PGD an attractive approach for couples carrying APC mutations [72].

The example of the PGD strategy for FAP mutations in the APC gene is presented in Fig. 3.33, showing PGD for the maternally derived mutation resulting from the 13 bp deletion in exon 15 of the gene. DNA testing in all the PGD cycles was performed by the multiplex nested PCR analysis, amplifying mutations simultaneously with linked markers both in blastomere and PB1 and PB2, with the set of primers listed in Table 3.21. Linkage analysis had been performed for each couple, and the maternal and paternal haplotypes were established to avoid a possible misdiagnosis, which still requires special attention because of the phenomena of allele-drop out (ADO) and preferential amplification, known to be frequent in a single-cell DNA analysis. For example, in a couple with FAP, two linked markers were found to be informative, including Rsa I restriction site in intron 11, and SspI restriction site in 3′ untranslated area of the APC gene (Fig. 3.33). As seen from this figure, three mutation-free oocytes were detected for transfer, resulting in an unaffected pregnancy, confirmed by follow-up prenatal diagnosis and a blood test after delivery of a child. However, a cord blood sample was not appropriate for confirmation analysis, as it was contaminated by maternal blood, clearly demonstrated by the presence of the mutant allele, as well as both maternal polymorphic markers in the cord blood sample. The fact that the baby has no mutant allele was further confirmed by testing of a heel stick blood from the child, which contained no trace of the mutant gene, also in agreement with the analysis of polymorphic markers.

**Table 3.21** Primers and reaction conditions for PGD of cancer predisposition

| Gene/ polymorphism | Upper primer | Lower primer | Annealing $T_m$ (°C) |
|---|---|---|---|
| APC 13 bp deletion | Outside | | 62–55 |
| | 5′ TCGACATGATGATAATAGGTC 3′ | 5′ TTTCTGTTGCTGGATGGTAG 3′ | |
| | Inside | | 58 |
| | 5′ GACAATTTTAATACTGGCAACA 3′ | 5′ TCCAAACTTCTATCTTTTTCAGA 3′ | |
| APC RFLP *RsaI* | Outside | | 62–55 |
| | 5′ GGTACCAGTTTGTTTTATTTTAG 3′ | 5′ CACAGGTTTTTATCAGTCATTG 3′ | |
| | Inside | | 62 |
| | 5′ GATGATTGTCTTTTTCCTCTTGC 3′ | 5′ CTGAGCTATCTTAAGAAATACATG 3′ | |
| APC RFLP *Ssp I* | Outside | | 62–55 |
| | CTATGCATTAAGAGTAAAATTCC 3′ | 5′ GTATTACCCTATCTGAGTGCC 3′ | |
| | Inside | | 62 |
| | 5′ CATTGAAGAAGACTGTTGCCAC 3′ | 5′ AAAAGGTTTTCCTCCCCAAATAC 3′ | |
| VHL (Nt 482) Creates *MboII* site | Outside | | 62–55 |
| | 5′ ACGGCGGGGAGGAGTCG 3′ | 5′ CTCAAGGGGCCTCAGTTC 3′ | |
| | Inside | | 58 |
| | 5′ GCGCCGAGGAGGAGATG 3′ | 5′ GGGGCTTCAGACCGTGC 3′ | |
| VHL D3S100 | Outside | | 62–55 |
| | 5′ GTCTGGTGGCCTGTGAAC 3′ | 5′ CTTTCTCTTCGGAATGGGAG 3′ | |
| | Inside | | 58 |
| | 5′ GAAAATGTGTTCATCATCCTC 3′ | 5′ TTATCTTATCCCTGCCTCAC 3′ | |
| hSNF5 (Nt443) Creates *Hpy188 I* site | Outside | | 62–55 |
| | 5′ GGGGGAGTTTGTCACCAC 3′ | 5′ GGAGGACGGAGCAAACAC 3′ | |
| | Inside | | 54 |
| | 5′ CCACCATCGCATACAGCA 3′ | 5′ GGAGGACGGAGCAAACAC 3′ | |
| D22S1144 (heminested) | Outside | | 62–55 |
| | 5′ AAATAGGCAGATGCTGAAA 3′ | 5′ ACAGAGCCTCTGGTCCTC 3′ | |
| | Inside | | 50 |
| | 5′ Hex GGAAAGCAACTTTGGTAAA 3′ | 5′ ACAGAGCCTCTGGTCCTC 3′ | |
| D22S1174 (heminested) | Outside | | 62–55 |
| | 5′ GGACATAGCAAACTCTTAGGG 3′ | 5′ GAATCTGCTGCTTGCTTTT 3′ | |
| | Inside | | 50 |
| | 5′ FamCACTTCTGAGTTGTTTGAATCTC 3′ | 5′ GAATCTGCTGCTTGCTTTT 3′ | |
| RB (GA del) | Outside | | 62–55 |
| | 5′ GTAGGCTTGAGTTTGAAGA 3′ | 5′ TGAAGTTGTTTTTAAAATGAGA | |
| | Inside | | 55 |
| | 5′ Fam TGATTTTACTGCATTATGTCAG 3′ | 5′ CTTACCAATACTCCATCCAC 3′ | |
| RB Intron 20 STR (CTTT)n | Outside | | 62–55 |
| | 5′ GACAGGCATTTGGACCAAG 3′ | 5′ GCAGTGAGCCGAGATTGC 3′ | |
| | Inside | | 56 |
| | 5′ Hex CCCTACTTACTTGTTAACTG 3′ | 5′ GGTAACAGAGTGAGACTCTA 3′ | |

Five cycles were performed for *VHL*, which is a cancer syndrome with age-related penetrance, characterized by hemangiomablastomas of the brain, spinal cord, and retina; bilateral renal cysts and renal carcinoma; pheochromocytoma; and pancreatic cysts. Depending on the combination of these clinical features, four different types of the disease have been described. The gene responsible for VHL syndrome consists of three exons and is located on chromosome 3 (3p26-p25), with specific VHL gene mutations correlating with the clinical phenotype. Its normal gene product is a

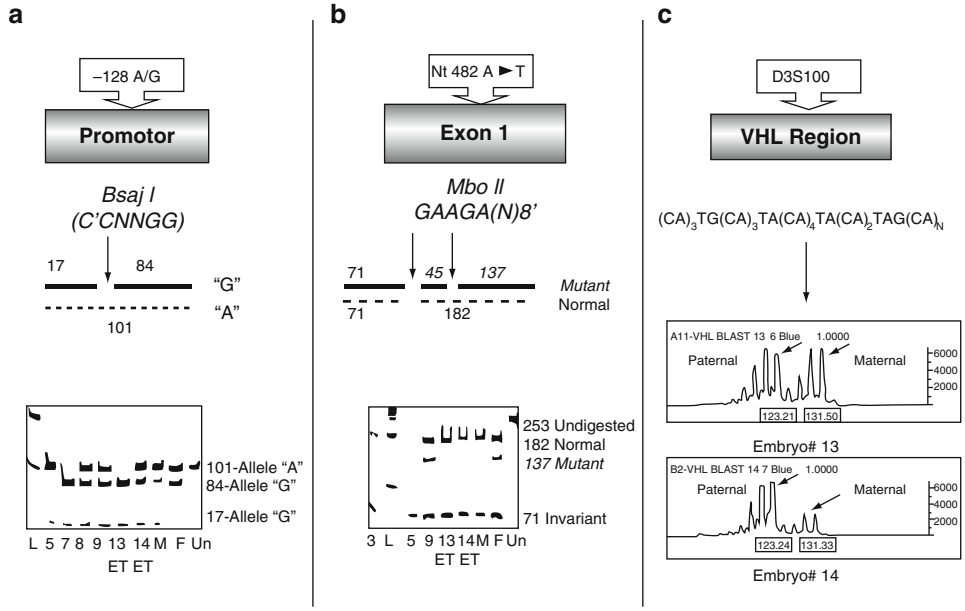



**Fig. 3.34** PGD for Nt 482 A-T mutation in exon 1 of VHL gene. (*Top*) Schematic diagram of the mutation, and linked markers; (**a**) Position of G/A SNP in promoter area of the gene and the restriction map, and the restriction digestion with *Bsaj*I enzyme differentiated "A" and "G" sequences (*middle*). (**b**) Position of nt482 A-T mutation in exon 1 of VHL gene and the restriction map (*middle*). Amplified sequence has invariant restriction fragment (71 bp). Enzyme *Mbo*II-digested mutant sequence into two fragments of 45 bp and 137 bp, with the size of the normal allele being 182 bp. (*Bottom*) Polyacrylamide gel electrophoresis of the *Mbo*II restriction-digested PCR products of blastomeres detected mutation-free embryos #13 and #14, evidenced by the presence of a 182 bp undigested fragment, as compared to the presence of a digested 137 bp fragment in the affected embryos. This is in agreement of the results of SNP analysis in the promoter (**a** *bottom*). (**c**) Position and sequence of D3S100 dinucleotide repeat in VHL region. Capillary electrophoregrams of fluorescently labeled dinucleotide repeat PCR products scored by Genotyper™. The presence of 123 bp paternal allele in embryos #13 and #14 confirmed the normal status of these embryos predicted by mutation analysis. *ET* embryo transfer, *M* maternal DNA, *P* paternal DNA containing the mutation, *L* 100 bp standard

tumor suppressor protein, which is expressed in most cells and has a variety of functions, including transcriptional and posttranscriptional regulation. More than 300 germ-line mutations have been identified in families with VHL syndrome, consisting of partial or complete gene deletions, and frameshift, nonsense, missense, and splice site mutations, most commonly affecting codon 167. Mutations in the VHL gene either prevent its expression completely or lead to the expression of an abnormal protein. Because only 20% of cases of VHL are sporadic, with the remaining 80% being familial, PGD is clearly an attractive option for couples carrying these mutations to avoid the inheritance of these tumor suppressor gene mutations to their potential children and have a mutation-free child, with no risk of developing cancer.

The example of PGD for paternally derived mutation causing VHL is shown in Fig. 3.34, performed for the male partner carrying A to T substitution in nucleotide 482 of exon 1. As seen from this figure, only one of four embryos in this cycle contained the mutant gene (embryo #9), the remaining three being normal. The haplotype analysis, an obligatory part of PGD for paternally derived dominant mutations, confirmed the presence of a normal paternal gene. As seen from Fig. 3.34, both embryos transferred in this cycle contained the polymorphic 123 bp marker, representing a dinucleotide repeat (CA)3TG(CA)3TA (CA)3TA(CA)2TAG(CA)n, which is strongly linked to the normal paternal gene, and therefore absolutely essential to be able to confirm the presence of the normal paternal gene in the

embryos selected for transfer, confirming the results of the mutation analysis. The transfer of these two normal embryos back to the patient, however, did not result in a clinical pregnancy. The affected embryo was reanalyzed, confirming the prediction of the Nt 482 (A-T) mutation in the embryo.

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

In each of the four cycles performed for three patients at risk for producing offspring predisposed to RB, nine unaffected embryos were preselected, resulting in clinical pregnancies in each cycle, with the birth of three healthy children free from the mutant gene predisposing to RB.

A single PGD cycle was performed for the patient carrying the *hSNF5* mutation predisposing to brain tumor, which is very rare, found in sporadic rhabdoid tumors of the central nervous system. Rhabdoid tumors are known to be highly malignant neoplasms usually occurring in children under 2 years of age. Although rhabdoid tumors determined by truncating mutations of the hSNF5 gene are mainly sporadic and have never been previously found in the parents of affected children, a first familial case of posterior fossa brain tumor has recently been described in two generations [73]. The proband presented at the age of 18 months with a cerebellar malignant rhabdoid tumor. Although the parents were healthy, the child's maternal uncle died at age 2 years from a posterior fossa choroids plexux carcinoma, and her grandfather's sibling also died as an infant from a brain tumor, suggesting the presence of a germ-line mutation. The couple presented for PGD in order to have a pregnancy free from the hSNF5 mutation, also avoiding the birth of a second child with brain tumor. As seen from Fig. 3.35, the mutation is due to G to A substitution in a donor splice site of exon 7, which alters the conserved GT sequence at the beginning of the intron violating the GT rule for splice site recognition. In this unique case, the mother was unaffected but her daughter who inherited the mutation had a brain tumor [51]. Because the mutation was also detected in DNA from her uncle's tumor, suggesting the risk of transmitting the mutation to the next child, PB1 and PB2 were removed in this case to preselect mutation-free oocytes in a standard IVF cycle.

Of only four oocytes available for testing, three have results of both PB1 and PB2 analysis, showing that only one oocyte (oocyte #4) could be predicted to be free of mutation, based on the heterozygous status of PB1 and homozygous mutant PB2. Of the remaining two oocytes, oocyte #2 was clearly mutant as evidenced by the heterozygous PB1 and homozygous normal PB2, suggesting that only the normal allele was left in the resulting oocyte. The results of mutation analysis in oocytes #2 and #4 were in agreement with both markers in PB1 and PB2 tested. On the other hand, while based on mutation analysis, oocyte #3 could have also been predicted as normal and transferred, as evidenced from the homozygous mutant status of PB1 and homozygous normal status of PB2, the presence of both alleles of D22s1144 marker linked to both the normal and mutant gene in the corresponding PB1 suggested a completely opposite (mutant) genotype of oocyte #3, despite the second polymorphic marker D22s1174 showing a correspondence with the mutation analysis. So the availability of

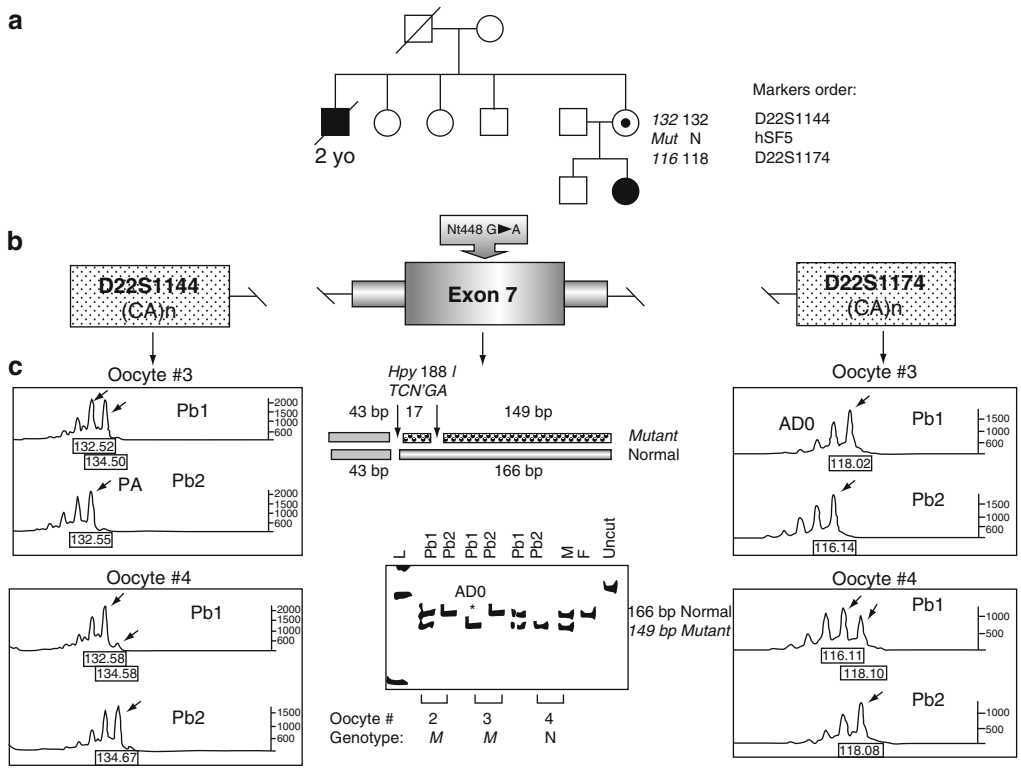



**Fig. 3.35** PGD for familial posterior fossa brain tumor (hSNF5). (**a**) Family pedigree with posterior fossa tumors in two generations: an unaffected carrier haplotype was determined by sequential PB1 and PB2 analysis. (**b**) Position of the Nt 443G--A mutation in exon 7 of hSNF5 gene and tightly linked dinucleotide polymorphic markers *D22s1144* and *D22s1174*. (**c**) (*Left*) Fluorescent PCR results of the sequential PB1 and PB2 analysis. One of the second-round PCR primers was labeled with Hex fluorescent dye. Oocyte #3 is predicted to be affected based on heterozygous (132/134) PB1 and 132 bp PCR product linked to normal maternal allele in PB2. Preferential amplification (PA) of 132 bp allele was detected in PB1 from oocyte #4 in agreement with mutation analysis and the second *D22s1174* marker, suggesting that the oocyte #4 may be predicted to be normal. (*Middle*) Restriction map and polyacrylamide gel analysis of *Hpy188 I* restriction digestion of PCR product from PB1 and PB2. Based on this analysis oocyte #2 is normal based on heterozygous PB1 and mutant PB2. Allele dropout (*ADO*) was detected in PB1 from oocyte #3 by polymorphic markers *D22s1144* and *D22s1174*. Without these markers, oocyte #3 genotype would have been predicted normal based on homozygous mutant PB1 and hemizygous normal PB2, which might have led to misdiagnosis. Oocyte #4 is predicted to be normal based on heterozygote PB1 and mutant PB2, in agreement with marker analysis as mentioned (*Right*) Fluorescent PCR (FL-PCR) results of sequential PB1 and PB2 analysis. One of the second-round PCR primers was labeled with Fam fluorescent dye. ADO of 116 bp allele was detected in PB1 from oocyte #3 by mutation analysis and *D22s1144* marker study. Heterozygous PB1 from oocyte #4 confirmed PA suspected during the *D22s1144* analysis. *N* normal, *M* mutant, *F* paternal DNA, *Mo* maternal DNA, *U* uncut PCR product, *Nt* nucleotide

the two polymorphic markers made it possible to avoid the transfer of the additional embryo, which may have led to misdiagnosis. Unfortunately, the transfer of only one unaffected embryo back to the patient yielded no clinical pregnancy. The follow-up testing of embryos resulting from oocytes #2 and #3 confirmed the diagnosis, further supporting the need for a simultaneous mutation and linked marker analysis for avoiding misdiagnosis in PB-based PGD. Therefore, as previously suggested, the priority in the preselection of mutation-free oocytes should be given to those with heterozygous PB1 and homozygous mutant PB2. If no such oocytes are available, at least three linked polymorphic markers are required to exclude ADO of one of the alleles in apparently

homozygous PB1, which is currently the major potential source of misdiagnosis due to ADO and preferential amplification in PB-based PGD.

With current progress in understanding of the molecular basis of cancers, and sequencing of the genes involved in malignancy, the inherited cancer predisposition will become one of the major emerging PGD indications, presently already representing approximately 10% of all PGD experience for Mendelian disorders. As mentioned, despite extensive discussions of the ethical and legal issues involved in PGD for late-onset disorders with genetic predisposition, an increasing number of patients regard the procedure not only as their favorable option but also the only possible reason for forgoing the pregnancy, which can be established free of mutation from the onset, avoiding their potentially difficult decision to have a pregnancy at high risk of being affected with the option of prenatal diagnosis and termination of pregnancy if the fetus would be diagnosed to carry a mutant gene. So the genetic counseling services may consider informing patients at risk of having children with a strong genetic predisposition to cancers about the presently available option for PGD, without which these couples may remain childless because of their fear to opt for prenatal diagnosis and possible pregnancy termination.

Because such diseases present beyond early childhood and even later may not be expressed in 100% of the cases, the application of PGD for this group of disorders is still highly controversial. However, initial experience in offering PGD for this indication shows that the availability of PGD allows couples forgoing pregnancy, which otherwise would never be attempted. This may be further demonstrated by the first case of PGD performed for genetic predisposition to Alzheimer disease (AD) [74].

### 3.7.2 Alzheimer Disease

Alzheimer disease (AD) is a rare autosomal-dominant familial predisposition to a presenile form of dementia. Three different genes were found to be involved in this form of AD, including presenilin 1 (PS1) located on chromosome 14 [75], presenilin 2 (PS2) on chromosome 1 [76], and amyloid precursor protein (APP) gene on chromosome 21 [77], which is well known for its role in the formation of amyloid deposits found in the characteristic senile plaques of patients with AD. The early-onset dementias associated with βAPP mutations are nearly completely penetrant, and, therefore, are potential candidates not only for predictive testing but also for PGD. Of ten APP mutations presently described, mutations in exons 16 and 17 were reported in the familial cases with the earliest onset. One of such mutations with as early onset as the mid- or late 30s has been reported to be due to a single G to C nucleotide substitution in exon 17, resulting in a valine-to-leucine amino acid change at codon 717 (V717L) [78]. This mutation was identified in three of five family members (siblings) tested, one of whom presented to PGD, described in this chapter, which resulted in a pregnancy and the birth of a healthy child free from the APP mutation.

The patient that presented for PGD was a 30-year-old woman with no signs of AD, carrying a V717L mutation, resulting from G to C substitution in exon 17 of the APP gene. The predictive testing in the patient was done because of the early onset of AD in her sister carrying this mutation, who developed symptoms of AD at the age of 38 [78]. This sister is still alive, but her cognitive problems progressed to the point that she was placed in an assisted living facility. Her father had died at the age of 42 and had also a history of psychological difficulties and marked memory problems. The V717L mutation was also detected in one of her brothers, who experienced mild short-term memory problems as early as the age of 35, with a moderate decline in memory, new learning, and sequential tracking in the next 2–3 years. The other family members, including one brother and two sisters, were asymptomatic, although predictive testing was done only in sisters, who appeared to be free from mutation in the APP gene (Fig. 3.36).

Two PGD cycles were performed, by testing for the maternal mutation using DNA analysis of PB1 and PB2. A multiplex nested PCR was

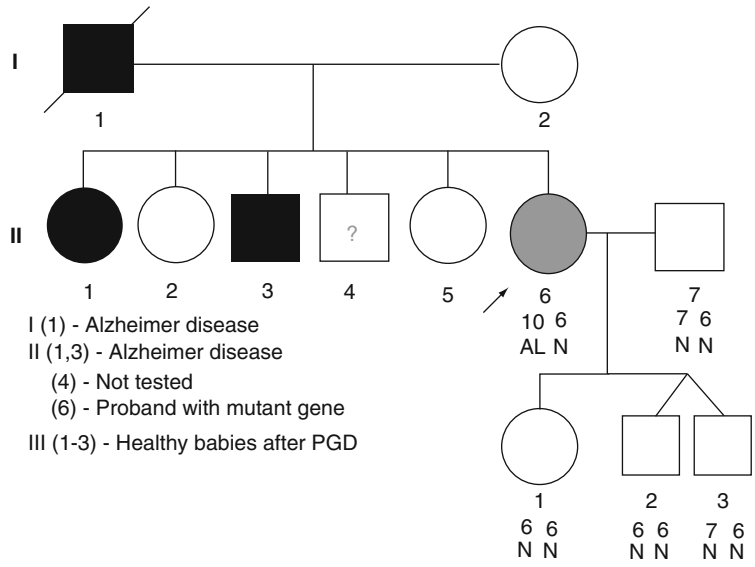



**Fig. 3.36** PGD for early-onset Alzheimer disease caused by mutation V717L: *Pedigree*. I Patient's parents, showing that her father was affecetd (I;1). The sister (II;1) and brother (II;3) were affected by early-onset AD, as well as the father (I;1). Two PGD cycles for asymptomatic carriers of the mutant gene (II;6), both resulting in the birth of unaffected children (III). Haplotype analysis shows that these children inherited normal maternal allele linked to the six repeats.

**Table 3.22** Primers and reaction conditions for PGD of Alzheimer disease

| Gene/ polymorphism | Upper primer | Lower primer | Annealing $T_m$ (°C) |
|---|---|---|---|
| APP V717L | Outside: APP-1 | APP-2 | 55 |
| | 5′ GTGTTCTTTGCAGAAGATG 3′ | 5′ CATGGAAGCACACTGATTC 3′ | |
| | Inside | | 55 |
| | 5′ GTTCAAACAAAGGTGCAATC 3′ | 5′ TCTTAGCAAAAAGCTAAGCC | |
| Intron 1 (GA) n(GT)n | Outside | | 55 |
| | 5′ CCTTATTTCAAATTCCCTAC 3′ | 5′ GATTGGAGGTTAAGTTTCTG 3′ | |
| | Inside | | 55 |
| | 5′ CAGCATCTGTCACTCAAG 3′ | 5′ AATATTTGTTACATTCCTCTC 3′ | |

performed, involving mutation testing simultaneously with the linked polymorphic marker, representing the short tandem repeat (STR) in intron 1 [(GA)n… (GT)n] [79].

The first-round amplification cocktail for the multiplex nested PCR system contained outer primers for both the APP gene and the linked marker, while the second-round PCR used inner primers for each gene. We designed the outer primers APP-1 and APP-102 (see primers in Table 3.22) for performing the first-round amplification, and the inner primers APP-101 and APP-103 for the second round of PCR. As shown in Fig. 3.36, second-round PCR produces a 115-base pair (bp) product, undigested by Mnl I restriction enzyme, corresponding to the normal allele, and two restriction fragments of 72 and 43 bp, corresponding to the mutant allele. There was also invariant fragment of 84 bp produced in both normal and mutant alleles, used as a control.

To perform nested PCR for specific amplification of the linked marker (GA)n… (GT)n in intron 1, we designed the outer primers In1–1 and In1–2 for the first round and the inner primers In1–3 and In1–4 for the second round of amplification. The haplotype analysis, based on the polar body genotyping, demonstrated that the affected allele was linked to the ten repeats, and the normal one to the six repeats.

A total of 23 oocytes were available for testing in two cycles, of which 15 were tested by both PB1 and PB2 (13 in one cycle and 2 in the other). The mutation and linked marker analysis in intron 1 revealed six normal oocytes, all in one cycle,

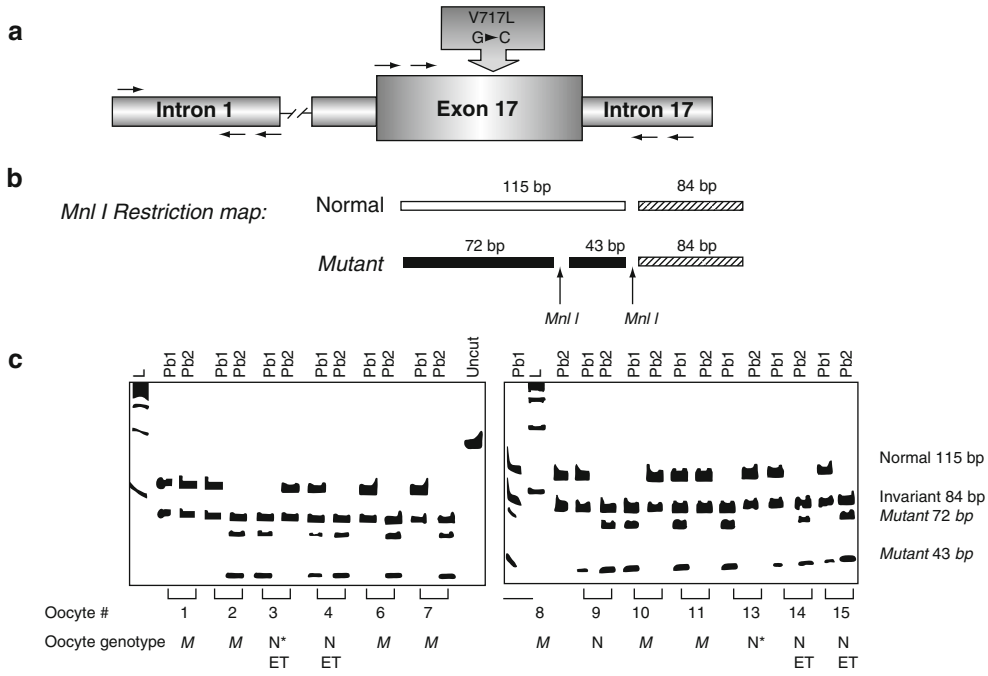



**Fig. 3.37** Preimplantation diagnosis for V717L mutation in the amyloid precursor protein gene, causing early-onset Alzheimer disease by polar body analysis. (**a**) Map of human APP gene, showing sites and location of V17L G-C mutation and polymorphic markers. (**b**) Restriction map for normal and abnormal alleles. (**c**) Polyacrylamide gel analysis of Mnl I restriction digestion, showing six unaffected (*N*) oocytes (#3, #4, #9, #13, #14, and #15), and seven mutant (*M*) oocytes (#1, #2, #6, #7, #8, #10, and #11). Four of 6 embryos resulting from unaffected oocytes (#3, #4, #14, and #15) were transferred back to the patient, resulting in an unaffected pregnancy. Three of these oocytes were with heterozygous PB1 and mutant PB2 (noted as *N*), and only one (#3) was with homozygous mutant PB1 and normal PB2, leaving 5% probability for misdiagnosis, noted as *N**. *ET* embryo transfer, *L* ladder (size standard), *Uncut* the undigested PCR product. *Arrows* indicate fully nested primer sets

and nine affected oocytes, including two in one cycle and seven in the other. The results of one of the cycles, resulting in the transfer, are presented in Fig. 3.37. As seen from this figure, oocytes #4, #9, #14, and #15 were clearly normal, because both mutant and normal genes were present in their PB1, with the mutant gene further being extruded with the corresponding PB2, leaving only the normal gene in the resulting oocyte. In addition, oocytes #3 and #13 were also normal, because their corresponding PB1s were homozygous mutant, suggesting, therefore, that the resulting oocytes should have been normal, as further confirmed by the presence of the normal gene in the extruded PB2s, also in agreement with the linked markers analysis. However, because only one linked marker was available for testing, a 5% probability for ADO of the normal gene in the corresponding PB1 could not be excluded in this particular PB1.

The remaining oocytes were predicted to be mutant, based on heterozygous PB1 and normal PB2 in five of them (oocytes #1, #2, #8, #10, and #11), and homozygous normal PB1 and mutant PB2 in two (oocytes #6 and #7). The follow-up study of the embryos resulting from these oocytes confirmed their affected status in all but one (oocyte #7). The latter may be explained by ADO of the mutant allele in the apparently heterozygous PB1, which was left undetected because of the amplification failure of the linked marker in this case.

To exclude any probability of misdiagnosis, the priority in the embryo transfer was given to the four of six normal embryos, resulting from the oocytes with heterozygous PB2 and mutant PB2.

However, only three of these embryos developed into the cleavage stage and could be transferred (#4, #14, and #15), so the additional embryo (#3) was preselected, originating from the oocyte with homozygous mutant PB1 and the normal PB2, as these results were also confirmed by the linked marker analysis. These four embryos were transferred back to the patient, yielding a singleton clinical pregnancy and the birth of an unaffected mutation-free child.

The presented results demonstrate feasibility of PGD for early-onset AD, providing a nontraditional option for patients who may wish to avoid the transmission of the mutant gene predisposing to the early AD in their potential children. This may appear for some patients the only reason of undertaking pregnancy, as the pregnancy may be established free from an inherited predisposition to AD from the very onset. Because the disease never presents at birth or early childhood and even later may not be expressed in 100% of the cases, the application of PGD for AD is still controversial. However, with no current prospect for treatment of AD, which may arise despite presymptomatic diagnosis and follow-up, PGD seems to be the only relief for the at-risk couples, such as in the presented case, and the cases of PGD for cancer predisposition described above.

Therefore, prospective parents with risk for AD and other relevant conditions should be informed about this emerging new technology, so they could make their choice between seizing their reproduction, and forgoing pregnancy free from AD. This seems to be ethically more acceptable than a denial of the information on the availability of PGD. Presented results of PGD for early-onset AD, together with previously described cases of PGD for the late-onset disorders with genetic predisposition and HLA typing (see below), demonstrate the extended practical implications of PGD, providing prospective couples at genetic risk with wider reproductive options for having unaffected children of their own.

So, for the diseases with genetic predisposition and with no current prospect for treatment, arising despite presymptomatic diagnosis and follow-up, PGD may be offered as the only relief for the at-risk couples.

### 3.7.3 Inherited Cardiac Diseases

As mentioned, PGD application has been expanding beyond traditional indications of prenatal diagnosis and currently includes common disorders with genetic predisposition described in detail in the previous section. This applies also to the diseases with no current prospect of treatment, such as inherited cardiac diseases, which may manifest despite presymptomatic diagnosis and follow-up, when PGD may provide the only relief for the at-risk couples to reproduce.

The first case of PGD for inherited cardiac disease was described for a couple at risk for producing offspring with Holt-Oram syndrome (HOS), which is an autosomal-dominant condition determined by mutation in the *TBX5* gene [80]. HOS is characterized by atrial septal defect and cardiac conduction disease, together with upper extremity malformations, although these clinical manifestations may be extremely variable, not usually being presented at birth, or presented only with a sinus bradycardia, as the only clinical sign which might be also left unnoticed.

As in PGD for other common disorders, the fact that inherited cardiac disorders may not be realized even during a lifetime makes the application of PGD controversial, perhaps explaining the limited application of PGD for inherited cardiac diseases at the present time. The majority of inherited cardiac disorders are dominant, for which no cure may be administered, because their first and only clinical occurrence may be a premature or sudden death. One of such conditions is the familiar hypertrophic cardiomyopathy (HCM), which clinically manifests at different ages, with no symptoms observed for years until provoked by different factors, such as excessive exercise. Different conditions leading to HCM have been reported, two of which, HCM4 and HCM7, will be described in this chapter. HCM4 is caused by mutation in the *MYBPC3* gene located on chromosome 11 (11p11.2), encoding the cardiac isoform of myosin-binding protein C, exclusively in the heart muscle (MIM ID#115197). HCM7 is caused by a mutation in the *TNNI3* gene located on chromosome 19 (19q13.4), leading to an asymmetric ventricular hypertrophy and defect in the

interventricular septum, with high risk of cardiac failure and sudden death (MIM ID#613690).

Hypertrophic cardyomyopathy is also one of the clinical manifestations of fatal infantile cytochrome C oxidase deficiency (MIM ID#604377), for which PGD is strongly indicated, as described below. In contrast to the above conditions, this is an autosomal-recessive cardiac disease, presented within the first month after birth and characterized by a generalized congenital muscular dystrophy, similar to spinal muscular atrophy (SMA), but with significant reduction or lack of cytochrome C oxidase in the muscles [81]. This devastating disease is caused by a defect in the *SCO2* gene located on chromosome 22 (22q13), although the same condition may be also determined by mutations in at least ten other genes involved in Cox activity.

The other condition, for which PGD is strongly indicated, is dilated cardiomyopathy (CMD), which is an autosomal-dominant disease, caused by different mutations in the *LMNA* gene located on chromosome 1 (1q21.2; MIM ID#115200). This cardiac disease is characterized by ventricular dilation and impaired systolic function, resulting in heart failure and arrhythmia, which causes premature or sudden death. While the large phenotypic variability of patients may be determined by different mutations in the *LMNA* gene, differences from one family to another may be observed within the same mutation, with possible involvement of skeletal muscles that leads to muscle weakness, similar to that in Emery-Dreifuss muscular dystrophy (EMD), which is an X-linked disease, also characterized by cardiomyopathy, although presented within the first year after birth (MIM ID# 310300).

The first cumulative experience of the PGD application is presented below for 18 cycles of inherited cardiac disorders that resulted in the birth of 7 healthy children free of the above predisposing gene mutation, demonstrating the utility of PGD for inherited cardiac disease. These 18 PGD cycles were performed for 9 couples at risk for producing an affected progeny with the above conditions, including 9 cycles for CMD, 3 for CMH4, 1 for CMH7, 3 for cardioencephalomyopathy, and 2 for EDMD (Table 3.23).

The couple at risk for producing a progeny with CMD, presented in Fig. 3.38, requested PGD prospectively, with no previous pregnancies attempted, because the male partner was the carrier of the *LMNA* mutation predisposing to CMD. He first experienced cardiac symptoms, such as palpitations, at the age of 22, and then was diagnosed to have a ventricular tachycardia in a 48-h Halter monitoring at the age of 26. To prevent the risk for the development of cardiomyopathy and arrhythmias, which can lead to sudden death, a cardioverter defibrillator had been implanted. As seen from Fig. 3.38, the patient's father passed away from sudden death at age 32, after experiencing heart failure due to cardiomyopathy. His father's side aunt also had been diagnosed with cardiomyopathy at the age 49, and his grandfather and great aunt and her son died at the age of 49–50 from cardiovascular complications.

The patient had dominant mutation in the *LMNA* gene as a result of C to T change in codon 1033 (c.1033C>T), leading to amino acid change from Arg to Trp in position 335 of the proteins lamin A and lamin B, involved in the heart muscles' work. This mutation was detected by *Msp*I digestion, which creates two fragments of 90 and 95 bp in the PCR product of the normal *LMNA* allele, leaving the mutant one uncut. As seen from Table 3.23, four polymorphic markers were also tested simultaneously with the mutation analysis, including *D1S2714*, *D1S82777*, *D1S2624*, and *D1S506*, to avoid misdiagnosis due to preferential amplification or allele drop out (ADO) of the genes tested.

Nine cycles were performed for four patients with CMH4 and CMH7, determined by mutation in *MYBPC3* and *TNNI3* genes respectively. Neither of these couples had previous progeny, but had a family history of premature or sudden death. As seen from Fig. 3.39a, CMH4 in one of the families was due to frameshift mutation *D1076 fs* in the *MYBPC3* gene, while CMH7 in the other family was caused by *A157V* mutation in the *TNNI3* gene (Fig. 3.39b). The *D1076 fs* mutation in the *MYBPC3* gene was detected by *RSA*I and *BsaH*I digestion, the first cutting the mutant gene into two fragments of 72 and 60 bp, and the second cutting the normal one into two fragments of

**Table 3.23** Reproductive outcome of PGD for cardiac diseases

| Disease | Gene (mutation) | Patient/cycle | Embryos | | | | Number of transfers | Number of embryos transferred | Pregnancies | Births |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Total received/ amplified | Normal/ carrier | Abnormal[a] | Inconclusive[b] | | | | |
| Cardioencephalomyopathy (AR) | SCO2 (*R262delCA*; *E140K*) | 1/3 | 33/32 | 16 | 13 | 3 | 3 | 7 | 2 | 1 |
| Cardiomyopathy dilated; CMD1(AD) | LMNA (*K270K*) | 1/ 4 | 51/ 47 | 20 | 26 | 1 | 4 | 9 | 2 | 3[c] |
| | LMNA (*R335T*) | 1/1 | 11/11 | 7 | 2 | 2 | 1 | 2 | 1 | 1 |
| | LMNA (*R189P*) | 1/1 | 2/2 | 1 | 1 | 0 | 1 | 1 | 0 | 0 |
| | LMNA (*T528K*) | 1/3 | 44/34 | 9 | 19 | 6 | 2 | 3 | 1 | 0 |
| Cardiomyopathy familial, hypertrophic 4; CMH4 (AD) | MYBPC3 (*D1076fs*) | 1/1 | 7/6 | 3 | 3 | 0 | 1 | 2 | 1 | 0 |
| | MYBPC3 (*IVS11–10C-A*) | 1/2 | 10/8 | 1 | 6 | 1 | 0 | 0 | 0 | 0 |
| Cardiomyopathy familial, hypertrophic 7; CMH7 (AD) | TNNI3 (*A157V*) | 1/1 | 11/10 | 3 | 7 | 0 | 1 | 1 | 0 | 0 |
| Emery–Dreifuss muscular dystrophy 1, X-linked | EMD | 1/2 | 31/31 | 17 | 14 | 0 | 2 | 5 | 2 | 2 |
| Total | | 9/18 | 190/181 | 77 | 91 | 13 | 15 | 30 | 9 | 7 |

[a]Including aneuploidies
[b]Shared markers in parents, making it impossible to exclude ADO
[c]Including one pair of twins

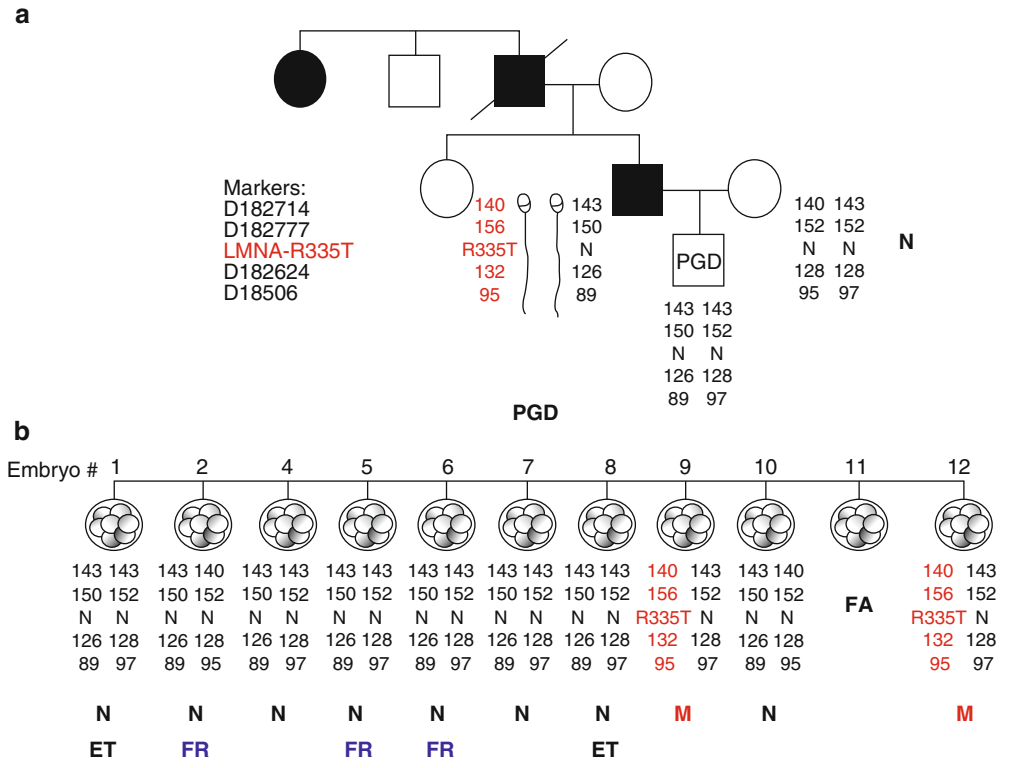



**Fig. 3.38** PGD for dilated cardiomyopathy (*CMD*), determined by dominant mutation in *LMNA* gene. (**a**) Family pedigree of a couple with affected husband carrying *R335T* mutation in *LMNA* gene. Paternal linked polymorphic markers are shown on the *left*, and maternal on the *right*, and the order of the markers and mutation in *LMNA* gene are shown on the *upper left*. (**b**) Blastomere results revealed two embryos carrying *R335T* mutation in *LMNA* gene (embryos #9 and #12), while the remaining nine were free of *R335T* mutation. Two of these embryos (#1 and #8) were transferred, resulting in a singleton pregnancy and the birth of a healthy child without the predisposing gene to CMD (as indicated in the family pedigree by PGD). *ET* embryo transfer, *FR* frozen embryos

the same size. In addition, five polymorphic markers were also used to exclude the possibility of ADO, including *D11S1978*, *D11S1344*, *D11S4117*, *D11S1350*, and *D11S4147*. The *A157V* mutation in the *TNNI3* gene was detected by the use of the two enzymes, *Hae*II, cutting the normal, and *Bsp*MI, cutting the mutant gene into two fragments, as presented in Table 3.24.

Two cycles were performed for cardioencephalomyopathy in the couple with a previous affected child with left ventricular hypertrophic cardyomyopathy, whose first symptoms were manifested as early as at 1.5 months, with a severe respiratory attack. Maternal mutation *E140K* of the *SCO2* gene in this case was detected by *Hind* III and *BsrB*I digestion, the first cutting the mutant and the second cutting the normal gene (see Table 3.24). The paternal mutation *R262* del (CA) was tested by sequencing, which resulted in detection of 139 bp fragment in normal and 137 bp fragment in the mutant gene. Five polymorphic markers, *D22S1153*, *D22S1160*, *D22S1161*, *D22S922*, and *SNP Nla*III, were also tested simultaneously, to avoid misdiagnosis due to ADO (Table 3.24).

Finally, two cycles were performed for a couple at risk for producing offspring with EMD, through testing for maternal mutation *IVS2+1G-T*, using *BpM*I digestion, which cuts the normal gene into two fragments of 115 and 6 bp, with the mutant one left uncut. In addition, five polymorphic markers, *DXS8103*, *DX1684*, *DXS8087*, *DXS1073*, and *DYS154*, were tested to exclude the presence of ADO (see Table 3.24).

All PGD cycles were performed using a standard IVF protocol coupled with

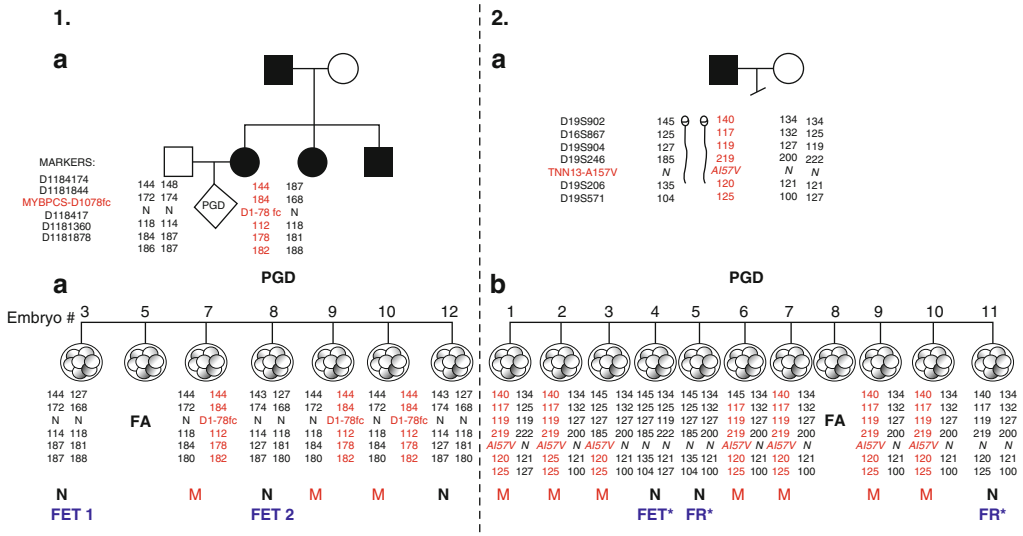



**Fig. 3.39** PGD for hypertrophic cardiomyopathy (CMH). (1) PGD for CMH4. (**a**) Family pedigree of a couple with affected mother carrying frameshift mutation *D1076 fs* in *MYBPC3* gene. Paternal linked polymorphic markers are shown on the *left*, and maternal on the *right*, and the order of the markers and frameshift mutation in *MYBPC3*gene are shown on the *upper left*. (**b**) Blastomere results revealed three embryos (embryos #7, #9, and #10) carrying the frameshift mutation *D1078*fr in *MYBPC3* gene, four unaffected, and 1 did not amplify. Two of the normal embryos were transferred (embryos #3 and #8), following the freezing (frozen embryo transfer (*FET*)), resulting in an unaffected pregnancy (as indicated in the family pedigree by PGD). (2) PGD for CMH7. (**a**) Family pedigree of a couple with affected father carrying *A157V* mutation in *TNNI3* gene. Paternal linked polymorphic markers are shown on the *left*, and maternal on the *right*, and the order of the markers and mutation in *A157V* mutation in *TNNI3* gene are shown on the *upper left*. (**b**) Blastomere results revealed three mutation-free embryos, based on the testing of the mutation and six polymorphic markers (embryos #4, #5, and #11), seven mutant ones, and one did not amplify. Unaffected embryos were tested for 24-chromosome aneuploidy at the blastocyst stage, of which one (embryo #4) was euploid and was transferred in the subsequent cycle. *FR* frozen, *FET* frozen embryo transfer, *FA* failed amplification

micromanipulation procedures for PB1 and PB2 sampling, and/or embryo biopsy, described in Chap. 2. The biopsied PBs and blastomeres were tested by the multiplex nested PCR analysis, involving the above-mentioned mutation and linked marker analysis in a multiplex heminested system. Except for the case of EMD for which PB biopsy procedure was performed, all others were tested by embryo biopsy at the cleavage stage. In cases of advanced reproductive age, aneuploidy testing by FISH analysis, described previously, or by microarray technique for 24 chromosomes, using array-CGH (see Chap. 2) was performed, the latter requiring a blastocyst biopsy and embryo freezing, with their transfer in a subsequent cycle.

As seen from Table 3.23, of 18 cycles performed for 9 at-risk couples, the cardiac disease predisposition-free embryos were preselected for transfer in 15 of them, resulting in 9 pregnancies and the birth of 7 disease-free or disease predisposition-free children.

In nine cycles performed for four patients with CMD, 15 mutation-free embryos were preselected for transfer in eight cycles, yielding the birth of three healthy children free from predisposition to sudden death. One of the cases of PGD for CMD, determined by dominant mutation in the *LMNA* gene, is demonstrated in Fig. 3.38, showing that of 10 of 11 embryos tested for mutation and four linked polymorphic markers, 2 were found to carry the *R335T* mutation in the *LMNA* gene, while the remaining 8 were free of the *R335T* mutation. Two of these embryos were transferred, resulting in a singleton pregnancy and the birth of a healthy child without a predisposing gene to CMD.

**Table 3.24** Primers and reaction conditions for PGD of cardiac diseases

| Disease | Gene/mutation polymorphism | Upper primer | Lower primer | Product size (bp) | Annealing $T_m$ (°C) |
|---|---|---|---|---|---|
| cardiomyopathy, dilated, CMD1A | LMNA- R335T | Outside: R335T-1<br>5′ GTCTCCTACACCGACCCACGT<br>3′ Inside: R335T-3<br>5′ GCTCACCAAACCCTCCCAC 3′ | Outside: R335T-2<br>5′ CGTGGATCTCCATGTCCAGG 3′<br>Inside: R335T-4<br>5′ GTCCAGAAGCTCCTGGTACTCGT 3′ | *Msp1* cuts Normal allele:90+95 bp; Mutant: 185 bp | 62–45 |
| | D1S2714 *Heminested* | Outside: 2714-1<br>5′ TGTGGGGGCTGAGATGAAT 3′<br>Inside:2714-3<br>Hex 5′ CCCAGGATTTTAAGACCAGC 3′ | 2714-2<br>5′ AGACTCTGGAGTAGCAGGGACTA 3′<br>2714-2<br>5′ AGACTCTGGAGTAGCAGGGACTA 3′ | 140–150 | 62–45<br><br>55 |
| | D1S2777 Heminested | Outside: 2777-1<br>5′ CACCACGGAACTCCAGTAT 3′<br>Inside 2777-1<br>5′ CACCACGGAACTCCAGTAT 3′ | 2777-2<br>5′ CAAGTAATCCTCCTGCCTCAG 3′<br>2777-3<br>Hex 5′ TGTTGGGATTACAGGTGTGAG 3′ | 140–150 | 62–45<br><br>55 |
| | D1S2624 Heminested | Outside: 2624-1<br>5′ GAGGCAGAGGCAGACACAGATG 3′<br>Inside:2624-3<br>Hex 5′ ATGGGGCTGACACTCTATGAGG 3′ | 2624-2<br>5′ GACTCAGCGTCCTGCACAGAGT 3′<br>2624-2<br>5′ GACTCAGCGTCCTGCACAGAGT 3′ | 120–130 | 62–45<br><br>55 |
| | D1S506 Heminested | Outside: 506-1<br>5′ CTGGACTCAGCCTGAGAAGAATATG 3′<br>Inside: 506-3<br>Fam 5′ AGAAAGGGAGGGATCGTTCAG 3′ | 506-2<br>5′ GCTATGCTGGGGCAAGGG 3′<br>506-2<br>5′ GCTATGCTGGGGCAAGGG 3′ | 80–100 | 62–45<br><br>55 |

(continued)

**Table 3.24** (continued)

| Disease | Gene/mutation polymorphism | Upper primer | Lower primer | Product size (bp) | Annealing $T_m$ (°C) |
|---|---|---|---|---|---|
| Cardiomyopathy, familial hypertrophic, CMH4 | MYBPC3 D1076fs | Outside:D1076fs-1<br>5′ CTGGTTGGCAGGGTGG 3′<br>Inside: D1076fs-3<br>5′ AGGCGTGGTGACCCAACTG 3′ | D1076fs-2<br>5′ TCTTCTTGTCGGCTTTCTGCA 3′<br>D1076fs-4<br>5′ TCCGTGTTGCCGACATCCT 3′ | *RsaI* cuts *Mutant*: *72+60 bp*; Normal –132 bp; BsaHI cuts normal: 72+60 bp; *Mutant – 132 bp* | 62–45<br><br>55 |
| | D11S1978 Heminested | Outside: 1978-1<br>5′ TGCACTCCACAAATACACACAATT 3′<br>Inside:1978-3<br>Hex5′ CAGAATGTTAGTATAAGTGTGCATGTG 3′ | 1978-2<br>5′ ACTTAGATGTCCATCGACAGATGAA 3′<br>1978-2<br>5′ ACTTAGATGTCCATCGACAGATGAA 3′ | 160–190 | 62–45<br><br>55 |
| | D11S1344 Heminested | Outside:1344-1<br>5′ GCCTCCTGTTCTGTTATTTCACTTA 3′<br>Inside:1344-3<br>Fam5′ TGACTTTAGCCTTGTGCTGAACTG 3′ | 1344-2<br>5′ CAGCGCCTGGCTTGTACATAT 3′<br>1344-2<br>5′ CAGCGCCTGGCTTGTACATAT 3′ | 130–180 | 62–45<br><br>55 |
| | D11S4117 Heminested | Outside: 4117-1<br>5′ TTGTCTTCTTTCTAATCTTCCTTCCA 3′<br>Inside:4117-1<br>5′ TTGTCTTCTTTCTAATCTTCCTTCCA 3′ | 4117-2<br>5′ GTGAGCAAGAGATCACGCCAC 3′<br>4117-3<br>Fam 5′ TGACAGAGCGAGACTCCATCTAAAA 3′ | 100–120 | 62–45<br><br>55 |
| | D11S1350 Heminested | Outside:1350-1<br>5′ CAAATTAAATCATTCTGGGGTCTTT 3′<br>Inside:1350-3<br>Fam 5′ AAACACCTGCTCTCCAAGAATATC 3′ | 1350-2<br>5′ AAACTACCAGCAGTAGAGCACACCT 3′<br>1350-2<br>5′ AAACTACCAGCAGTAGAGCACACCT 3′ | 180–200 | 62–45<br><br>55 |
| | D11S4147 Heminested | Outside: 4147-1<br>5′ AGCTTTTCCCTTGTGGGTGTT 3′<br>Inside:4147-3<br>Fam 5′ AAGGGGAAGACGGACATAAAAC 3′ | 4147-2<br>5′ GCCAGCCTATCTAAACTGTATAATT 3′<br>4147-2<br>5′ GCCAGCCTATCTAAACTGTATAATT 3′ | 130–150 | 62–45<br><br>55 |

| | | | | | |
|---|---|---|---|---|---|
| Cardiomyopathy, familial hypertrophic, CMH7 | TNNI 3 A157V | Outside: A157V-1<br>5′ AAAAAGGAGTGTAGGATGGAGGAGT 3′<br>Inside: A157V-3<br>5′ GGTGTGCGGGAAATGGAAG 3′ | A157V-2<br>5′ TTCCCCTCAGCATCCTCTTTC 3′<br>A157V-4<br>5′ TTCTCGGTGTCCTCCTTCTTCA 3′ | *HaeII cuts* Normal: 150+26; Mutant: 226; *BspMI cuts* Mutant: 190+35+1; Normal: 134+56+35+1 | 62–45<br><br>55 |
| | D19S867 | Outside:867-1<br>5′ CAATGAAAATGCTTTGTAAAACTCTT 3′<br>Inside: 867-1<br>5′ CAATGAAAATGCTTTGTAAAACTCTT 3′ | 867-2<br>5′ TTGGTTTCCTTCTGTCATGTCATC 3′<br>867-3<br>Fam 5′ TCAGAGGTGACCAGTTCTTTCATAC 3′ | 110–130 | 62–45<br><br>55 |
| | D19S 904 | Outside:904-1<br>AATCACACCATTGTACTCCAGCC<br>Inside:904-3<br>HEX 5′ AGGGCAAGACTCCGTTTCAA 3′ | 904-2<br>TCGGAGATGTTAAAATGTGAAAAAC<br>904-2<br>TCGGAGATGTTAAAATGTGAAAAAC | 115–130 | 62–45<br><br>55 |
| | D19S 246 | Outside:246-1<br>5′ GTGAGCCAAGACTACGCCACT<br>Inside: 246-3<br>Fam 5′ AGAGTGAGATTCCACCTTTCAAAAA 3′ | 246-2<br>5′ CCAGAAACACATCATTTACCCACTT 3′<br>246-2<br>5′ CCAGAAACACATCATTTACCCACTT 3′ | 200–230 | 62–45<br><br>55 |
| | D19S 206 | Outside:206-1<br>5′ TTTTCCTATTTTATCTGGCGGG 3′<br>Inside:206-3<br>FAM 5′ AAGTGAAAGCCGAAGTCTTTTCA 3′ | 206-2<br>5′ TCATCAAGTCTGTTCCAGCCAA 3′<br>206-2<br>5′ TCATCAAGTCTGTTCCAGCCAA 3′ | 120–140 | 62–45<br><br>55 |
| | D19S 571 | Outside:571-1<br>5′ TGAACTCCAGCCTGGGTGAG 3′<br>Inside:571-1 | 571-2<br>TTGACAGCATGTATTTGAAATATGG<br>571-3HEX | 100–130 | 62–45<br><br>55 |

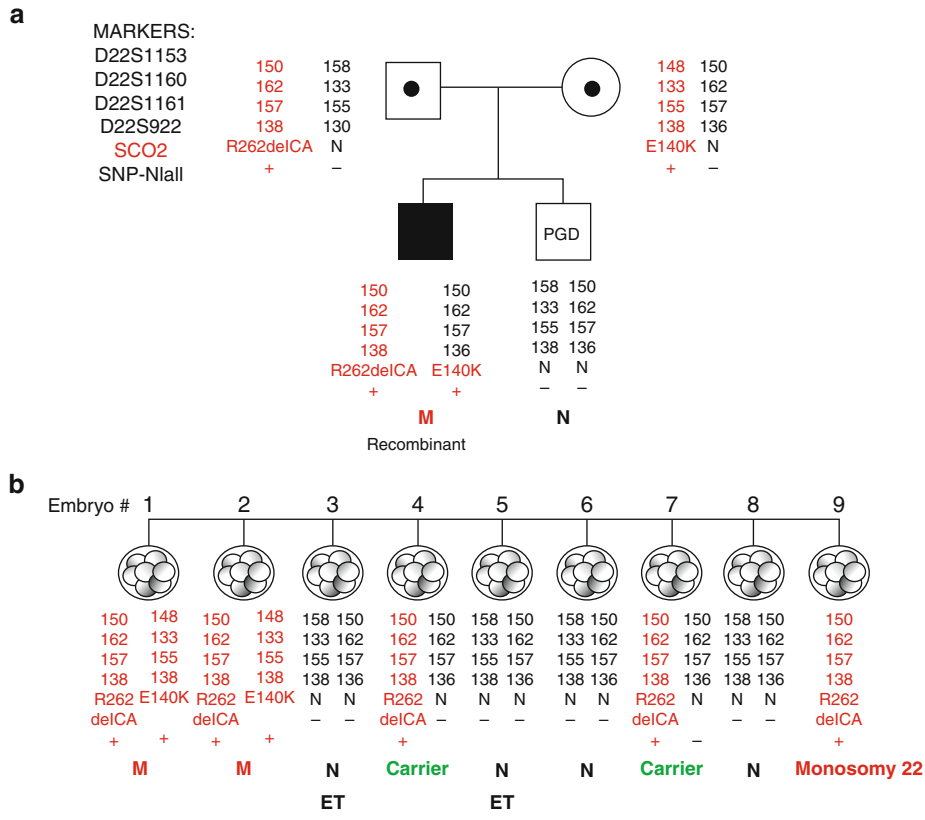



**Fig. 3.40** PGD for cardioencephalomyopathy. (**a**) Family pedigree of a couple with a previous affected child, who was double-heterozygous for *E140K* and *R262* del (CA) in the *SCO2* gene. Paternal polymorphic markers are shown on the *left*, and maternal on the *right*, with the order of the markers and mutation shown on the *upper left*. (**b**) Blastomere results revealed two embryos (embryo #1 and #2) homozygous affected, two (embryos #4 and #7) carriers of the paternal mutation, four mutation-free embryos (embryos #3, #5, #6, and #8), and one monosomic for chromosome 22, based on the testing of the mutation and 6 polymorphic markers. Two mutation-free embryos (embryos#3 and #5) were transferred, resulting in a singleton pregnancy and the birth of an unaffected child (as indicated in the family pedigree by PGD). *ET* embryo transfer

Of four cycles performed for three couples at risk for producing offspring with CMH, three embryos were preselected for transfer in two cycles, resulting in a singleton pregnancy, which is presented in Fig. 3.39-1. Of seven embryos tested, three (embryos #7, #9, and #10) were carriers of the frameshift mutation *D1078*fr in the *MYBPC3* gene, three were unaffected, and one did not amplify. Two of the normal embryos were transferred following freezing, resulting in an unaffected pregnancy.

The results of the PGD cycle for the patient at risk for producing the offspring with CMH7 is presented in Fig. 3.39-2. Of 11 tested embryos, 10 amplified, of which 3 (embryos #4, #5, and #11) were unaffected, based on the testing of the mutation and 6 polymorphic markers. Because these embryos were also tested for 24 chromosome aneuploidy by array-CGH analysis at the blastocyst stage, the embryos were frozen and one of them (embryo #3), which was also aneuploidy-free, was transferred in the subsequent cycle.

Of three cycles performed for cardioencephalopathy, seven unaffected embryos were found unaffected and transferred, resulting in two unaffected pregnancies and the birth of a healthy child free from cardioencephalopathy. The results of one of these cycles are shown in Fig. 3.40, showing that of nine embryos tested, two embryos

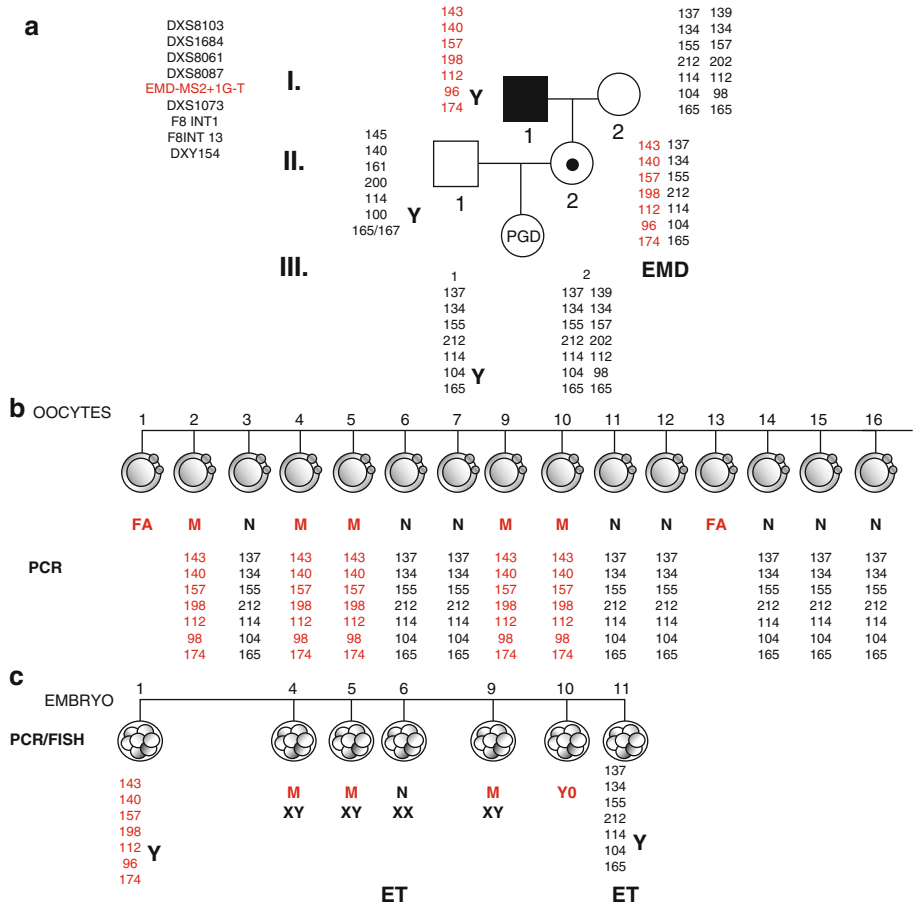



**Fig. 3.41** PGD for Emery–Dreiffus muscular dystrophy (*EMD*). (**a**) Family pedigree of a couple with the mother (II-2) carrying an X-linked EMD- IVS2+1G-T mutation, inherited from her father (I-1). Maternal polymorphic markers are shown on the *right*, paternal on the *left*, with the order of the markers and mutation shown on the *upper left*. The haplotypes for the patient's father (I-1) are also shown on the *left*. (**b**) Sequential first and second polar body analysis results in eight mutation-free oocytes, five mutant (#2, #4, #5, #9, and #10) and two did not amplify (#1 and #13). (**c**) Blastomere results of seven resulting embryos for gender determination by FISH and PCR showed that embryos resulting from mutant oocytes #4, #5, and #9 were males, and therefore affected, so only embryos #6 and #11, originating from mutation-free oocytes, regardless of XY genotype, were transferred, resulting in a singleton pregnancy and the birth of an unaffected child (as indicated in the family pedigree by PGD). *ET* embryo transfer

(embryo #1 and #2) were homozygous affected, two (embryos #4 and #7) carriers of the mutant gene, one (embryo #9) monosomic for chromosome 22, and four (embryos #3, #5, #6, and #8) free of the mutation. Two of these embryos (embryos #3 and #5) were transferred, resulting in a singleton pregnancy and the birth of an unaffected child.

Of two PGD cycles performed for EMD, five disease-free embryos were preselected for transfer, yielding an unaffected pregnancy in each cycle and the birth of two EMD-free children. One of these cycles is presented in Fig. 3.41, demonstrating the results of sequential PB1 and PB2 analysis for IVS2+1G-T mutation, followed by mutation and aneuploidy testing at the cleavage stage. Only one of these embryos was free of mutation and aneuploidy (embryo #12) and was transferred, resulting in the birth of an unaffected child.

The presented results show that PGD may be a realistic option for couples at risk for producing offspring with cardiac disease, determined by

inherited predisposition. Inheritance of such susceptibility factors places the individual at risk of serious cardiac disease, clinically manifested from as early as the first year of life such as in cardioencephalopathy, to as late as later in life, with the only clinical realization of premature or sudden death, as in CMD and CMH.

Among the conditions in the family history of the couples at risk that may indicate a possible need of PGD may be a heart attack and sudden death at young age, family members with pacemakers or internal cardiac defibrillators, arrhythmia, and heart surgery. The chances that the offspring of these patients will develop the same heart disease will differ depending on the mode of inheritance, but their penetrance is difficult to predict, because many inherited cardiac conditions are difficult to diagnose and will develop with age and may be induced by certain medications or activities, such as excessive exercise, which may lead to cardiac arrest or sudden death, justifying the parents' requests for PGD.

In fact, in some cases a common, apparently "milder" disease susceptibility gene may contribute to premature death, major disability, or hardship in a family. However, only personal experience may alter a family's perception of severity of the condition, as the basis for their decision to undertake PGD. Many couples already going through IVF for fertility treatment may have questions about the implications of genetic susceptibility factors for offspring, the option to test embryos, and the appropriateness of using PGD in testing for susceptibility to inherited cardiac disease.

Because the symptoms of inherited cardiac disease may be easily overlooked, as seen from the description of the cases above, the family history may be the only reason to test for the presence of predisposing gene mutations and consideration about the need for PGD, which may appear as the life-saving procedure for individuals at risk. So with the future identification of the genes predisposing to inherited cardiac disease, PGD might appear as a useful tool for couples at risk to avoid the risk for producing offspring with inherited cardiac diseases with high probability of premature or sudden death during their life span.

## 3.8 Blood Group Incompatibility

Although the at-risk pregnancies for blood group incompatibility, including that caused by Kell (KI) genotype or RhD, may be detected by prenatal diagnosis, in order to be treated by an intrauterine transfusion, the potential complication for the fetus cannot be completely excluded even after the procedure. Pregnancy termination in such cases will also be unacceptable, as the antibodies to K1, for example, are developed only in 5% of persons obtaining incompatible blood. On the other hand, some of the at-risk couples have had so unfortunate an experience of hemolytic disease of the newborn (HDN), resulting in neonatal death, that they regard PGD as their only option to plan another pregnancy. This makes PGD attractive for patients at risk for alloimmunization, although such conditions have rarely been an indication for prenatal diagnosis.

We performed the first PGD for maternal fetal incompatibility caused by K1 genotype, which is presented below.

The K1 system is one of the major antigenic systems in human red blood cells, comparable in importance to RhD, as it may cause maternofetal incompatibility leading to severe hemolytic disease of the newborn (HDN) in sensitized mothers. The K1 allele is present in 9% of the populations, in contrast to its highly prevalent allelic variant K2. The gene is located on chromosome 7 (7q33), consisting of 19 exons, with the only C to T base substitution in exon 6 in K1 compared to the K2 antigen, which leads to a threonine to methionine change at amino acid residue 193, preventing *N*-glucosylation [82, 83]. C to T base substitution also creates a BsmI restriction enzyme site, providing a reliable DNA test for diagnosis of KEL genotype.

In case of pregnancy by the K1 fetus in the K2 mother, antibodies to K1 may be developed leading to maternofetal incompatibility causing severe HDN. Although prenatal diagnosis is available for identification of pregnancies at risk for HDN, this may not always prevent the potential complications for the fetus, stillbirth, or neonatal death, making PGD a possible option for preventing both Kell and Rhesus hemolytic diseases.

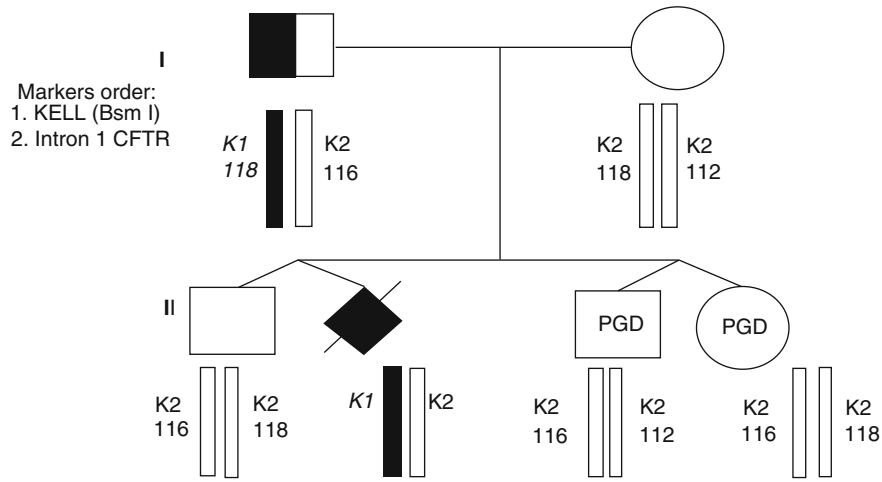



**Fig. 3.42** Preimplantation genetic diagnosis for Kell genotype: family pedigree. (*Upper panel*) The father (*upper left*) has *K1*/*K2* genotype, *K1* allele linked to 118 bp repeats, and *K2* allele to 116 bp repeats of intron 1 of *CFTR* polymorphic marker, while the mother (*upper right*) has *K2*/*K2* genotype, one allele linked to 118 bp repeats, and the other to 112 bp repeats of intron 1 of *CFTR* polymorphic marker. (*Lower panel*) Reproductive outcomes of this couple, including previous twin pregnancy resulting in the death of one of the twins near birth due to HDN. Two healthy twins with *K2*/*K2* genotype resulting from PGD were born confirmed also by linked polymorphic markers

Two couples presented for PGD, both with paternal K1/K2 genotype, that is, heterozygous for C to T base substitution in exon 6, creating a BsmI restriction enzyme site. In one couple, a 36-year-old mother had a previous dizygotic twin pregnancy, resulting in the death of one of the twins carrying the K1 allele near birth, due to HDN (Fig. 3.42).

In the second couple, a 37-year-old mother had three previous pregnancies, of which the first resulted in the birth of a healthy boy, carrying the K1 allele, the second in the birth of a normal K2/K2 boy, and the third in a premature delivery of a 32-week female carrying the K1 allele, who died the next day after birth with the clinical features of a severe HDN.

To establish paternal haplotypes, a single-sperm analysis was performed, to be able to undertake the linked marker analysis, in addition to KEL genotyping. Short tandem repeats (STR) associated with the cystic fibrosis (CFTR) gene were used, which are known to be located close to the K1 and K2 alleles, with extremely rare recombination rates [84]. This analysis showed the presence of one informative linked marker (CFTR Intron 1) in the first and three (D7S550, CFTR Intron 6, and CFTR Intron 8) in the second couple. The K1 allele was linked to 118 (CFTR Intron 1) repeat in the first (Fig. 3.43), and to the 158 (D7S550), 7 (CFTR Intron 6), and 124 (CFTR Intron 8) repeats in the second couple. Thus, in PGD cycles for both couples, multiplex nested PCR analysis was performed, by testing the BsmI restriction site simultaneously with the linked polymorphic markers, including the CFTR Intron 1 in the first, and the D7S550, CFTR Intron 6, and CFTR Intron 8 in the second couple. Outside and inside primer sequences and primer melting temperatures for DNA analysis in both couples are shown in Table 3.25 [85–87]. PCR products were identified by restriction digestion using BsmI for Kell gene (Fig. 3.43), and by capillary electrophoresis and scoring by GynotyperTM for STRs.

Overall, five PGD cycles were performed, including one for the first and four for the second couple, using blastomere biopsy. Based on both BsmI restriction digestion and STR analysis, K1 allele-free embryos were preselected for transfer back to patients, while those predicted to contain the K1 allele were exposed to confirmatory analysis using genomic DNA from these embryos, in order to evaluate the PGD accuracy.

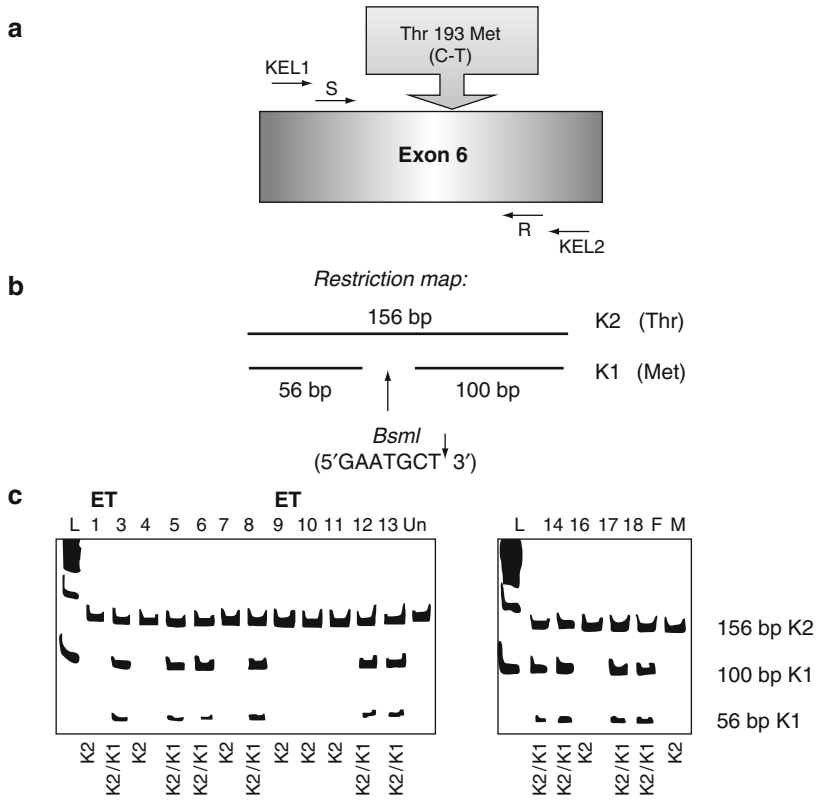



**Fig. 3.43** Preimplantation genetic diagnosis for Kell genotype. (**a**) Schematic diagram showing C>T substitution in exon 6 of *KEL* gene on chromosome 7: *black arrows* demonstrate the positions of nested primers. (**b**) Restriction map for *Bsm*I digestion, showing the gain of *Bsm*I site by the *K1* allele (*lower line*). (**c**) Polyacrylamide gel electrophoresis of the *Bsm*I digested PCR products of 16 blastomeres from the first PGD couple, demonstrating *K1* allele-free genotype in embryos #1, #4, #7, #9, #10, #11, and #17, from which embryos #1 and #9 were transferred, resulting in a twin pregnancy and the birth of healthy *K1* allele-free children. The remaining nine embryos have *K1*/*K2* genotype. *L* standard, *F* paternal DNA amplified from sperm, *M* maternal normal amplified DNA, *Un* undigested PCR product, *K1*/*K2* affected blastomere, *K2*/*K2* normal blastomere

In a single PGD cycle for the first couple, 16 embryos at day 3 were tested, of which 9 were excluded from transfer due to the presence of the K1 allele (Fig. 3.43). The remaining 7 embryos did not contain the K1 allele (embryos #1, #4, #7, #9, #10, #11, and #17), also in agreement with the absence of the linked intron 1 CFTR (118 repeats) marker, but showed the presence of the paternal K2 allele, evidenced also by the presence of the intron 1 CFTR (116 repeats) marker, linked to the paternal K2 allele. One of the K1 allele-free embryos (embryo #11) did not develop, 4 were frozen (embryos #4, #7, #10, and #17), while the remaining 2K1 allele-free embryos (embryos #1 and #9) were transferred back to the patient, resulting in a clinical twin pregnancy and the birth of a healthy boy and girl homozygous for K2 genotype (Fig. 3.42).

In the second couple, a total of 20 embryos at day 3 were tested for the K1 allele in four PGD cycles (8 in the first, 5 in the second, 3 in the third, and 4 in the fourth cycle). Nine of these embryos were heterozygous for K1 allele, that is, unsuitable for transfer, while the remaining 11 embryos were predicted to be free from this allele. Seven of these embryos were transferred, including 2 in the first, 1 in the second, 1 in the third, and 3 in the fourth cycle, however, yielding no clinical pregnancy.

The follow-up analysis of the K1 allele-containing embryos, excluded from transfer, was possible in a total of nine cases from both couples. Although the predicted genotypes were

**Table 3.25** Primers and reaction conditions for PGD of Kell genotype

| Gene/ polymorphism | Upper primer | Lower primer | Annealing $T_m$ (°C) |
|---|---|---|---|
| Kell gene (NESTED PCR) | Outside | | 62–55 |
| | 5′ TCAGCCCCCTCTCTCTCCTT 3′ | 5′ GTGTCTTCGCCAGTGCATCC 3′ | |
| | Inside [63] | | 50 |
| | 5′ AAGCTTGGAGGCTGGCGCAT 3′ | 5′ CCTCACCTGGATGACTGGTG 3′ | |
| D7s550 (Heminested PCR) | Outside: | Outside: | 62–55 |
| | 5′ ACTATCATCCACAATCCACTCC 3′ | 5′ GCAGTTGGGTTATTTCAAGTCT 3′ | |
| | Inside: | Inside: | 56 |
| | 5′ ACTATCATCCACAATCCACTCC 3′ | 5′ HexGATGTTGTGATTAGAGTTGCTGTA 3′ | |

confirmed overall, six cases of recombination between K alleles and two linked markers (CFTR Intron 6 and CFTR Intron 8) was observed, suggesting a limited value of these two markers on their own. However, no recombination was observed between the gene and one linked marker in both cases (intron 1 CFTR in the first and D7S550 in the second couple), which allowed verifying the absence of the KI allele and detecting both maternal and paternal K2 alleles in the embryo, thus improving considerably the reliability of the diagnosis. For example, the preselection of the K1-free embryos shown in Fig. 3.42 was based not only on the absence of the K1 allele, which may be also explained by ADO, but also on the absence of the linked intron 1 CFTR (118 repeats) marker, and the presence of both the paternally and maternally derived K2 alleles, evidenced by the presence of polymorphic markers linked to the paternal and maternal K2 alleles. In other words, the absence of the K1 allele together with the presence of both paternal and maternal K2 alleles allowed reliably preselecting the KI allele-free embryos for transfer.

The presented cases are the only PGD cycles resulting in the birth of unaffected K1-free children in the worldwide PGD experience. A number of attempts have been undertaken also to perform PGD for Rhesus disease, which initially did not resulted in a clinical pregnancy [88]. The most recent attempt, however, yielded a clinical pregnancy and the birth of a healthy girl confirmed to be blood type Rh-negative [89]. A couple with Rh-negative mother and RhD-positive father had two children, one of whom was affected by HDN, with hyperbilirubinemia and neonatal jaundice, as well as significant hemolitic anemia. Because of RhD alloimmunization, the couple was presented with the dilemma of whether to attempt further pregnancy, as there is a tendency for rhesus disease to worsen with each subsequent rhesus-incompatible pregnancy in sensitized women. Using blastomere biopsy and direct PCR amplification with analysis by capillary electrophoresis of fluorescently labelled amplicons, RhD-negative embryos were preselected for transfer, yielding an unaffected pregnancy and the birth of a healthy child.

Both Kell and Rh disease are quite prevalent in the populations, taking into consideration approximately 15% frequency for RhD and 9% for KEL antigen, presenting the risk for alloimmunization that may lead to HDN in some of the at-risk couples. Therefore, PGD may be a practically useful option for these couples to avoid the establishment of the RhD or K1 pregnancy in the sensitized mothers.

Thus, PGD for Kell genotype and other red blood group systems is feasible, providing a novel approach for sensitized mothers to avoid the risk of having children with HDN [90]. Therefore, PGD may be a useful option for these couples to avoid the establishment of the RhD or K1 pregnancy in sensitized mothers.

## 3.9 Congenital Malformations

Congenital malformations are highly prevalent (29.3/1,000 live births) and are usually sporadic. As described in Chap. 1, the major reduction of congenital malformations may be expected from population-based preventive measures, such as folic acid fortification of major foodstuffs, which may result in prevention of birth of tens of

thousands of children with congenital malformations in North America. However, this will not have sufficient impact on the prevention of inherited forms, for which PGD might be an important option. In fact, with the progress of the human genome project, an increasing number of inherited forms are being described, which, therefore, may be avoided through PGD. One example of the first application of PGD for congenital diseases was PGD for the sonic hedgehog (SHH) gene mutation, which is presented below.

The SHH gene is a human homolog of the Drosophila gene encoding inductive signals involved in patterning the early embryo, known to be (functionally) highly conserved in many species. The gene was mapped to chromosome 7 (7q36), previously designated as the locus for the gene involved in holoprosencephaly (HPE3) [91].

The available data provide the evidence that SHH mutations may cause the failure of cerebral hemispheres to separate into distinct left and right halves, leading to HPE, which is one of the most common developmental anomalies of the forebrain and midface [92]. Although the majority of HPE are sporadic, familial cases are not rare, with clear autosomal-dominant inheritance.

A great intrafamilial clinical variability of HPE from alobar HPE and cyclopia to cleft lip and palate, microcephaly, ocular hypertelorism, and even normal phenotype suggests the interaction of the SHH gene with other genes expressed during craniofacial development and the possible involvement of environmental factors. This may explain the fact that almost one-third of the carriers of SHH mutations may be clinically unaffected. Therefore, even in familial cases, the detection of SHH mutations in prenatal diagnosis might not justify pregnancy termination, making preimplantation genetic diagnosis (PGD) a more attractive option for couples at risk for producing a progeny with HPE.

In our first case, the couple presented for PGD with two children showing the clinical signs of HPE [93] (Fig. 3.44). One of them, a female with severe HPE and cleft lip and palate died shortly after birth. The chromosomal analysis performed using peripheral blood lymphocytes of both this

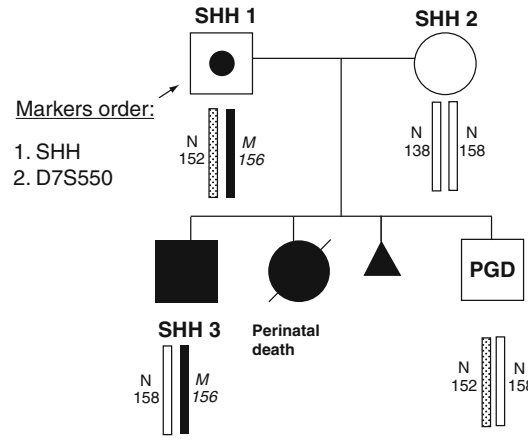



**Fig. 3.44** Preimplantation diagnosis for sonic hedgehog (*SHH*) mutation: family pedigree. (*Upper panel*) The father has a gonadal mosaicism for SHH mutation, which is linked to 156 bp dinucleotide C-A repeat allele of D7S550 polymorphic marker, while the mother is normal, with one normal allele linked to 158 bp repeat and the other to 138 bp repeat alleles. (*Lower panel*) Reproductive outcomes of this couple, including three previous pregnancies, one resulting in the birth of an affected child with holoprosencephaly, carrying the mutant gene (*lower left*), one in perinatal death, also carrying the mutant gene (*lower middle* (*circle*)), and one in a spontaneously aborted fetus with Turner syndrome, free from SHH mutation (*lower middle* (*triangle*)). The *lower right* (PGD) shows the outcome of preimplantation diagnosis, resulting in an unaffected clinical pregnancy and the birth of a healthy child, following confirmation of the mutation-free status by amniocentesis

child and the parents was normal, but DNA analysis in the child's autopsy material demonstrated the presence of SHH nonsense mutation due to GAG>TAG sequence change leading to premature termination of the protein at position 256 (Glu256→stop) [92] (Fig. 3.45). SHH protein is an intercellular signalling molecule, which is synthesized as a precursor undergoing autocatalytic internal cleavage into a highly conserved domain (SHH-N) with signaling activity, and a more divergent domain (SHH-C), which in addition to precursor processing acts as an intramolecular cholesterol transferase crucial for proper patterning activity in animal development. Although the effect of the above nonsense mutation on SHH function is unknown, the resulting protein may fail fulfilling the expected signaling function in early morphogenesis [16, 92].

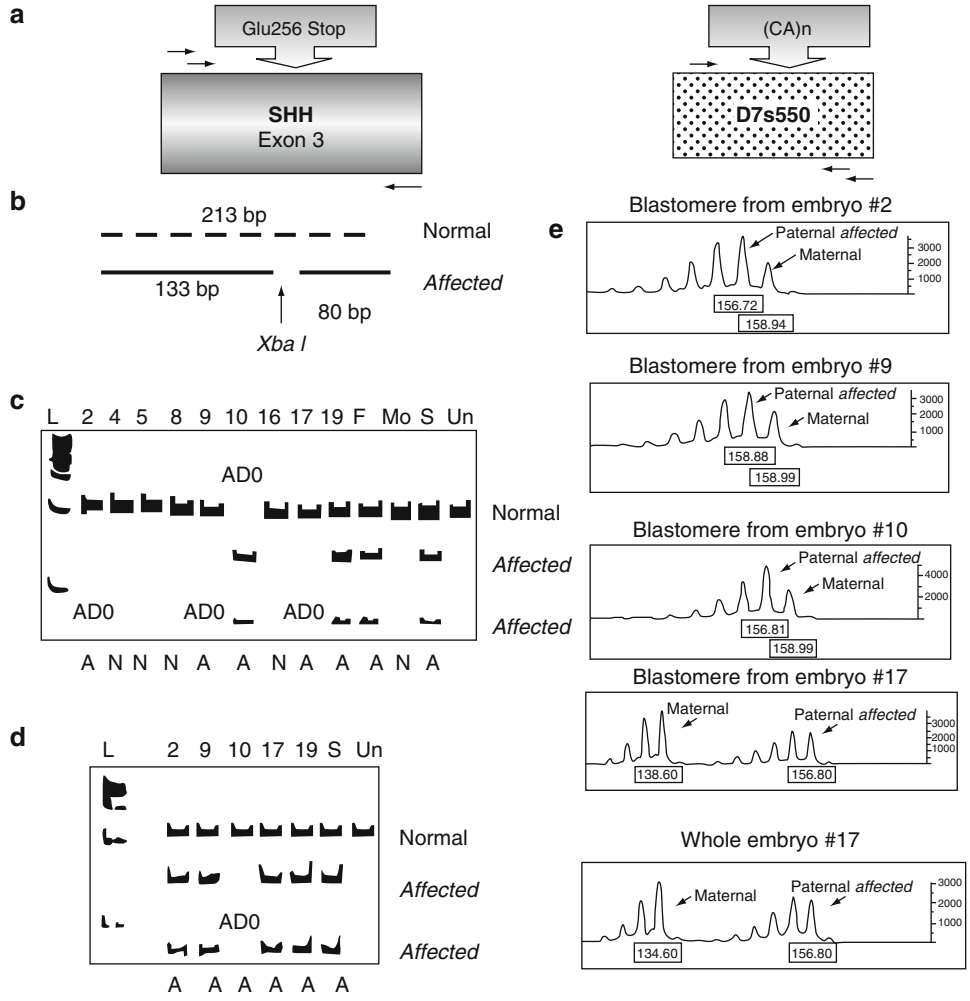



**Fig. 3.45** PGD for sonic hedgehog (*SHH*) mutation involving confirmation of the presence of both maternal and paternal normal genes in preselected mutation-free embryo. (**a**) Schematic diagram of the mutation and D7S550 linked marker on chromosome 7. *Black arrows* demonstrate the positions of heminested primers. (**b**) Restriction map for *Xba*I digestion, showing the gain of *Xba*I site by the mutant allele (*lower line*). (**c**) Polyacrylamide gel electrophoresis of the *Xba*I digested PCR products of nine blastomeres from PGD cycle. (**d**) Follow-up DNA analysis of genomic DNA from five embryos predicted to be affected by blastomere testing. (**e**) Capillary electrophoregrams of fluorescently labeled PCR product of tightly linked marker D7S550. Paternally derived 156 bp dinucleotide C-A repeat linked to SHH mutation are shown by arrow (noted as "paternal affected") in blastomeres of embryos #2, #9, #10, and #17 and the genomic DNA of the whole embryo #10, in which ADO of mutant gene was seen in the follow-up study (see *panel* **b**, embryo #10). Maternally derived 158 bp dinucleotide C-A repeats of D7S550 polymorphic marker are shown by *arrow* (noted as "maternal") in blastomeres of embryos #2, #9, and #10, while the other maternally derived 138 bp repeats of D7S550 are shown in blastomeres of embryo #17. *L* 100 bp standard, *ADO* allele dropout, *F* paternal DNA amplified from sperm, *Mo* maternal normal amplified DNA, *S* amplified DNA from affected baby, *Un* undigested PCR product, *A* affected blastomere, *N* normal blastomere

The same mutation was found in their 5-year-old son, who was born after a full-term normal pregnancy weighing 6 lb, with a birth length of 18 1/3 inches. This child has less severe facial dismorphisms, which included microcephaly, Rathke's pouch cyst, single central incisor, and choanal stenosis (the latter was treated surgically after birth with dilatation). There was also

**Table 3.26** Primers and reaction conditions for the detection of Glu256Stop mutation in sonic hedgehog gene and linked marker D7S550

| Gene/polymorphism | Upper primer | Lower primer | Annealing $T_m$ (°C) |
|---|---|---|---|
| SSH (heminested) | Outside: | Outside: | 62–55 |
| | GAGCAGGGCGGCACCAA | GGCCGAGTCGTTGTGC | |
| | Inside: | Inside: | 56 |
| | GGCACCAAGCTGGTGAAG | GGCCGAGTCGTTGTGC | |
| D7S550 (heminested) | Outside: | Outside: | 62–55 |
| | ACTATCATCCACAATCCACTCC | GCAGTTGGGTTATTTCAAGTCT | |
| | Inside: | Inside: | 56 |
| | ACTATCATCCACAATCCACTCC | GATGTTGTGATTAGAGTTGCTGTA | |

clinodactily of the fifth fingers and incurved fourth toes bilaterally. The child's growth was slow in the first 2 years, but then he has been maintaining a reasonably good growth and presently has normal social and cognitive development.

The couple had another pregnancy, which has ended in spontaneous abortion due to Turner syndrome (45, X), showing no inheritance of the SHH mutation. The mutation was not found in either parent's genomic DNA, although the paternity testing showed that the father was in fact a biological father of both affected children. This clearly suggested a de novo gonadal mutation in one of the parents, which has been identified by a single-sperm genotyping in the present study (see below).

Two PGD cycles were performed based on single blastomere analysis removed from the 8-cell embryos and tested by multiplex nested PCR analysis, involving specific mutation testing simultaneously with linked marker analysis. Of 15 embryos in the first cycle, 12 were available for blastomere biopsy at the 8-cell stage. Blastomeres from four embryos failed to amplify, leaving eight with available data for mutation analysis. Seven of these eight embryos appeared to contain the mutant allele, while only one embryo was mutation-free and transferred, yielding no clinical pregnancy.

The second PGD cycle was performed in 1 year's time, in which 19 embryos were available, of which 10 were acceptable for blastomere biopsy and DNA analysis. Of these 10 biopsied single blastomeres, only one failed to amplify, the remaining 9 being with available data for the SHH gene and the marker, to identify the mutation-free embryos for transfer (Fig. 3.45).

Prior to PGD cycles, a single-sperm testing was performed, which identified mosaicism for SHH mutation. As the mutation was shown to lead to the gain of an XbaI restriction site [92], the normal allele was identified as undigested PCR product, the mutant allele being represented by two fragments, as a result of XbaI digestion (Fig. 3.45).

To avoid misdiagnosis in mutation analysis due to ADO, which exceeds 10% in single blastomere DNA analysis, a closely linked microsatellite DNA marker D7S550 was tested in the same reaction as the internal control. The list of primes used in the first- and second-round PCR for mutation and linked marker analysis and reaction conditions are presented in Table 3.26. A haplotype analysis showed that the mutant allele was linked to 156 bp dinucleotide CA repeat, while the normal gene was linked to 152 bp repeat allele in 7q36 (Figs. 3.44 and 3.45). Although other linked markers have also been described [91], they were not informative in the present couple.

As seen from Fig. 3.45, four ADOs were observed in the mutation analysis, including ADO of the mutant allele in embryos #2, #9, and #17, and ADO of the normal allele in embryo #10. This was based on the marker analysis, showing that in all four cases the embryos were heterozygous. In other words, three of these four embryos (#2, #9, and #17) could have been misdiagnosed as normal without linked marker analysis. In addition to these three embryos, embryo #19 also contained the mutant gene.

The remaining four embryos were free of the mutant gene, as confirmed by marker analysis,

showing that all these embryos contained two normal alleles, including the paternal one linked to 152 repeat, and either normal maternal allele linked to 138 repeat (embryos #4 and #5) or the other normal maternal allele linked to 158 bp repeat (embryos #8 and #16). Two of these embryos (embryos #4 and #5) were transferred back to the patient, resulting in a singleton pregnancy and the birth of a healthy child following confirmation of the mutation-free status by amniocentesis. The other two mutation-free embryos (embryos #8 and #16) were frozen for further use by the couple.

The presented data demonstrate a diagnostic accuracy of the multiplex PCR-based blastomere analysis, despite the well-known high ADO rate in this type of single cells. As mentioned, ADO is an important limitation of PCR analysis in single cells, due to allele-specific amplification failure, which is particularly high in single-blastomere analysis shown to be at least two times higher than in single fibroblasts and polar bodies. As shown by the follow-up analysis of the preselected mutant embryos, PGD results were confirmed in all resulting embryos available for study, which is in accordance with extensive data, described above, based on testing of hundreds of oocytes and embryos for different single-gene disorders. Although ideally three linked markers are needed to completely exclude the risk for misdiagnosis due to ADO, the use of only one linked marker in the present study was quite reliable, probably because the preselection of mutation-free embryos was based not only on the presence of the paternally derived normal allele, but also on the presence of the second normal allele linked to the maternal-linked marker. In other words, the absence on the mutant gene together with the presence of the two normal alleles, identified by different linked makers, led in this case to the correct identification of embryos as normal or carrying the SHH mutation.

The presented case demonstrates the clinical relevance of PGD for familial HPE [93]. Because of the high prevalence of congenital craniofacial anomalies, this approach may have practical implications for at-risk couples. A great intrafamilial clinical variability of HPE from alobar HPE and cyclopia to cleft lip and palate, microcephaly, ocular hypertelorism, and even normal phenotype suggests the interaction of the SHH gene with other genes expressed during craniofacial development and the possible involvement of environmental factors. This may explain the fact that almost one-third of carriers of SHH mutations may be clinically unaffected. Therefore, even in familial cases, the detection of SHH mutations in prenatal diagnosis might not justify pregnancy termination, making PGD a more attractive option for couples at risk for producing a progeny with HPE, as demonstrated by the first PGD for this mutation mentioned, which resulted in the birth of a healthy mutation-free baby.

Of other congenital malformations, PGD was performed for Currarino triad, Crouson and Holt-Oram syndrome [94, 95], all resulting in the birth of mutation-free children. In these cases the application of prenatal diagnosis may be limited by the factors that modify clinical manifestations and confound prediction of an individuals' phenotype making PGD an attractive choice, as shown in the example of PGD for Crouson syndrome and Currarino triad [95], presented below.

**Currarino Triad**. Currarino syndrome (CS) is a severe autosomal-dominant disorder caused by homeobox gene HLXB9 mutation, involving partial sacral agenesis, presacral mass, and anorectal malformations, which are one of the commonest digestive anomalies requiring neonatal surgery [16, 17]. This homeobox gene is located on chromosome 7q36, between microsatellite DNA markers D7S559 and D7S2423 [96]. The abnormalities observed in CS are caused by the disturbances in early embryonic development of the human tail bud, leading to the formation or positioning defects of neural tube, notochord, somites, and hindgut. More than two dozens of different HLXB9 intragenic mutations and microdeletions were detected in patients with CS, including frameshift and nonsense mutations in intron 1 and missense mutations in homeodomain, resulting in a nonconservative substitution of a highly conserved amino acid [16, 97–99].

Although a carrier screening and prenatal diagnosis of CS is currently available, the decision

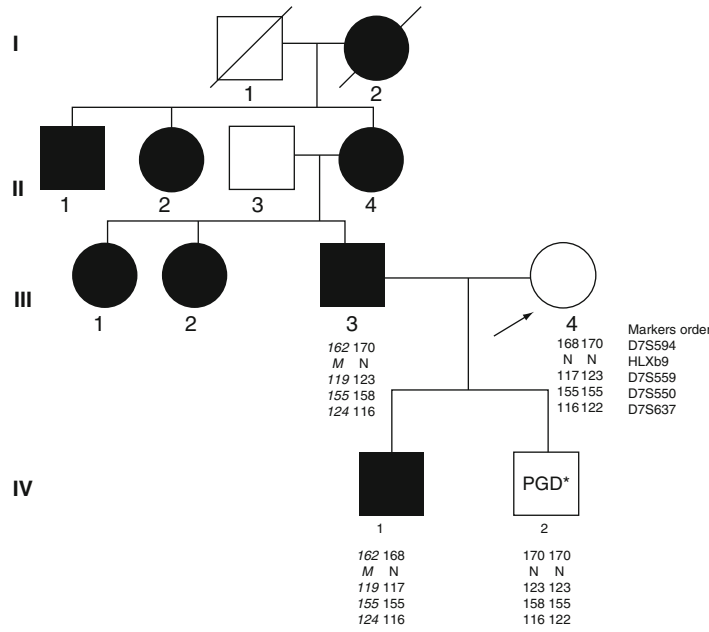



**Fig. 3.46** Preimplantation diagnosis for Currarino syndrome: Family pedigree. I & II Patient's parents & grand parents with signs of symptoms of the disease in his mother and grand father. Affected father (III, 3) has two affected sisters; homeobox gene HLXB 9 mutation was inherited from their mother (II, 4), who also had two affected siblings (brother II, 1, and sister II. 2). Reproductive outcome is shown in *lower panel*, with the previous affected child (IV. 1) and the healthy baby boy born after PGD (IV. 2)

about termination of pregnancy will be controversial, as approximately half of the carriers of the mutation are asymptomatic [99]. Therefore, the absence of the genotype–phenotype correlation and an extremely high variability of phenotype in the carriers, from the above severe triad to minor sacral abnormalities undetectable without X-ray or even a completely asymptomatic carrier status, make PGD an important alternative for the at-risk couples wishing to avoid CS in their offspring.

A couple presented for PGD with a previous child diagnosed to have CS. The child was born with imperforate anus, an anterior meningocele, and a typical sickle-shaped hemisacrum revealed by X-ray of the sacrum region (Fig. 3.46; *IV 1*). The father was also born with an anal stricture (Fig. 3.46; *III 3*), requiring anal dilatation, and also had sacral defect detected by X-ray, involving a central anomaly from S2 downward. One of his two sisters (Fig. 3.46; *III 1*) was born with imperforate anus and anterior meningocele and also had rectovaginal fistula, and a vesico-ureteric reflux resulting in the need of renal transplantation; a sacral X-ray showed the same central defect with absence of the distal one-third of the sacrum. The other sister (Fig. 3.46; III 2) was clinically asymptomatic, but sacral X-ray revealed no coccyx and a MRI scan disclosed an anterior meningocele. His mother had an undeveloped coccyx and urinary tract bilateral ureteropelvic junction obstruction (Fig. 3.46; *II 4*). Of her three siblings, only one of two brothers had anal stricture (Fig. 3.46; *II 1*), while the asymptomatic sister (Fig. 3.46; *II 2*) was identified as a carrier of the mutation because of the finding of an imperforate anus in her grandson. As seen from the pedigree, the father inherited the mutation from his grandmother (Fig. 3.46; *I 2*), who was probably the first affected member of the family, known to have constipation but normal sacral X-ray.

DNA analysis in this family demonstrated the presence of homeobox HLXB9 mutation due to frameshift insertion of a cytosine into a stretch of six cytosines at positions 125–130 in exon 1 of the gene, leading to the introduction of premature termination codon [96]. The primer sequences for mutation testing and their positions are presented in Fig. 3.47 and listed in Table 3.27.

A PGD cycle was performed using single blastomeres, removed from the 8-cell embryos and tested by multiplex nested PCR analysis, involving specific mutation testing simultaneously with linked marker analysis. The PCR product was identified by fragment-length analysis using capillary elecrophoresis (Fig. 3.47).

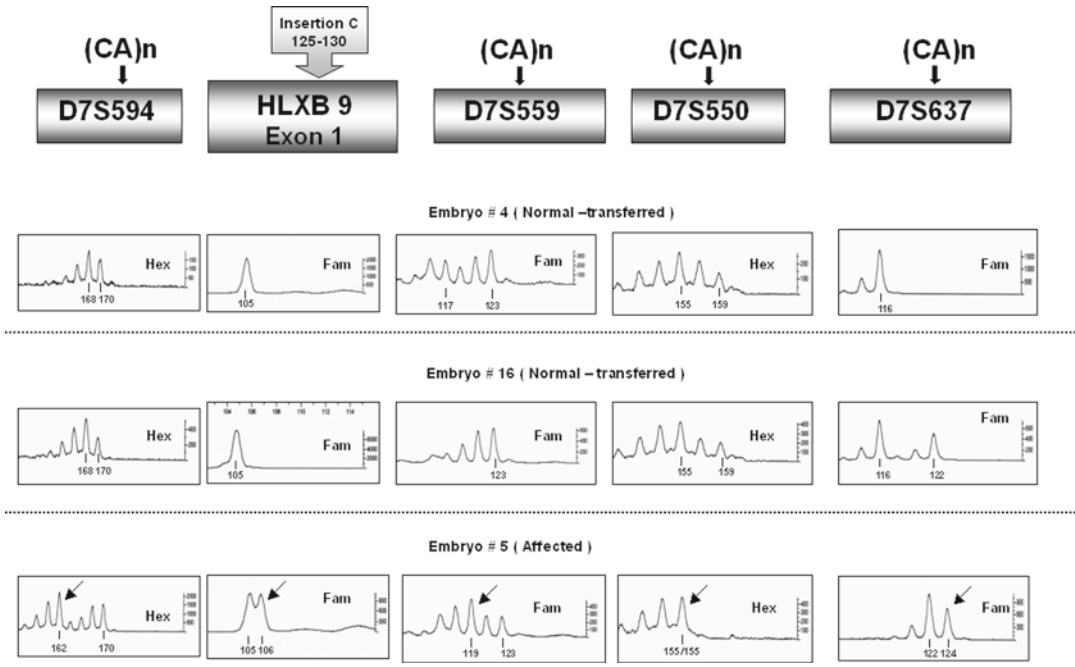



**Fig. 3.47** Preimplantation diagnosis for homeobox gene HLXB 9 mutation in Exon 1 causing Currarino syndrome. *Upper panel* shows the location of the mutation in HLXB9 gene and linked markers on chromosome 7. The *other three panels* show capillary elecrophoregrams of fluorescently labeled PCR products of HLXB9 alleles and each of the four linked markers. Paternally derived mutant allele is shown by *arrow* in embryos #5 (*lower panel*), in agreement with paternally derived markers (CA repeats) linked to the mutant gene. The mutant allele is absent in embryos # 4 and #16 (*middle panels*), also in agreement with all four markers. These embryos have been transferred back to the patient resulting in the birth of mutation-free baby. According to marker analysis the baby originates from the transfer of embryo #16

Prior to initiating PGD cycles a single-sperm testing was performed to establish paternal haplotypes. To be able to identify a possible allele drop out (ADO) in the mutation analysis, four closely linked microsatellite dinucleotide DNA markers, D7S559, D7S550, D7S637, and D7S594, were tested in the same reaction with the HLXB9 gene [96]. As seen from Fig. 3.47 and Table 3.27, the mutant allele was linked to 119, 155, 124, and 162 bp, and the normal allele to 123, 159, 116, and 168 bp repeats of D7S559, D7S550, D7S637, and D7S594 markers, respectively. Testing for these markers in the affected child and the mother showed that the parents shared the same size of two of four markers linked to the normal allele, making theses markers of limited value for preselection of mutation-free embryos for transfer.

A total of 17 embryos were available for testing in a single PGD cycle. Single blastomeres were removed from these embryos, of which 3 failed to amplify either HLXB9 alleles or polymorphic markers (embryos #1, #9, and #14), suggesting the lack of a nucleus in these blastomeres, and 2 showed amplification of polymorphic markers only with no signal detected for the HLXB9 gene (embryos #8 and #12) (Table 3.28). Of a total of 12 embryos with results for the mutation and linked marker analysis, 11 were with conclusive results, of which 5 were predicted to contain the mutant allele in agreement with the presence of repeat markers (embryos #3, #5, #6, #11, and #13). One of these embryos contained only paternal alleles (embryo #13), which may be explained by the absence of the maternal chromosome 7, which may be due to mosaicism, known to be very frequent at the cleavage stage.

The remaining six embryos were predicted to be free of mutation, but only in three of them (embryos #2, #4, and #16) ADO of the mutant allele may have been excluded based on the linked marker analysis (Table 3.28; Fig. 3.47). Two of these embryos were transferred, yielding a

**Table 3.27** Primers and reaction conditions for PGD of Currarino triad

| Gene/polymorphism | Upper primer | Lower primer | Annealing $T_m$ (°C) |
|---|---|---|---|
| HLXb9 Insertion C 125–130 (Heminested PCR) | Outside: 5′ TGGCAAATAACCACCATAACAA 3′ | 5′ CGAGCGACGTGACCAAGG 3′ | 62–55 |
| | Inside: 5′ FamCGAGCCGATGGAAAAATCC 3′ | 5′ CGAGCGACGTGACCAAGG 3′ | 55 |
| D7s559 (Heminested PCR) | Outside: 5′ GACCACAGTAAGAAATAACCCATTA 3′ | 5′ AAAAATAATAAAACAGAAGATGCCA 3′ | 62–55 |
| | Inside: 5′ GACCACAGTAAGAAATAACCCATTA 3′ | 5′ FamAAGTACTGTCTTGTTAAATTTTTGC 3′ | 55 |
| D7S637 (Heminested PCR) | Outside: 5′ GCACAGAACTAAATACACCCCAT 3′ | 5′ CTTCCCCTAAAGTTAACATCTTG 3′ | 62–55 |
| | Inside: 5′ GCACAGAACTAAATACACCCCAT 3′ | 5′ FamAACTATAGTACAATATCAAAACCAAGA 3′ | 55 |
| D7s594 (Heminested PCR) | Outside: 5′ CAGCCTGTGTATGGTTACTTTTTA 3′ | 5′ GAAGTTCTACAGGCTGAAATCAA 3′ | 62–55 |
| | Inside: 5′ HexAAAAGTATGCATTTATGTATTTTTT 3′ | 5′ GAAGTTCTACAGGCTGAAATCAA 3′ | 55 |
| D7s550 (Heminested PCR) | Outside: 5′ ACTATCATCCACAATCCACTCC 3′ | 5′ GCAGTTGGGTTATTTCAAGTCT 3′ | 62–55 |
| | Inside: 5′ ACTATCATCCACAATCCACTCC 3′ | 5′ HexGATGTTGTGATTAGAGTTGCTGTA 3′ | 55 |

**Table 3.28** Summary of results of PGD for Currarino triad

| Blastomere number | D7S594 | HLXb9 | D7S559 | D7S550 | D7S637 | Predicted genotype | ET |
|---|---|---|---|---|---|---|---|
| 1 | **FA** | FA | **FA** | **FA** | FA | Embryo: INCONCLUSIVE | **NO** |
| 2 | 170 | N | **123** | 159/155 | **116/122** | **Embryo: NORMAL** | **YES** |
| 3 | **162**/170 | M/**N** | **119**/123 | **155**/155 | **124**/122 | **Embryo:** AFFECTED | **NO** |
| 4 | 170/168 | **N** | 123/117 | 159/155 | 116/116 | **Embryo: NORMAL** | **YES**[a] |
| 5 | **162**/170 | M/**N** | **119**/123 | **155**/155 | **124**/122 | **Embryo:** AFFECTED | **NO** |
| 6 | **162**/168 | **M/N** | **119**/117 | **155**/155 | ADO/116 | **Embryo:** AFFECTED | **NO** |
| 7 | 170 | N | 123 | 159/155 | 116/122 | **Embryo: NORMAL** | **YES**[b] |
| 8 | 170 | FA | 123 | **155**/155 | **124**/122 | Embryo: INCONCLUSIVE | **NO** |
| 9 | **FA** | FA | **FA** | FA | **FA** | Embryo: INCONCLUSIVE | **NO** |
| 10 | 170 | N | 123 | 159/155 | 116/122 | **Embryo: NORMAL** | **YES**[b] |
| 11 | **162**/168 | **M/N** | **119**/117 | **155**/155 | **124**/116 | **Embryo: AFFECTED** | **NO** |
| 12 | 170/168 | FA | **119**/117 | **155**/155 | **124**/116 | Embryo: INCONCLUSIVE | **NO** |
| 13 | **162** | **M** | **119** | **155** | **124** | **Embryo: AFFECTED** | **NO** |
| 14 | **FA** | **FA** | **FA** | **FA** | **FA** | Embryo: INCONCLUSIVE | **NO** |
| 15 | 170 | N | 123 | 155 | 116/122 | **Embryo: NORMAL** | **YES**[b] |
| 16 | 170/168 | FA | 123/ADO | 159/155 | 116/122 | **Embryo: NORMAL** | **YES**[a] |
| 17 | 170 | FA | FA | 159/155 | 116/122 | **Embryo: INCONCLUSIVE** | **NO** |
| Father | **162**/170 | **M**/N | **119**/123 | **155**/159 | **124**/116 | | |
| Mother | 168/170 | N/N | 117/123 | 155/155 | 116/122 | | |
| Affected child | **162**/168 | **M** / N | **119**/117 | **155**/155 | **124**/116 | | |

*ADO* allele dropout, *FA* failed amplification, *ET* embryo transfer, *M* mutant allele, *N* normal
Affected haplotype is bolded
[a]Transferred embryos
[b]Chance of misdiagnosis due to potential ADO or recombination

singleton pregnancy and the birth of a mutation-free child, following confirmation of diagnosis by amniocentesis (Fig. 3.48). In the other three embryos predicted to contain the normal allele (embryos #7, #10, and #15), ADO of the mutant gene could not be excluded, because of the parents' sharing the same size of polymorphic markers linked to the normal allele. For example, one of these embryos (embryo #15) was informative only for one linked marker, D7S637 (116/122 bp), which may have suggested the presence of both paternal and maternal normal alleles, assuming that both of these alleles could not be of maternal origin. However, this may have been also due to the uniparental disomy 7 of maternal origin, which was not supported by the other linked markers.

The probability of ADO of the mutant gene in the other two embryos (embryos #7 and #10) could have not been excluded either, despite the fact that these embryos were heterozygous for the two linked markers, D7S637 (116/122) and D7S550 (159/155), because the detected alleles (116 and 159 bp), linked to the paternal normal gene, may have also derived from the mother who shares the same size of linked marker, linked to the normal allele. Finally, one of the embryos (embryo #12) showed the failure of amplification of HLXB9 alleles, but may have been predicted to be mutant, based on the presence three of four polymorphic markers linked to the mutant gene. However, this was not in agreement with the presence of 170 bp repeat of the D7S594 marker, linked to the normal paternal allele, which may probably be due to recombination of the paternal alleles.

Unfortunately, due to the parents sharing two of four markers, linked to the normal allele, only three of six potentially normal embryos could have been preselected for transfer, because of the inability to completely exclude the risk for misdiagnosis due to ADO of the mutant paternal allele.

The presented case is the first PGD for homeobox-containing gene mutations, demonstrating

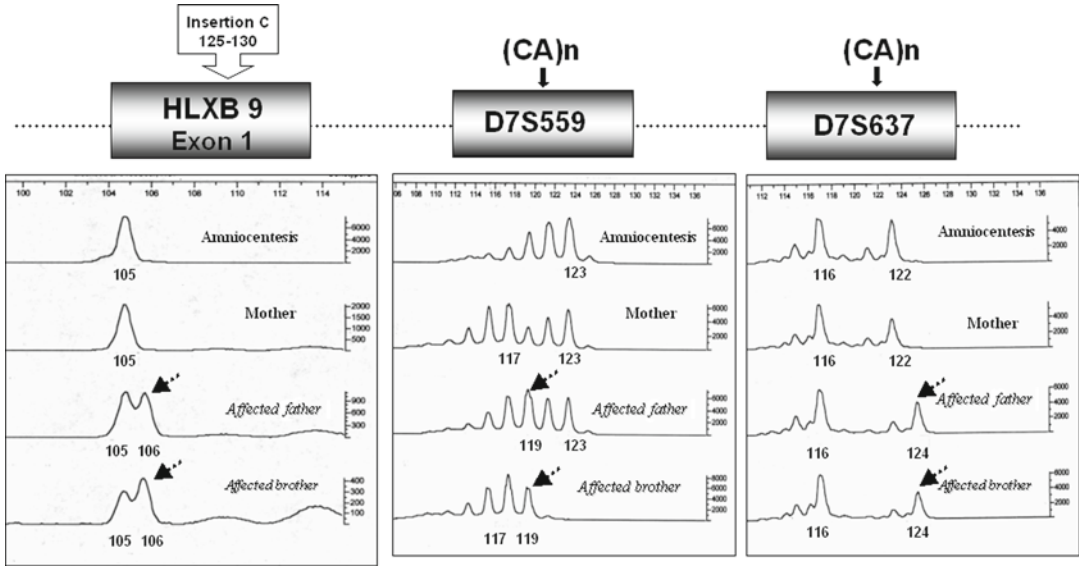



**Fig. 3.48** Confirmation of Currarino triad free pregnancy by amniocentesis. *Upper panel* shows capillary electrophoregrams of fluorescently labeled PCR products of HLXB9 gene and two linked markers in amniotic fluid, evidencing the mutation-free status of the fetus, confirming the results of PGD. *Two lower panels* show patterns for affected father and the first affected child, in which paternal mutant allele is shown by arrow, in agreement with paternally derived markers (CA repeats) linked to the mutant gene. The *second panel from the top* shows the normal pattern for the mother

**Table 3.29** Results and outcomes of PGD for congenital malformations

| Disease | Number of patients | Number of cycles | Cycle by PB | Cycle by PB & BL | Cycle by BL | Number of ET | Number of embryos | Pregnancy | Birth |
|---|---|---|---|---|---|---|---|---|---|
| Craniofacial dysostosis, type I (CFD1) | 2 | 3 | 0 | 2 | 1 | 3 | 8 | 2 (1)[a] | 1 |
| Currarino syndrome | 1 | 1 | 0 | 0 | 1 | 1 | 2 | 1 | 1 |
| Sonic hedgehog (SHH) | 1 | 2 | 0 | 0 | 2 | 2 | 3 | 1 | 1 |
| Treacher–Collins–Franceschetti syndrome (TCOF) | 1 | 1 | 1 | 0 | 0 | 1 | 2 | 1 (1)[a] | 0 |
| Total | 5 | 7 | 1 | 2 | 4 | 7 | 15 | 5 (2)[a] | 3 |

[a]Ongoing pregnancies; number of fetuses is shown in parentheses

the clinical relevance of PGD for this group of genes involved in transcriptional regulation during early embryonic development. Because of the high prevalence of congenital anomalies determined by the mutations causing the formation and positioning defects, this approach may have practical implications for the at-risk couples carrying such mutations, and also mutations causing other groups of familial dysmorphologies, as a principally new option for a large group of couples to avoid the risk of having children with a wide range of the formation and positioning abnormalities at different stages of embryogenesis. The data on the stage-specific expression of homeobox genes observed in CS will be also of relevance for the development of PGD for these transcriptionally relevant anomalies to offer different options for the couples at risk for producing progeny with inherited predisposition to congenital malformations.

Our experience of PGD for congenital malformations (Table 3.29) also includes Crouson syndrome (CFD1), which is described below. It is a dominantly inherited craniosynostosis

caused by mutations in the fibroblast growth factor receptor 2 gene (*FGFR2*) on chromosome 10q [16, 100]. It is quite rare, with birth prevalence of 15–16 per million [101]. PGD was previously applied to testing of G/A base substitution at codon 568 in a couple with one affected child who died aged 18 months during corrective surgery. Of two PGD cycles performed, one resulted in the birth of an unaffected child [94]. We performed three PGD cycles for two couples at risk of producing offspring with Crouson syndrome, of which two resulted in unaffected clinical pregnancies (primers and reaction conditions are presented in Table 3.30). In one of these cases, the mother was a carrier of the C3422Y mutation in exon B of the FGFR2 gene and had also one previous affected child with Crouson syndrome (Fig. 3.49). Two clinical cycles were performed for this patient, using PB1 and PB2 testing, based on restriction digestion with HpyCH4 V, cutting the normal allele into 74 and 20 bp fragments and leaving the mutant allele intact. Two informative linked markers, D10S190 and Msp I polymorphism, were amplified simultaneously with the causative gene for testing PB1 and PB2, allowing the preselection of mutation-free oocytes for transfer. Of nine oocytes tested in the first cycle, five appeared to be mutation-free, but the transfer did not result in clinical pregnancy. In the other cycle, 22 oocytes were available for testing, of which 9 were mutation-free (Fig. 3.50). The transfer of 2 of these embryos resulted in a singleton pregnancy and the birth of an unaffected child, confirmed to be free of the causative gene.

## 3.10 Dynamic Mutations

Dynamic mutations, which represent trinucleotide repeat expansion, are currently among the most frequent indications for PGD, following X-linked disorders, *CF*, and hemoglobin disorders, although PGD for this group of diseases was introduced only in 1995, initially offered to the couples at risk for producing offspring with myotonic dystrophy (DM) [102, 103]. PGD for XRM1, another important example of the dynamic mutations, was described in Sect. 3.3, demonstrating the complexity of performing PGD for this group of diseases [104, 105].

In addition to DM and FRAXA, the current PGD practices include PGD for Huntington disease (HD), spinal and bulbar muscular atrophy (SBMA), and spino-cerebellar ataxia (SCA) type 2, 3, 6, and 7. The majority of these cases were done for DM, XRM1, and HD. The accuracy of PGD for dynamic mutations has reported to be improved with the application of fluorescent PCR with the expanded long template (ELT) kit, which enabled reducing the ADO rate from 30% to 35% in both conventional and fluorescent PCR to as low as 5% in the testing for DM [104]. The other attractive approach for improving the accuracy of PCR analysis for this group of disease, similar to those mentioned in Chap. 2, involved the application of real-time PCR, which was found to reduce the ADO rate by half, in comparison to conventional or fluorescent PCR. The application of these approaches together with the simultaneous testing of a sufficient number of linked markers may allow avoiding the risk for misdiagnosis completely in PGD for dynamic mutations.

Table 3.31 presents the first 95 PGD cycles of our initial experience for 60 couples at risk for producing offspring with DM, FRAXA, Huntington disease (HD), SCA2, SCA3, SCA6, and SCA7, which is representative of our overall experience. Among these conditions, almost half (43 cycles) were performed for FRAXA, also described in Sect. 3.3, which resulted in 37 embryo transfers yielding 16 unaffected pregnancies and 15 healthy children born. The second largest group was DM, involving 33 PGD cycles performed for 19 at-risk couples, of which 22 resulted in the transfer of the mutation-free embryos, yielding 11 clinical pregnancies and 8 births of healthy children. Overall, 168 (2.3 per transfer on an average) unaffected embryos were selected for transfer in 74 (77.8%) of 95 cycles, resulting in 32 (43%) unaffected pregnancies and the birth of 25 healthy children.

As the expanded alleles are usually not amplified in single-cell PCR, PGD for dynamic mutation is mainly based on the identification of normal alleles based on the testing of a sufficient number of linked markers. To avoid misdiagnosis, at least three closely linked markers should be

**Table 3.30** Primer sequences and reaction conditions for PGD of Crouzon syndrome

| Gene/polymorphism | Upper primer | Lower primer | Annealing Tm (°C) |
|---|---|---|---|
| FGFR2 Cys 342 Tyr (G—A) (Heminested; mismatched inside primer; Hpy CH4V cuts normal allele) | Outside | | 62–45 |
| | 5′ TAACACCACGGACAAAGAGA 3′ | 5′ ACCCAGAGAGAAAGAACAGTAT 3′ | |
| | Inside | | 50 |
| | 5′ TAACACCACGGACAAAGAGA 3′ | 5′ AATAGAATTACCCGCCA*T*G 3′ | |
| D10S190 (Heminested) | Outside | | 62–45 |
| | 5′ AGTGTATGGTTCCTTCCCCTG 3′ | 5′ CAGGCATGTAACACAGCAAAAG 3′ | |
| | Inside | | 55 |
| | 5′ HexCTGGTTGTTTAAGTGTATGGTTCC 3′ | 5′ CAGGCATGTAACACAGCAAAAG 3′ | |
| D10S 1483 (Heminested) | Outside | | 62–45 |
| | 5′ CCAATGCTATCCCGGCTAT 3′ | 5′ AAGCGTGTAACATTTGGTATGC 3′ | |
| | Inside | | 55 |
| | 5′ CCAATGCTATCCCGGCTAT 3′ | 5′ HexCAAATGCAATAACATTAAACCATT 3′ | |
| D10S 209 (Heminested) | Outside | | 62–45 |
| | 5′ TCATATCCTGCTAACATTACCAACA 3′ | 5′ CCCACAGGTCACTATGCTTACTT 3′ | |
| | Inside | | 55 |
| | 5′ FamCTGAGCCAGTGGGATGAGAG 3′ | 5′ CCCACAGGTCACTATGCTTACTT 3′ | |
| D10S 1230 (Nested) | Outside | | 62–45 |
| | 5′ GCTCTGGTTATTTGCTGCCT 3′ | 5′ GCAGCAGCTTTGTTTTCCA 3′ | |
| | Inside | | 55 |
| | 5′ HexTTTCACCTGACTTTCCTAATAATCC 3′ | 5′ AGAGCAAGCAACTAAATTATTTTTC 3′ | |
| D10S 1757 (Heminested) | Outside | | 62–45 |
| | 5′ CAAAGACAATTTAGGAAATTCAAAA 3′ | 5′ CATTCCCTCGTGTATCAGCC 3′ | |
| | Inside | | 55 |
| | 5′ CAAAGACAATTTAGGAAATTCAAAA 3′ | 5′ Fam CTTTCTGCCTAAAACATATCCCT 3′ | |
| D10S 1679 (Heminested) | 5′ GCAGTGCCTGAGGCTTGTG 3′ | 5′ CCATGAGGGTACTATAGAAAGTTG 3′ | 62–45 |
| | 5′ Hex CCATGAGGGTACTATAGAAAGTTG 3′ | 5′ CCATGAGGGTACTATAGAAAGTTG 3′ | 55 |

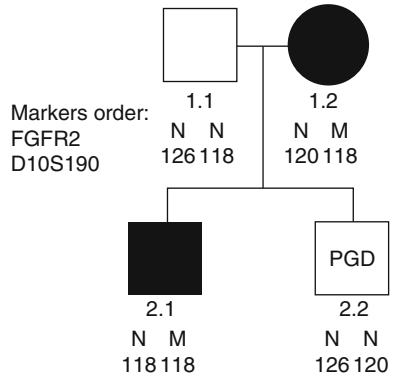



**Fig. 3.49** PGD for Crouson syndrome: family pedigree. The mother (1.2) is the carrier of a mutation in FGFR2 gene and had a previous child with Crouson syndrome (2.1). PGD resulted in the birth of a healthy unaffected boy (2.2)

present, confirming the inheritance of the normal allele from the affected parents. In practice, the presence of normal alleles from both parents should be confirmed by the analysis of the maternal and paternal haplotypes, as demonstrated by the example of PGD for DM and Machado-Joseph disease (SCA3), presented in Figs. 3.51 and 3.52.

As seen from Fig. 3.51, the affected mother with DM (*DMPK*) has an expanded allele linked to 159 and 151 repeat markers. Of six embryos tested, using three closely linked markers, three were affected (embryos #2, #9, and #15), including one with trisomy 19 (embryo #2), evidenced by the presence of both mutant and normal maternal alleles, and one set of paternally derived mark-

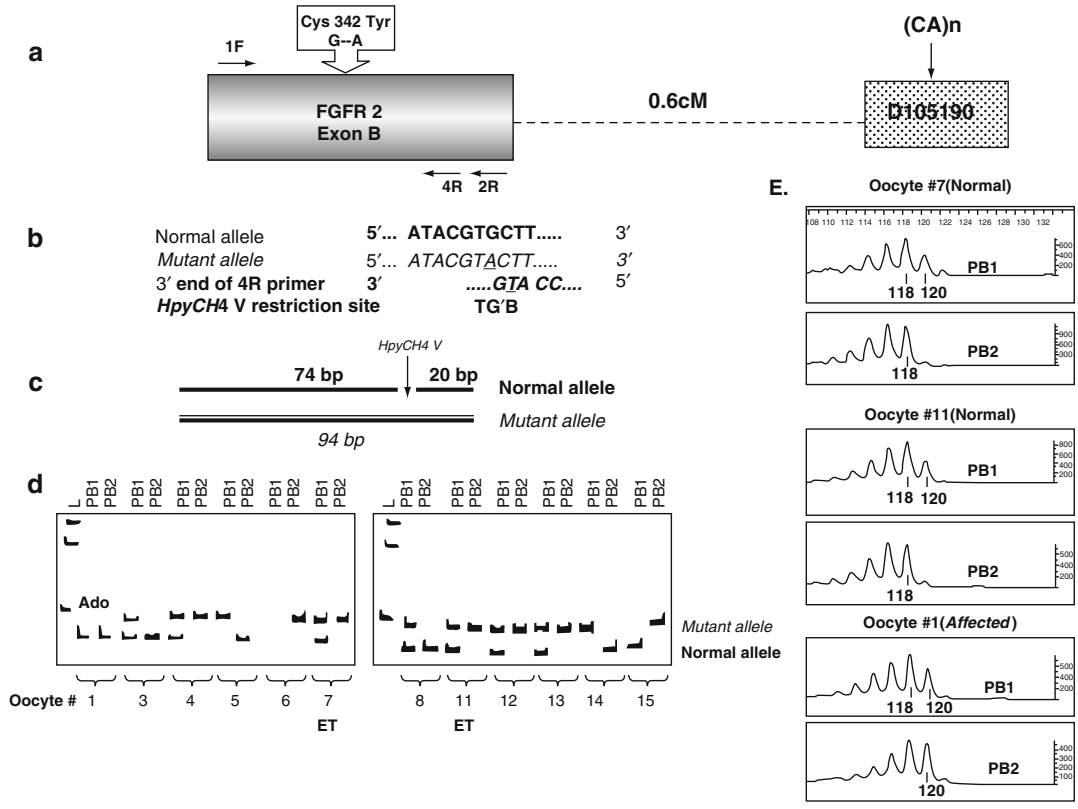



**Fig. 3.50** PGD for mutation in FGFR2 gene causing Crouson syndrome. (**a**) Map of human FGFR2 gene, showing sites and location of C342Y mutation and position of linked D10s190 dinucleotide STR. Horizontal arrows show primer sets for heminested PCR. (**b**) Primer design and (**c**) restriction map for normal and abnormal alleles. (**d**) Mutation analysis of 12 oocytes by sequential first (PB1) and second (PB2) polar bodies. Allele dropout (*ADO*) of the mutant allele (*) was detected both by sequential PB1 and PB2 study (identical genotype of both) and linked marker analysis (presence of 118 bp and 120 bp bands). STR profile for PB1 and PB2 from oocytes #1, #7, and #11 confirmed the results of mutation analysis (**e**), suggesting that the latter two are normal and may be transferred. *L* size standard, *bp* base pair, *ET* embryo transfer

**Table 3.31** Results and outcomes of PGD for dynamic mutations

| Disease | Number of patients | Number of cycles | Number of cycles performed by PBs | Number of cycles performed by PB + Blast | Number of cycles performed by Blast | Number of ET | Number of embryos | Pregnancy | Birth |
|---|---|---|---|---|---|---|---|---|---|
| DM | 19 | 33 | 6 | 13 | 14 | 22 | 48 | 11 | 8 |
| FRA X | 29 | 43 | 16 | 24 | 3 | 37 | 86 | 16 | 15 |
| HD | 6 | 10 | 0 | 1 | 9 | 8 | 17 | 3 | 0 (1)[a] |
| SCA2 | 3 | 4 | 1 | 3 | 0 | 4 | 11 | 1 | 2 |
| SCA3 | 1 | 2 | 1 | 0 | 1 | 1 | 2 | 1 | 2 |
| SCA6 | 1 | 2 | 1 | 1 | 0 | 1 | 2 | 0 | 0 |
| SCA7 | 1 | 1 | 1 | 0 | 0 | 1 | 2 | 1 | 0 |
| Total | 60 | 95 | 26 | 42 | 27 | 74 | 168 | 33 | 27 |

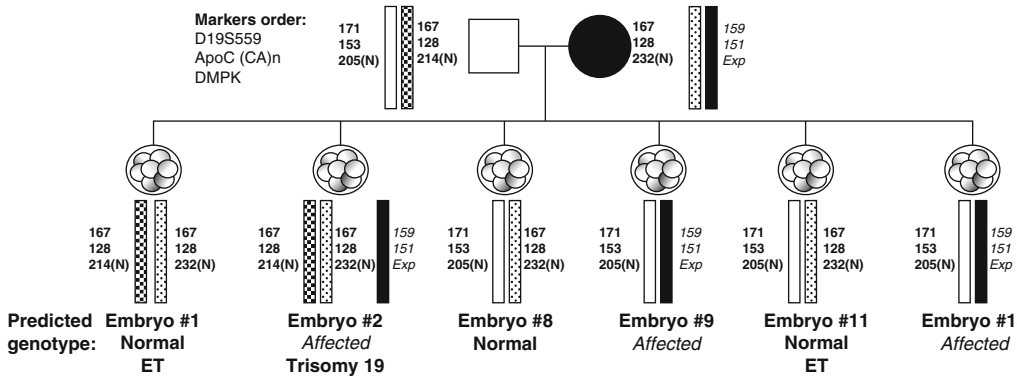



**Fig. 3.51** PGD for myotonic dystrophy combined with chromosome 19 aneuploidy testing. (*Top*) Maternal haplotype based on PB1 and PB2 multiplex DNA amplification of the normal allele of DMPK gene (19q13.2–13.3) and linked markers (*right*). Paternal haplotype based on blastomere analysis (*open and darker*). As the expansion of $(CTG)^n$ repeat in DMPK gene is not detectable at single-cell level (*darker bar* in maternal haplotype corresponds to the affected allele), linked polymorphic markers are used for PGD; *clearer bar* represents the normal allele and tightly linked markers. (*Bottom*) Six embryos tested for the presence of the normal number of $(CTG)^n$ repeat in DMPK gene using polymorphic marker pattern. Embryos #1, #8, and #11 were predicted normal based on the presence of the normal maternal chromosome (haplotype) (*clearer bar*) and one paternal chromosome 19. Embryos #2, #9, and #15 were affected evidenced by the presence of the maternal affected haplotype (*darker bar*). Embryo #2 contains two sets of maternal and one set of paternal polymorphic markers, suggesting trisomy 19 of maternal origin. Therefore, amplification of only $(CTG)^n$ repeat in DMPK gene would have revealed the normal status based on the presence of only normal maternal and paternal alleles, which would have led to misdiagnosis. The follow-up FISH analysis confirmed trisomy 19, as predicted, and also incidental trisomy 13 (data not shown). *DMPK* dystrophic myotonia protein kinase gene, *ET* embryo transfer

ers. The fact that this embryo is trisomic for chromosome 19, to which the gene for *DMPK* is assigned (19q13.2–q13.30), was also confirmed by FISH analysis using a specific fluorescent probe for chromosome 19 and 13, showing three signals for this chromosome as well as three signals for chromosome 13, suggesting double trisomy 13 and 19. The remaining three embryos (embryos #1, #8, and #11) were normal, in which all three markers were in agreement with the presence of the maternal normal allele, together with the presence of one of the paternal alleles. So two of these embryos (embryo #1 and #11) were transferred, resulting in an unaffected clinical pregnancy.

The other condition caused by dynamic mutation, Machado-Joseph disease, for which PGD is shown in Fig. 3.52, is a neurodegenerative disorder characterized by cerebellar ataxia

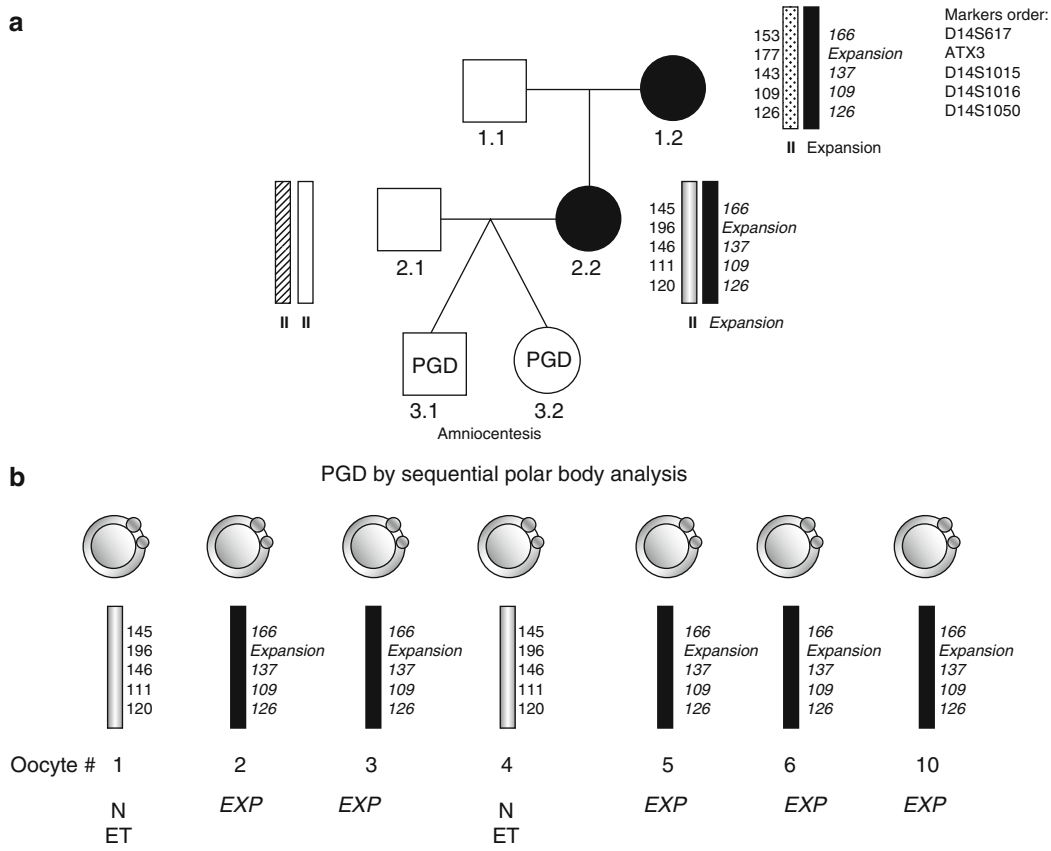



**Fig. 3.52** PGD for Machado–Joseph disease (SCA3) resulted in the birth of healthy twins. (**a**) Family pedigree with haplotype analysis, showing that the mother (2.2) inherited the expanded allele (*darker bar*) from her mother (1.2). PGD resulted in the birth of two unaffected twins (3.1 and 3.2), following amniocentesis. (**b**) Seven oocytes were tested for the presence of the expanded allele by sequential PB1 and PB2 analysis, showing that only two oocytes (oocyte #1 and #4) were free of the expansion. Both embryos resulting from these mutation-free oocytes were transferred yielding the birth of unaffected children

3 (SCA3), pyramidal and extapyramidal signs, peripheral nerve palsy, external ophthalmoplegia, facial and lingual fasciculation, and bulging. The mother was a carrier of the expanded allele, inherited from her mother, closely linked to 166, 137, 109, and 126 repeat markers of D14S617, D14S1015, D14S1016, and D14S1050, respectively. PGD was performed by sequential PB1 and PB2 analysis (primer sequences are listed in Table 3.32) in 7 oocytes, 5 of which appeared to be affected and only two without expansion, which were transferred back to patients, yielding an unaffected twin pregnancy and the birth of healthy children, following confirmation of PGD by amniocentesis.

Finally, a nondisclosure PGD has been considered for couples at risk for HD, involving the transfer of the disease-free embryos while the prospective parents do not learn their own status. Parents receive no information about the number of oocytes obtained after hormonal stimulation, the number of embryos formed, and the number of embryos available for transfer. However, there might be no unaffected embryos for transfer, or no affected embryos might be found, suggesting the parent is genetically normal. The other alternative in HD is an exclusion-PGD testing, but embryos with a detected grandparental allele would be excluded from transfer notwithstanding that only half of these embryos would contain the

**Table 3.32** Primers and reaction conditions for PGD of Machado-Joseph Disease

| Gene/polymorphism | Upper primer | Lower primer | Annealing Tm (°C) |
|---|---|---|---|
| Mutation in ATX3 gene | Outside | | 62–45 |
| | 5′ TACCAGTGACTACTTTGATTCGTGA 3′ | 5′ GCTCCTGAACTGGTGGCTG 3′ | |
| | Inside | | 55 |
| | 5′ TACCAGTGACTACTTTGATTCGTGA 3′ | 5′ FamTCTGTCCTGATAGGTCCCCC 3′ | |
| D14S995 (Heminested) | Outside | | 62–45 |
| | 5′ TCTGGAAGGCAGAGAGGTTG 3′ | 5′ GTAGATAATATGTTTGGCGAGGG 3′ | |
| | Inside | | 58 |
| | 5′ HexCCACTGCACTCCAGCCTG 3′ | 5′ GTAGATAATATGTTTGGCGAGGG 3′ | |
| D14S1050 (Heminested) | Outside | | 62–45 |
| | 5′ AGACAGGAGTGGGCAGAAAT 3′ | 5′ ATCTCCCATACTGACTCTCCCT 3′ | |
| | Inside | | 58 |
| | 5′ AGACAGGAGTGGGCAGAAAT 3′ | 5′ HexGTGACAATTTAGAGGGGGGAC 3′ | |
| D14S1015 (Heminested) | Outside | | 62–45 |
| | 5′ TATTTTGTCCTTTTTTATGGCTG 3′ | 5′ AACTACAGAAAAAATTGAAAGTAGAAC 3′ | |
| | Inside | | 58 |
| | 5′ TATTTTGTCCTTTTTTATGGCTG 3′ | 5′ HexCTATTTGATTCAGAAATCCCATTAC 3′ | |
| D14S1016 (Heminested) | Outside | | 62–45 |
| | 5′ TAAAAAGGGAAAGTAGTTAGAATGG 3′ | 5′ GAAGATGAACCTGAACATGGC 3′ | |
| | Inside 5′ | | 58 |
| | HexGTTATGACATATATTTTTTCCCCAC 3′ | 5′ GAAGATGAACCTGAACATGGC 3′ | |
| D14S617 (Heminested) | Outside | | 62–45 |
| | 5′ ATTCTCCTCTTATTATAGGACTTTA 3′ | 5′ GCAACAGAACAAGATCTGTCTC 3′ | |
| | Inside | | 58 |
| | 5′ FamTGTGGAGAAATTAAGTTTTAGGTG 3′ | 5′ GCAACAGAACAAGATCTGTCTC 3′ | |

affected allele. However, in our experience, a direct testing of embryos from known disease gene carriers was performed, currently including ten PGD cycles for six couples at risk, resulting in the transfer of 17 mutation-free embryos in eight cycles, yielding three unaffected clinical pregnancies.

## 3.11 Overall Experience of PGD for Mendelian Disorders

The presented experience is the world's largest series of PGD for Mendelian disorders, presented in Tables 3.1, 3.2, and 3.3, which comprises 2,158 PGD cycles performed in the period of over 20 years. The number of conditions for which PGD is being performed is expanding gradually, with the present number being within 300. As can be seen from Tables 3.2 and 3.3, the outcome of PGD for single-gene disorders was even more favorable than in a routine IVF, resulting in 733 pregnancies (41.2% pregnancy rate), despite transfer of only two embryos per cycle on an average. It is of note that unaffected embryos for transfer were available in 1,778 of 2,158 PGD cycles (82.4%), so only in 380 cycles (17.6%) either no unaffected embryos could have been detected or the embryos predicted to be normal did not develop properly to consider their transfer on day 3 or day 5. Overall, 731 children were born following the procedure, with only three misdiagnoses (0.2%) observed during the whole period of over 20 years, all due to ADO, one in PGD for CFTR, involving one misdiagnosis of compound heterozygote embryos as heterozygous at the very beginning of the introduction of PGD, when the phenomenon of ADO was not yet appreciated, and the other two in PGD for FRM1 and myotonic dystrophy, involving the transfer of the embryos with the predicted over 5% risk of misdiagnosis, which the couple decided to transfer in addition to others with 100% accuracy, to improve their chances to become pregnant. Assuming that the overall experience has involved the genetic testing of more than 20,000 oocytes and embryos, which resulted in preselection and transfer of 3,437 unaffected embryos in 1,778 cycles, the applied technique may be considered to be highly accurate and reliable. The distribution of different conditions for which PGD was performed have been changing gradually, with an increase in the proportion of common diseases with genetic predisposition and non-disease testing, including preimplantation HLA matching, described below in Chap. 4.

Although some of the cases have been done exclusively by PB approach, as it is sufficient to perform PGD with high accuracy, other approaches, such as single-blastomere removal at the cleavage stage, or blastocyst biopsy, should be available to ensure PGD application in complex cases, and to avoid the transfer of affected embryos, determined by paternally derived mutations. In addition, these methods also provide a confirmatory diagnosis following the PB diagnosis. The choice of additional methods will differ depending on circumstances, and a reliable diagnosis may require using two or even three different methods, especially when there is more than one indication for PGD, such as PGD for single-gene disorders together with HLA typing, or preimplantation HLA typing together with aneuploidy testing. The combined testing is required with the expanding range of PGD indications when testing is performed for causative gene, linked markers, HLA typing, and aneuploidy in the same case (see Chap. 4 and 5).

The above overall experience also includes the world's largest PB-based PGD series of *938* PGD cycles for Mendelian disorders performed for 553 patients at risk for producing offspring with inherited disorders. These PB-based PGD cycles were performed for 146 monogenic conditions (Table 3.33), resulting in the preselection and transfer of 1,578 unaffected embryos originating from mutation-free oocytes in 790 of 988 cycles (84%), demonstrating the safety, reliability, and extremely high accuracy of the procedure. While PB sampling was sufficient for making decisions on embryo transfer in 188 of 237 of these cycles, additional blastomere and/or blastocyst biopsy was required in the remaining 602 of 701 cycles involved. A total of 9,036 oocytes were tested, of which 7,841 (86.8%) were with both PB1 and PB2, with the results of sequential PB1 and PB2

testing obtained in 97.6% of these oocytes. This made it possible to preselect for transfer as many as 1,578 embryos originating from these oocytes (1.99 on the average), in 790 (84.2%) cycles, resulting in 329 pregnancies (41.6%) and the birth of 342 healthy children.

Additional embryo biopsy was applied in 701 of these cases, to confirm the diagnosis when necessary, or identify unaffected embryos for transfer in the absence of embryos originating from mutation-free oocytes, such as heterozygous carrier embryos, originating from mutant oocytes.

Of a total of 9,036 oocytes tested, amplification of both PB1 and PB2 was successful in 7,650 PB1/PB2 sets, suggesting an extremely high (97.6%) amplification efficiency. This allowed transferring embryos in 84.2% of initiated cycles, with the priority in preselection of mutation-free oocytes given to the oocytes with heterozygous PB1, that is, with both normal and mutant genes amplified, ideal for further testing, despite the fact that their potential transfer depended entirely on the identification of the mutant gene in the sequential analysis of PB2. In the absence of DNA contamination, this indicates the absence of ADO of either normal or mutant allele, and allows avoiding the potential misdiagnosis due to ADO. Although most of the transferred embryos were preselected using this particular strategy, the embryos originating from homozygous normal oocytes, inferred from homozygous mutant status of PB1 and hemizygous normal status of PB2, may be also transferred, provided that ADO could be excluded using a sufficient number of linked polymorphic markers. In our experience of PB-based PGD, we observed only two misdiagnoses in 790PB-based PGD transfer cycles, suggesting an extremely high accuracy rate of 99,7%.

As seen from Tables 3.3 and 3.33, autosomal-recessive disorders were the most frequent indication for PGD by PB analysis, performed for 81 recessive conditions in 504 (53.7%) of an

**Table 3.33** List of Mendelian disorders for which PGD was performed by PB-based approach

| Genetic disorder | Gene | Inheritance |
|---|---|---|
| ACYL-CoA dehydrogenase, medium-chain, deficiency | ACADM | AR |
| ACYL-CoA dehydrogenase, long-chain, deficiency | ACADL | AR |
| ACYL-CoA dehydrogenase, very long-chain (ACADVL) | ACADVL | AR |
| Argininosuccinic aciduria | ASL | AR |
| Ceroid lipofuscinosis, neuronal 2, late infantile (CLN2) | CLN2 | AR |
| Citrullinemia | ASS | AR |
| Congenital adrenal hyperplasia (CAH) | CYP21A2 | AR |
| Deafness, neurosensory, autosomal-recessive 1 (DFNB1) | GJB2 | AR |
| Cystic fibrosis (CF) | CFTR | AR |
| Cystinosis, nephropathic (CTNS) | CTNS | AR |
| Ectodermal dysplasia, hypohidrotic | EDA | AR |
| Epidermolysis bullosa dystrophica | COL7A1 | AR |
| Fanconi anemia, complementation group A | FANCA | AR |
| Fanconi anemia, complementation group F | FANCF | AR |
| Fanconi anemia, complementation group J | FANCJ | AR |
| Gaucher disease, type I | GBA | AR |
| Glutathione synthetase deficiency | GSS | AR |
| Glycogen storage disease II | GAA | AR |
| Hemoglobin-alpha locus 1 (HBA1) | HBA1 | AR |
| Hemoglobin-beta locus (HBB) | HBB | AR |
| Hurler syndrome | IDUA | AR |
| Hypophosphatasia, infantile | ALPL | AR |
| Isovaleric acidemia (IVA) | IVD | AR |
| Krabbe disease | GALC | AR |

**Table 3.33** (continued)

| Genetic disorder | Gene | Inheritance |
|---|---|---|
| Leukoencephalopathy with vanishing white matter (VWM) | EIF2B2 | AR |
| Homocystinuria due to deficiency of N(5, 10)-methylenetetrahydrofolate reductase activity | MTHFR | AR |
| Nephrosis 1, congenital, Finnish type (NPHS1) | NPHS1 | AR |
| Neuropathy, hereditary sensory and autonomic, type III; HSAN3 | IKBKAP | AR |
| Oculocutaneous albinism, type I; OCA1 | TYR | AR |
| Osteopetrosis, autosomal-recessive | TCIRG1 | AR |
| Polycystic kidney disease, autosomal-recessive; ARPKD | PKHD1 | AR |
| Propionic acidemia | PCCA | AR |
| Sandhoff disease | HEXB | AR |
| Sickle cell anemia | HBB | AR |
| Smith-Lemli-Opitz syndrome (SLOS) | DHCR7 | AR |
| Spinal muscular atrophy, type I (SMA1) | SMN1 | AR |
| Tay-Sachs disease (TSD) | HEXA | AR |
| Thrombotic thrombocytopenic purpura, congenital (TTP) | ADAMTS13 | AR |
| Tyrosinemia, type I | FAH | AR |
| Zellweger syndrome (ZS) | PEX1 | AR |
| Ataxia telangiectasia (AT) | ATM | AR |
| Adenomatous polyposis of the colon (APC) | APC | AD |
| Angioedema, hereditary (HAE) | AD | AD |
| Brain tumor, posterior fossa of infancy, familial | SMARCB1 | AD |
| Breast cancer, familial | BRCA1 | AD |
| Breast-ovarian cancer, familial | BRCA2 | AD |
| Cardiomyopathy, dilated, 1A (CMD1A) | LMNA | AD |
| Charcot-Marie-Tooth disease, axonal, type 2E | NEFL | AD |
| Charcot-Marie-Tooth disease, demyelinating, type 1A | PMP22 | AD |
| Charcot-Marie-Tooth disease, demyelinating, type 1B | MPZ | AD |
| Craniofacial dysostosis, type I (CFD1) | FGFR2 | AD |
| Darier-White disease (DAR) | ATP2A2 | AD |
| Diamond-Blackfan anemia (DBA) | RPS19 | AD |
| Dystrophia myotonica 1 | DMPK | AD |
| Epiphyseal dysplasia, multiple, 1 (EDM1) | COMP | AD |
| Huntington disease (HD) | HTT | AD |
| Li-Fraumeni syndrome 1 (LFS1) | TP53 | AD |
| Loeys-Dietz syndrome (LDS) | TGFBR2 | AD |
| Machado-Joseph disease (MJD) | ATX3 | AD |
| Marfan syndrome (MFS) | FBN1 | AD |
| Migraine, familial hemiplegic, 1 (FHM1) | CACNA1A | AD |
| Multiple endocrine neoplasia, type I (MEN1) | MEN1 | AD |
| Neurofibromatosis, type I (NF1) | NF1 | AD |
| Neurofibromatosis, type II (NF2) | NF2 | AD |
| Oculocutaneous albinism, type II (OCA2) | OCA2 | AD |
| Omenn syndrome | RAG1 | AD |
| Optic atrophy 1 (OPA1) | OPA1 | AD |
| Osteogenesis imperfecta congenita (OIC) | COL1A1 | AD |
| Polycystic kidney disease 1 (PKD1) | PKD1 | AD |
| Popliteal pterygium syndrome (PPS) | IRF6 | AD |
| Retinoblastoma (RB1) | RB1 | AD |

(continued)

**Table 3.33** (continued)

| Genetic disorder | Gene | Inheritance |
|---|---|---|
| Spinocerebellar ataxia 1 (SCA1) | ATXN1 | AD |
| Spinocerebellar ataxia 2 (SCA2) | ATX2 | AD |
| Spinocerebellar ataxia 6 (SCA6) | CACNA1A | AD |
| Spinocerebellar ataxia 7 (SCA7) | SCA7 | AD |
| Stickler syndrome, type I (STL1) | COL2A1 | AD |
| Symphalangism, proximal (SYM1) | NOG | AD |
| Torsion dystonia 1, autosomal-dominant (DYT1) | DYT1 | AD |
| Treacher Collins-Franceschetti syndrome (TCOF) | TCOF | AD |
| Tuberous sclerosis type 1 | TSC1 | AD |
| Tuberous sclerosis type 2 | TSC2 | AD |
| Von Hippel-Lindau syndrome (VHL) | VHL | AD |
| Adrenoleukodystrophy (ALD) | ABCD1 | XL |
| Agammaglobulinemia, X-linked (XLA) | BTK | XL |
| Albinism, ocular, type I (OA1) | OA1 | XL |
| Alport syndrome, X-linked (ATS) | AMMECR1 | XL |
| Charcot-Marie-Tooth disease, X-linked, 1 (CMTX1) | GJB1 | XL |
| Choroideremia (CHM) | CHM | XL |
| Emery-Dreifuss muscular dystrophy, X-linked (EDMD) | EMD | XL |
| Fabry disease | GLA | XL |
| Fragile site mental retardation 1 | FMR1 | XL |
| Granulomatous disease, chronic, X-linked (CGD) | CYBB | XL |
| Hemophilia A | F8 | XL |
| Hemophilia B | F9 | XL |
| Hydrocephalus, X-linked (L1CAM) | L1CAM | XL |
| Immunodeficiency with hyper-IgM, type 1 (HIGM1) | CD40LG | XL |
| Immunodysregulation, polyendocrinopathy, and enteropathy, X-linked (IPEX) | FOXP3 | XL |
| Incontinentia pigmenti (IP) | IKBKG | XL |
| Mucopolysaccharidosis type II (Hunter) | IDS | XL |
| Muscular dystrophy, Duchenne type (DMD) | DMD | XL |
| Myotubular myopathy 1 (MTM1) | MTM1 | XL |
| Norrie disease (NDP) | NDP | XL |
| Ornithine transcarbamylase deficiency | OTC | XL |
| Pelizaeus-Merzbacher-like disease (PMLD) | PLP1 | XL |
| Rett syndrome (RTT) | MECP2 | XL |
| Wiskott-Aldrich syndrome (WAS) | WAS | XL |

overall 938 PB cycles. PB analysis is the method of choice for autosomal-recessive disorders, because for avoiding the transfer of embryos with autosomal-recessive conditions, it is sufficient to preselect the embryos originating from the mutation-free oocytes. The largest group of autosomal-recessive disorders performed by PB analysis were hemoglobinopathies, applied extensively in Cyprus [9, 10].

As a result of PB testing performed in 504 PGD cycles for recessive conditions, a total of 882 (2.1 embryos on the average) unaffected embryos were preselected for transfer in 428 (84.9%) of these cycles, yielding 168 (39.3%) clinical pregnancies and 187 healthy children born, with no misdiagnosis (Table 3.3). As can be seen from Fig. 3.53, presenting PB-based PGD for congenital disorder of glycosylation (mutation in PMM2 gene), the sequential PB1 and PB2 analysis provides a robust procedure for selection of embryos originating from mutation-free oocytes (oocytes #2, #5, #9, #13, and #15). The

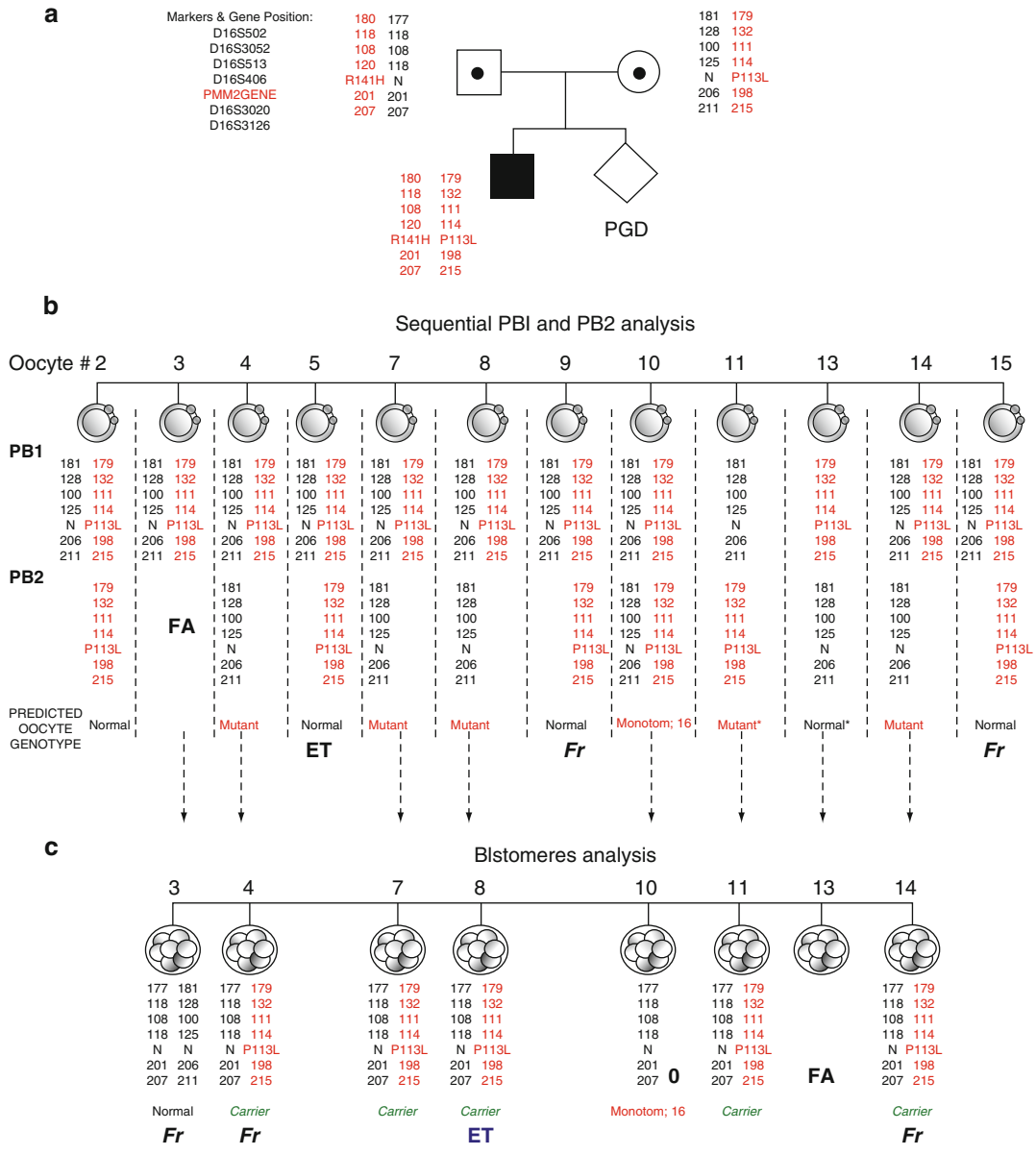



**Fig. 3.53** PGD by sequential PB1 and PB2 and blastomere analysis for congenital disorder of glycosylation (PMM2 gene). (**a**) Pedigree and table with PGD results of testing for PMM2 gene and six linked markers. As can be seen from the pedigree, the mother and father are carriers of different mutations (P113L and R141H, respectively), their affected child being double-heterozygous for PMM2 mutation. (**b**) Sequential PB1 and PB2 analysis of 12 oocytes available for testing, resulted in prediction of oocyte status in 11 of them (PB2 of oocyte #3 failed to amplify). Of 12 PB1s tested, oocyte #11 appeared to be homozygous normal, and oocyte #13 homozygous mutant, confirmed by PB2 testing, and further testing by blastomere analysis. The remaining oocytes were heterozygous, so based on the sequential testing of PB2 mutant, four of them (oocytes #2, #5, #9, and #15) were predicted free of PMM2 mutation, requiring no further blastomere testing. (**c**) A follow-up blastomere testing of embryos originating from mutant oocytes or the oocyte with failed amplification of PB2, showing the preselection of additional six unaffected embryos, of which carrier embryo #8 and embryo originating from oocyte 5, with heterozygous PB1 and mutant PB2, were transferred, resulting in unaffected ongoing pregnancy

case also shows the utility of additional blastomere testing to identify the unaffected embryos originating from the mutant oocytes, such as embryos #4, #7, #8, #11, and #14. One of these embryos (carrier embryo #8) was transferred, together with embryo #5, originating from oocytes with heterozygous PB1 and mutant PB2, resulting in an unaffected ongoing singleton pregnancy.

X-linked conditions were the second largest group of indications for performing PB analysis, involving PGD for 24 different conditions (Table 3.3 and 3.33). A total of 270 cycles were performed, which resulted in the transfer of 411 unaffected embryos (1.9 embryos on an average) in 220 (81.5%) cycles, yielding 94 (42.7%) clinical pregnancies and the birth of 91 children (Table 3.3), with one misdiagnosis for FMR1 mentioned. This involved indirect testing using linkage analysis, as no test is available for direct analysis of the expanded alleles. So the mutation-free oocytes in this case were inferred from the presence of the linked markers for the normal allele, and misdiagnosis was due to the fact that a number of markers amplified were not of sufficiently high accuracy for diagnosis. However, despite the predicted 10% error rate in one of the embryos, the couple selected transferring this particular embryo, in addition to two embryos diagnosed with higher accuracy, which in fact turned out to be heterozygous because of an undetected ADO of both alleles linked to the normal gene (see Sect. 3.3).

PB analysis is obviously the method of choice in PGD for X-linked disorders, because preselection of oocytes free of X-linked disorders allows avoiding any micromanipulation of the embryo. As seen from Fig. 3.54, demonstrating PB-based PGD for X-linked adrenoleukodystrophy, embryo #3, originating from the oocyte with mutant PB1 and normal PB2, confirmed with simultaneous testing of six linked markers, required no further testing and was transferred irrespective of gender or the paternal genetic contribution, resulting in the birth of an unaffected child.

In contrast to autosomal-recessive and X-linked disorders, the PB-based approach is applicable to only maternally derived dominant disorders. As listed in Tables 3.3 and 3.33, we performed PB-based PGD for 41 different dominant conditions of maternal origin, for which PB analysis was concentrated in detection and transfer of embryos deriving from oocytes with heterozygous PB1 and hemizygous mutant PB2. The example is demonstrated in Fig. 3.55, presenting PB-based PGD for myotonic dystrophy, in which the embryo originating from oocyte #23 was predicted to be free from the expanded allele, based on the presence of both normal and expanded alleles in PB1 and an expanded allele in PB2. ADO of the normal allele could not be totally excluded in cases of oocytes with homozygous mutant PB1 in oocytes #3, #5, and #6, so to transfer the embryos originating from these oocytes, a follow-up embryo biopsy was required, involving tracing of the maternal and paternal normal haplotypes.

The PB approach was applied in a total of 164 PGD cycles for dominant disorders, which resulted in the transfer of 285 unaffected embryos (2.0 embryos on the average) in 142 (86.6%) of cases, yielding 65 (45.8%) clinical pregnancies and the birth of 64 children (Table 3.3), with one misdiagnosis. The latter represented PGD for myotonic dystrophy, which is always a challenge, because the diagnosis is based on indirect testing, requiring a sufficient number of linked markers, while only one such marker was available for testing.

The presented data show that the PB-based approach is an integral part of PGD, which makes it possible to perform preselection of mutation-free oocytes and complete PGD prior to fertilization. This provides the possibility for pre-embryonic diagnosis for couples objecting to embryo biopsy because of their social or religious attitudes, as described in Chap. 2. Although the approach is currently limited to PGD for autosomal-recessive conditions, X-linked disorders, and dominant mutations of maternal origin, the future progress in testing of paternal mutations prior to fertilization may allow performing pre-embryonic diagnosis for any disease. On the other hand, the PB approach is a component of PGD for couples with two or more PGD indications and should be available for testing of

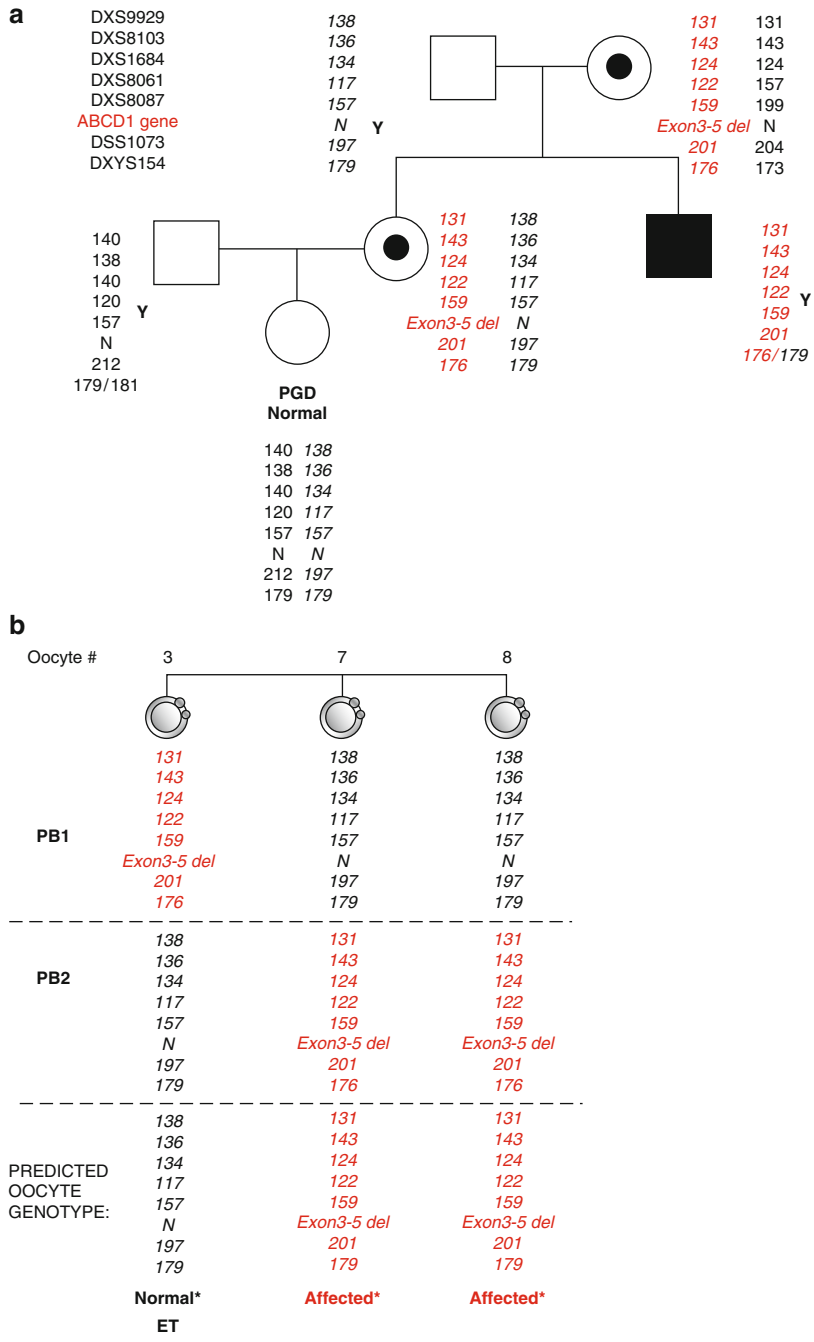



**Fig. 3.54** PB-based PGD for X- linked adrenoleukodystrophy (ABCD1 gene). (**a**) Pedigree and table with PGD results of testing for ABCD1 gene and seven linked markers. Haplotypes of the mother and grandmother carrying a mutation are shown in red in the *left column* and the normal haplotypes shown in black on the *right column*. The only maternal brother is affected with mutant allele inherited from their mother (shown in *red*). On the left of the pedigree, an unaffected child, born as a result of PGD, is shown carrying only a normal maternal allele (linked to seven markers). (**b**) As can be seen from the sequential analysis of PB1 and PB2, oocyte #7 and #9 were diagnosed as affected, based on homozygous normal PB1 and mutant PB2, while oocyte #3 was predicted as normal, as evidenced by mutant PB1, confirmed by all seven linked markers, and normal PB2, in agreement with all the linked markers. This embryo was transferred, resulting in a normal singleton pregnancy and the birth of an unaffected child, mentioned

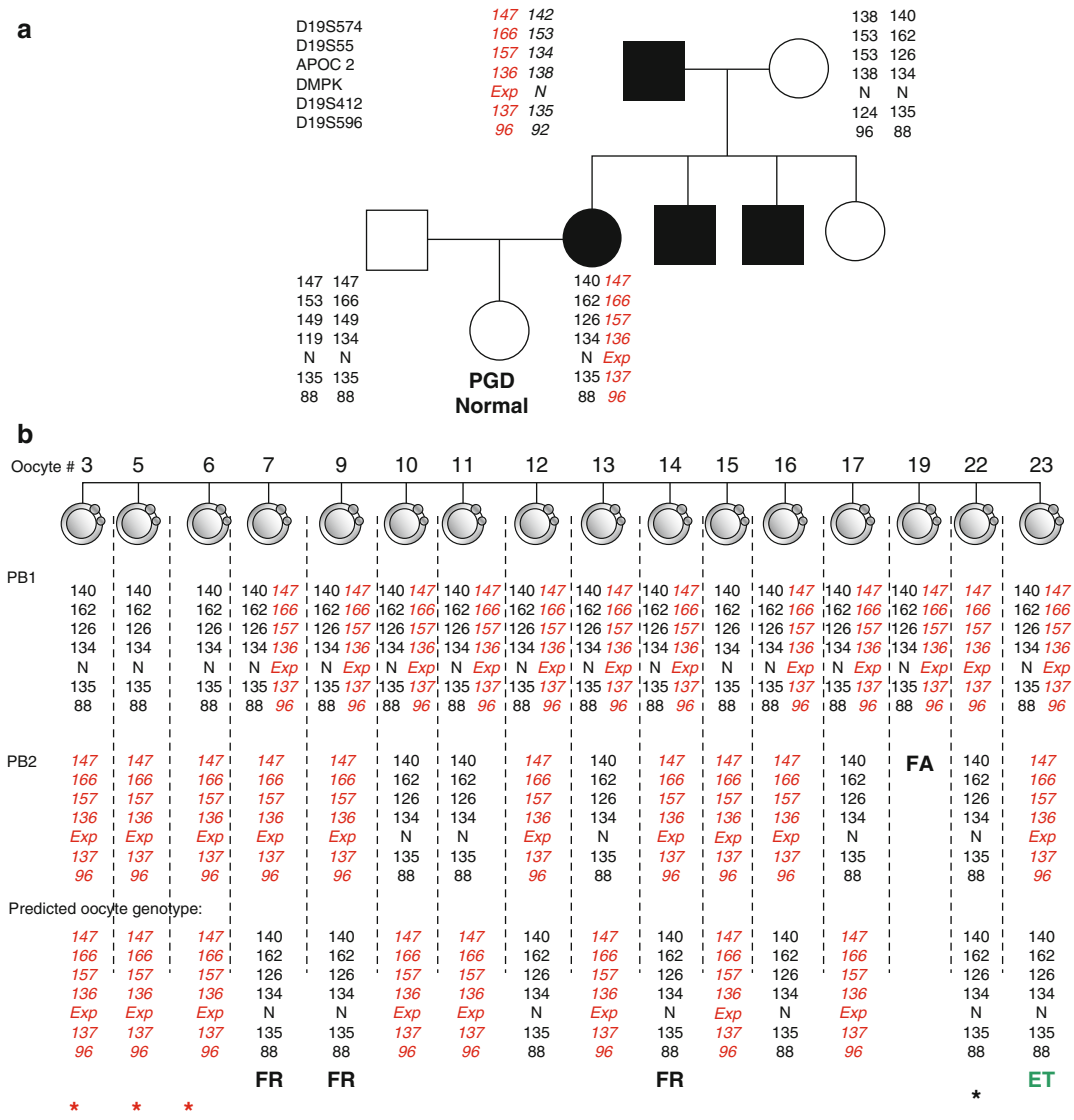



**Fig. 3.55** PGD for myotonic dystrophy by sequential PB1 and PB2 analysis. (**a**) Pedigree showing haplotypes of the mother, who inherited an expanded allele of DMPK gene (shown in *red*) from her father (normal haplotype is shown in *black*). Her two brothers are also affected, while the only sister is normal. (**b**) Sixteen oocytes were tested by PB1 (*top*) and PB2 (*middle*) analysis for the presence of the expanded allele in DMPK gene by six polymorphic markers. Embryos, originating from oocytes #7, #9, #12, #14, #16, #22, and #23 were predicted normal based on the presence of the normal maternal chromosome (haplotype) (*black bar*). Embryos #3, #5, #6, #10, #11, #13, #15, and #17 were affected evidenced by the presence of the maternal affected haplotype (*red bar*). PB1 in oocyte #19 was heterozygous, but PB2 did not amplify, so genotype of this oocyte could not be predicted. One embryo originating from oocyte #23 predicted free of expanded allele, based on the presence of heterozygous PB1 and hemizygous affected PB2, was transferred, resulting in a singleton pregnancy and the birth of a normal child (see pedigree)

complex conditions, including Mendelian disorders and chromosomal aneuploidy, originating predominantly from female meiosis. A wider application of the PB approach to PGD of single-gene disorders may be expected with the present tendency of using PB biopsy for testing of chromosomal aneuploidy by microarray technology [106–108], which makes it possible to

perform PGD simultaneously for multiple conditions, including chromosomal aneuploidy and single-gene disorders. So the presented results demonstrate that the PB approach is highly efficient and reliable, providing an extremely high accuracy of PGD for Mendelian disorders. This together with the application of embryo biopsy technique provides a comprehensive diagnostic package, which allows efficient and reliable PGD with extremely high accuracy. As described in Chap. 2, and demonstrated above in the description of our experience, this will in future be applied together with 24-chromosome aneuploidy testing, to ensure the improvement of reproductive outcome of PGD for Mendelian disorders.

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# PGD for HLA Typing

# 4

Preimplantation HLA matching has never been indicated for prenatal diagnosis because of a possible clinical pregnancy termination after finding the fetus HLA-unmatched. However, PGD for such purposes should be acceptable because only a limited number of embryos are usually preselected for transfer anyway, which in this case will represent unaffected embryos with a perfect match for affected siblings in need of a transplant. The world's first case of preimplantation HLA typing was introduced in combination with mutation analysis for Fanconi anemia (FA), with the objective of establishing an unaffected pregnancy yielding a potential donor progeny for transplantation in an affected sibling [1, 2]. This historical case, which has opened a new chapter in reproductive medicine, is presented below.

## 4.1 Fanconi Anemia – World's First PGD for HLA Typing

One of the most severe congenital disorders requiring stem cell transplantation from a family member is FA, which actually was also the world's first disease for which cord blood transplantation was introduced [3]. FA is an autosomal-recessive disorder, characterized by inherited bone marrow failure, congenital malformations, and an increased predisposition to the development of leukemia. It is genetically heterogeneous, involving different complementation groups (FANCA, FANCB, FANCC, FANCD, and FANCE), one of the most severe being the FANCC mutation leading to aberrantly spliced transcripts (IVS4+4A-T), which result in inactivating the FANCC protein [4–6]. Bone marrow transplantation is the only treatment which restores definitively hematopoiesis in FA patients. However, because any modification of the conditioning is too toxic for these patients, leading to a high rate of transplant-related mortality, the HLA identical cord blood transplantation from a sibling is particularly valuable for FA, to avoid late complications due to severe GVH [7, 8].

A couple presented for PGD, with both parents being unaffected carriers of IVS 4+4A-T mutation in the FANCC gene. Their affected 6-year-old daughter had two copies of this mutation, requiring an HLA-compatible donor for bone marrow transplantation. The couple requested PGD for FANCC, together with HLA testing of embryos, in order to have an unaffected child who may also be a compatible cord blood donor for their affected daughter.

PGD was performed using a standard IVF protocol combined with micromanipulation procedure to biopsy single blastomeres from the day 3 cleaving embryos, as described in Chap. 2. Blastomeres were tested for IVS 4+4A-T mutation in the FANCC gene using polyacrylamide gel analysis of PCR product digested with *ScaI* restriction enzyme, according to the method of single-cell PCR analysis, also described in Chap. 2.

The outer primers, IVS4–1 (5′-GTCATAAAA GGCACTTGCAT-3′) and IVS4–2 (5′-GGCACA TTCAGCATTAAACA-3′), were designed for

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performing the first round of amplification, while using the previously described inner primers [9] for the second round of PCR. The second-round PCR produces a 131-bp product, undigested by the *ScaI* restriction enzyme, corresponding to the mutant allele, and two restriction fragments of 108 and 23-bp, corresponding to the normal allele, due to introduction of the restriction site by application of 4R inner primer with single base modification of T to G.

Nested PCR for specific amplification of HLA-A gene exons 2 and 3 was performed using gene-specific outer primers Asp5 (5′GCCCCG AACCCTC(CT)TCCTGCTA 3′) and Asp3 (5′CC GTGGCCCCTGGTACCCGT 3′) [10], followed by four separate second-round PCRs with allele-specific inner primers 085 (5′TCCTCGTCCCC AGGCTCT 3′) and 98 (5′GCAGGGTCCCCA GGTCCA 3′) for A2 allele, 140 (5′GGTTCTCAC ACCATCCAGATA 3′) and 142 (5′ CAGGTATCT GCGGAGCCCG 3′) for A1 allele[21], 140 (5′ GGT TCTCACACCATCCAGATA 3′) and 126 (5′CC ACTCCACGCACGTGCCA 3′) for A3 allele, and 118 (5′TCCATGAGGTATTTCTACACC 3′) and 145 (5′GCAGGGTCCCCAGGTTCG 3′) for allele A26 [11]. As haplotype analysis for the father, mother, and affected child showed different polymorphic short tandem repeat (STR) marker (GAAA)n (C2_4_4) located in between HLA-A and HLA-B (in HLA-E–HLA-C region), a heminested PCR system was designed to study the number of repeats in blastomeres from different embryos [12]. The first-round amplification cocktail for this system contained outer primers P1–1 (5′-GGCTTGACTTGAAACTCAGAG-3′) and P1–3 (5-TATCTACTTATAGTCTATCAC G-3′), while the second-round PCR used in addition to P1–1, the inner primer P1–2 (5′-CTTC AAACAATACGCAATGACA-3′). The nested PCR system for HLA-B allele discrimination included outer primers Bout 1(5′-GAGGGT CGGGCGGGTCTCAG-3′) and Bout 2 (5′-TG GGGGATGGGGAGTCGTGAC-3′) for the first round of amplification. The second round of amplification for HLA-B35 was performed using inner PCR primers CG4 (5′-GACGACAC CCAGTTCGTGA-3′) and 35in (5′-GAAGATC TGTGTGTTCCGG-3′). Accordingly, the second round of amplification of HLA-B41 was performed with primers CG3 (5′-CTCTGGTTGTAG TAGCCGC-3′) and 41 up (5′-CCACGAGTCCG AGGAAGG-3), and HLA-B44 with primers 41up and GC2 (5′-GCTCTGGTTGTAGTAGC GGA-3′) [13].

Blastomere genotyping for IVS 4+4A-T mutation in the FANCC gene was performed in four clinical cycles, involving mutation analysis in 33 embryos, including 7 in the first, 4 in the second, 8 in the third, and 14 in the fourth cycle. Of 30 embryos with results, 19 were heterozygous carriers, 6 were homozygous affected, and 5 were homozygous normal. Of 14 embryos tested for mutation in the last cycle, only 1 was homozygous affected, 3 were homozygous normal, 2 did not amplify, with the remaining being heterozygous unaffected.

Testing for HLA-A (A2, A26) and HLA-B (B35, B44) in these 24 unaffected embryos, including 19 heterozygous and 5 homozygous normal embryos, revealed 5 heterozygous unaffected embryos for transfer with HLA match for the affected sibling, requiring transplantation of stem cells. The results of HLA typing in 14 embryos in the last cycle revealed only 1 unaffected heterozygous embryo being HLA identical to the affected child, which therefore was transferred back to the patient. Similarly, 2 unaffected HLA-matched embryos were available for transfer in the first, 1 in the second, and one in the third cycle. However, only the transfer in the last cycle resulted in a clinical pregnancy and birth of a healthy carrier of the FANCC gene, following confirmation of the results of both mutation analysis and HLA matching by CVS. Umbilical cord blood of the baby was collected at birth and transplanted to the affected sibling, resulting in a successful hematopoietic reconstitution. Nine embryos predicted normal but carrying HLA genes different from the sibling developed to the blastocyst stage and were frozen, while five affected embryos were exposed to PCR analysis, confirming the blastomere diagnosis.

As mentioned, the practical application of PGD has recently been extended for new indications, which appeared to be different from those used in prenatal diagnosis, such as the late-onset

disorders with genetic predisposition, which could have hardly been considered candidates for prenatal genetic diagnosis. HLA testing was the most recent and most unexpected addition to the indications for PGD. The results of this first case demonstrated feasibility of preimplantation HLA matching as part of PGD, with a prospect for the application of this approach to the other inherited conditions, such as thalassemias and other congenital disorders, also requiring an HLA-compatible donor for bone marrow transplantation. Although initially this was the first and only experience of PGD for HLA testing, it provided a realistic option for the couples desiring to avoid the birth of an affected child, together with the establishment of a healthy pregnancy, potentially providing an HLA match for an affected sibling. The data showed that the HLA testing in single blastomeres was accurate, and may also be applied as primary indication, that is, in cases not requiring mutation testing, such as for couples having affected children with leukemia or other cancers, awaiting an HLA-compatible donor with no success for years. These new indications make PGD a genuine alternative to conventional prenatal diagnosis, providing patients with important prospects not only to avoid an inherited risk without facing termination of pregnancy, but also to establish a pregnancy with particular genetic parameters to benefit the affected member of the family.

Our experience of PGD with HLA typing is presented in Table 4.1, showing that among conditions requiring HLA-compatible stem cell transplantation, thalassemia is the most prevalent one, representing the commonest autosomal-recessive diseases in the Mediterranean region, Middle East, and South East Asia, with heterozygous frequency of thalassemia mutations reaching over 14% in Greece and Cyprus.

## 4.2 Thalassemia

As described in Chap. 3, beta-thalassemia is an autosomal-recessive disease affecting the production of beta-globin chains resulting in a severe anemia, which makes the patients transfusion-dependant starting from 6 months after birth, so bone marrow transplantation is the only option for radical treatment. At present, over 400 different mutations have been described in the beta-globin gene, located on chromosome 11 (11p15.5), causing congenital anemia of variable severity [14, 15]. Prenatal diagnosis has been applied widely for almost three decades resulting in considerable reduction of new cases of thalassemia up to 70% in many populations, including large countries in the Eastern Mediterranean region such as Turkey and Iran [16, 17]. A considerable progress has been achieved also in the treatment of the disease by bone marrow transplantation [18], the application of which is still limited to the availability of HLA-matched stem cells, making PGD an attractive option for couples with thalassemic children. PGD for thalassemia has already been provided for 15 years [19], so HLA typing is presently offered in the same framework, allowing couples not only to avoid the birth of another child with thalassemia, but also producing an unaffected child who may be an HLA match to the thalassemic sibling, and thus a potential stem cell donor.

Thalassemias are presently one of the major indications for PGD. PGD for thalassemia was first performed for couples who had previously undertaken prenatal diagnosis but had to terminate the pregnancy with an affected fetus on repeated attempts [19]. Then, it was offered as a primary option to the patients with infertility problems, and to those who could not accept the risk for prenatal diagnosis and termination of pregnancy [19–21]. This was followed by PGD for the couples with existing thalassemic children requiring HLA-compatible bone marrow transplantation [22–25]. The objective of PGD in these cases was not only to have a thalassemia-free child, but also to ensure that the resulting baby could serve as an HLA-compatible donor for bone marrow transplantation for the affected siblings.

In our experience, of a total of 293 PGD cycles for 161 couples at risk for producing offspring with thalassemia, 144 cycles were performed for HLA typing. PB or blastomere biopsy was performed to identify thalassemia mutations, and blastomere sampling was also used for

**Table 4.1** Experience in PGD with HLA typing

| Disease | Patients | Cycles | No. of embryo transfers | No. of embryos transferred | Pregnancy | Birth |
|---|---|---|---|---|---|---|
| Thalassemia/sickle cell disease | 51 | 149 | 82 | 130 | 20 | 15 |
| FANCA,FANCC, FANCD2,FANCF, FANCI, FANCJ | 17 | 53 | 34 | 52 | *7* | 4 |
| WAS | 2 | 2 | 2 | 4 | *1* | 1 |
| X-ALD | 2 | 5 | 1 | 1 | 0 | 0 |
| Hyper-IgM | 5 | 8 | 6 | 9 | *3* | 2 |
| HED + ID; IP | 2 | 9 | 6 | 8 | *2* | 3 |
| DBA | 3 | 5 | 3 | 6 | *2* | 2 |
| Krabbe | 1 | 1 | 1 | 2 | 1 | 2 |
| DM | 1 | 2 | 1 | 2 | 1 | 2 |
| Chronic granulomatous disease | 1 | 3 | 3 | 5 | 1 | 1 |
| Total | 85 | 237 | 139 | 219 | 38 | 32 |

HLA testing, in order to identify the embryos containing the maternal and paternal chromosome 6 identical to the sibling with thalassemia, as described in detail elsewhere and summarized below [22, 25, 26].

HLA genes were tested simultaneously, using the short tandem repeats in the HLA region, by applying a multiplex heminested PCR system, involving only closely linked polymorphic short tandem repeat (STR) markers located throughout the HLA region [12], including D6S426, D6S291, Ring 3 CA, TAP1, G51152, D6S2447, LH1,DN, D6S273, 9N-2, TNF a,b,c,d; 62, MIC A, MIB, D6S276, D6S439, D6S1624, D6S265, D6S510; D6S248, RF, MOG a,b,c,d, D6S 258, D6S306, D6S464, D6S299, D6S461 (Fig. 4.1). The choice of alleles and markers was based on the information they provided about the presence of maternal and paternal matching or non-matching chromosomes. For each family, heterozygous alleles and markers were selected not to be shared by the parents. Such markers provided information about the origin of chromosome 6. A haplotype analysis for the father, mother, and affected child was performed for each family prior to preimplantation HLA typing. This allowed detecting and avoiding misdiagnosis due to preferential amplification and allele dropout (ADO), exceeding 10% in PCR of single blastomeres, potential recombination within the HLA region (see below), and a possible aneuploidy or uniparental disomy of chromosome 6, which may also affect the diagnostic accuracy of HLA typing of the embryo. The multiplex nature of the first round of PCR required a similar annealing temperature as the outside primers. Thirty cycles of PCR were performed with denaturation step at 95°C for 20 s, annealing at 62–50°C for 1 min and elongation at 72°C for 30 s [27]. Twenty minutes of incubation at 96°C was performed before starting cycling, and after cycling, 10 min of elongation at 72°C was performed. Annealing temperature for the second round was programmed at 55°C. The applied strategy provided a 100% HLA match, because the embryos with the same paternal and maternal chromosome 6 as in the affected siblings were preselected. Figure 4.2 presents PGD for HLA in the case of parents carriers of different mutations IVSI-5 and Cd8. Using PB1 and PB2 analysis, six mutation-free oocytes were identified prior to blastomere analysis, used mainly for selection of paternal mutation-free embryos (embryos #1, #3, #5, and #9) and HLA matching, simultaneously with linked marker analysis. In addition, HLA typing was also done in PB1 and PB2 to identify the oocytes with maternal HLA match, which is useful for interpretation of HLA-matching results in blastomeres. Only embryos #2 and #6 appeared to be HLA-matched to the affected sibling, which were transferred, resulting in the birth of a healthy child, who was confirmed to be thalassemia-free as well as HLA-matched to the affected sibling with thalassemia. The chances to identify

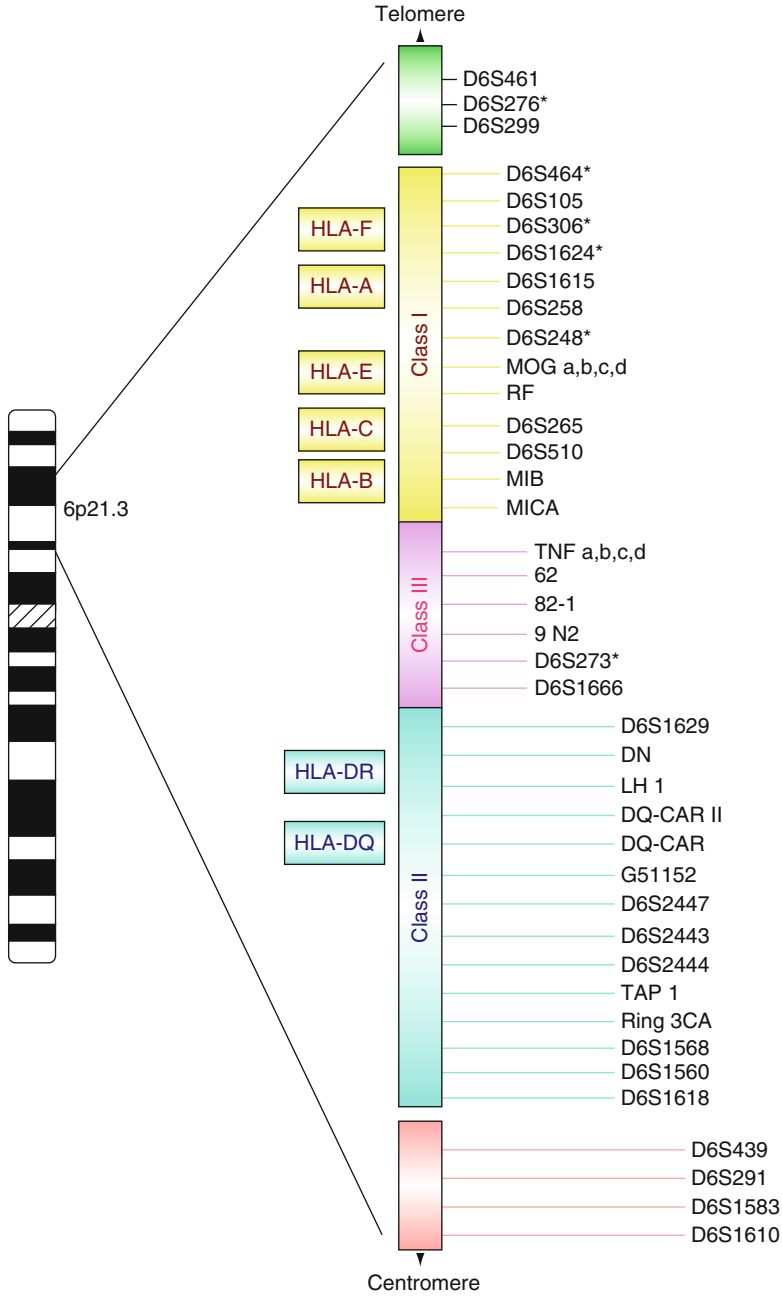



**Fig. 4.1** Polymorphic markers in HLA region applied for preimplantation HLA typing

unaffected embryos fully matched to thalassemic siblings is 18.75% and, as for other autosomal-recessive conditions, there is a 25% chance of HLA match and 75% chance of having an unaffected embryo (see below).

While the HLA-matched and thalassemia-free embryos were preselected for transfer back to the patient, based on the information about the mutation testing and polymorphic markers, those embryos predicted mutant or with insufficient

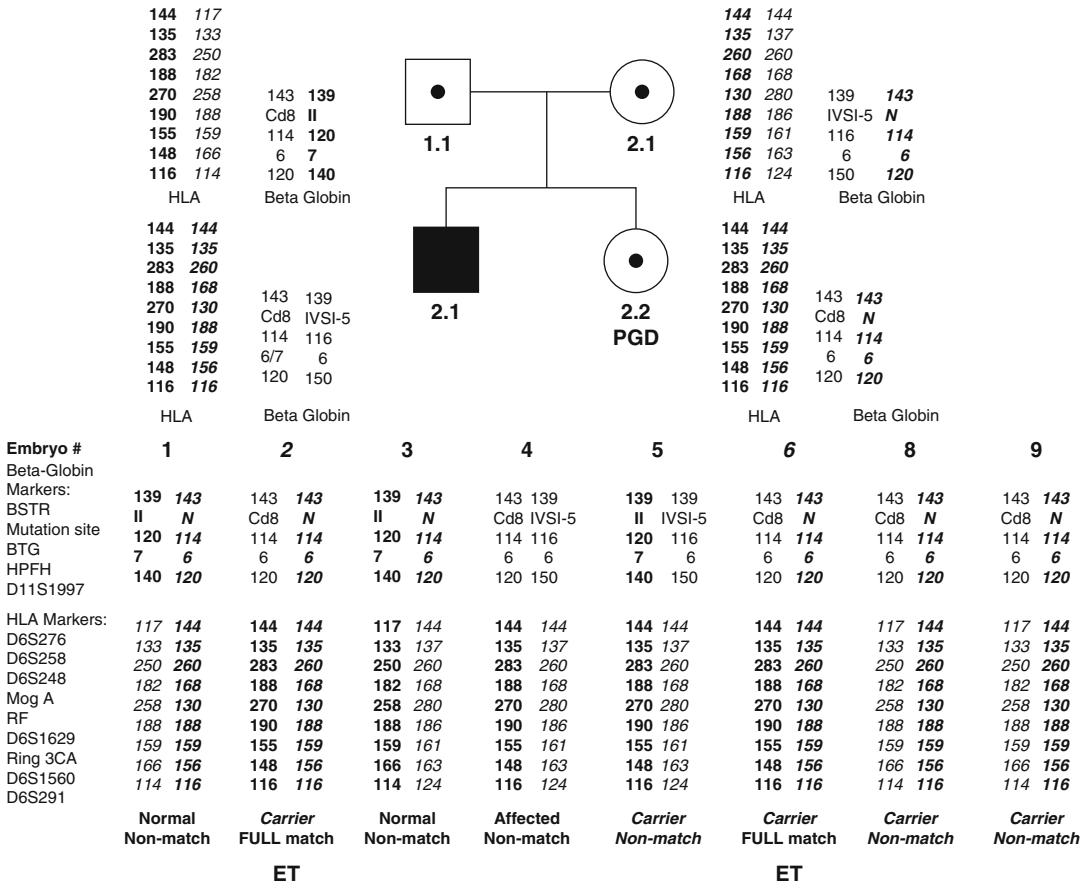



**Fig. 4.2** Preimplantation HLA matching combined with PGD for thalassemia. (*Top panel*) Family pedigree with HLA haplotype analysis based on parental (1.1 and 1.2) and affected child's (2.1) genomic DNA testing. HLA marker order is presented on the *upper left* for the father and *upper right* for the mother. *Dark bars* represent the matching paternal and maternal HLA haplotypes, and the *non-bold bars* non-matching haplotypes. (*Middle panel*) Maternal mutation and linked polymorphic markers were first assessed by sequential multiplex polar body (*PB*) analysis. Two oocytes (#4 and #5) had affected alleles, while the remaining six (#1, #2, #3, #6, #8, and #9) were normal (data not shown). Based on blastomere results, one embryo was affected (#4), two were homozygous normal (#1 and #3), and five were carriers of paternal (#2, #6, #8, and #9) or maternal (#5) mutations. As seen from HLA typing blow (see *lower panel*), embryos #2 and #6 are also fully HLA-matched to the sick sibling (2.1; *upper panel*). (*Bottom panel*) HLA typing by short tandem repeats (*STRs*) along with mutation analysis was performed on blastomeres from eight embryos, two of which (#2 and #6) were predicted to be HLA-matched to that of the affected sibling (2.1), although carrying the paternal mutation (also see above). Prenatal testing confirmed these results, and a healthy baby girl with HLA type matching that of the sick sibling was born. Cord blood stem cells were collected during the delivery and frozen for the stem cell transplantation

marker information were exposed to confirmatory analysis. Non-matched unaffected embryos were frozen for future use by the couple.

Of more than two dozens of different beta-globin gene mutations tested, the most frequent ones were IVSI-110 mutation – 100 cases (33%), followed by IVS I-6 – 39 cases, IVSII-745 – 23 cases, codon 8 – 20 cases, IVSI-1 – 18 cases, and codon 39 and IVSI-5 –16 cases each (see Table 3.4). Among other mutations were IVSII-2, Codon 5, Codon 6, Codon 41/2, E121K, -29 (A-G) -87, R30T, Cap 1, deletion 69 kb, and deletion 13.4 kb. Mutation testing resulted in detection and transfer of 476 unaffected embryos (approximately two embryos per transfer) in 240 (81.9%) of 293 clinical cycles, yielding 67 (27.9%) unaffected

pregnancies and the birth of 70 thalassemia-free children. PGD for thalassemias currently represents 15% of our overall experience of 2,158 PGD cycles performed for single-gene disorders [28].

A total of 144 of these PGD cycles were performed for HLA typing, which allowed detecting and transferring unaffected HLA-matched embryos in 78 of them (Table 4.2). Of 824 embryos with conclusive results for testing of beta-globin gene mutations and HLA type, 602 (73.0%) were predicted to be unaffected carriers or normal, of which only 126 (15.3%) appeared to be HLA-identical to the affected siblings, which is not significantly different from the expectation [26]. As many as 123 of these embryos developed appropriately to be acceptable for transfer, resulting in 18 unaffected HLA-identical pregnancies and the birth of 13 healthy children. Umbilical cord blood was collected at birth of these children and transplanted or pending, resulting in a successful hematopoietic reconstitution in all of them.

Figure 4.3 demonstrates the case of PGD for HLA typing for a couple with two thalassemic children, resulting in preselection and transfer of unaffected embryos matched to each of the affected children. HLA typing showed that one of the embryos was matched to one of the affected siblings, another to the other affected sibling (embryos #4 and #9), and three were non-matched (embryos #6, #7, and #8), including one with a single

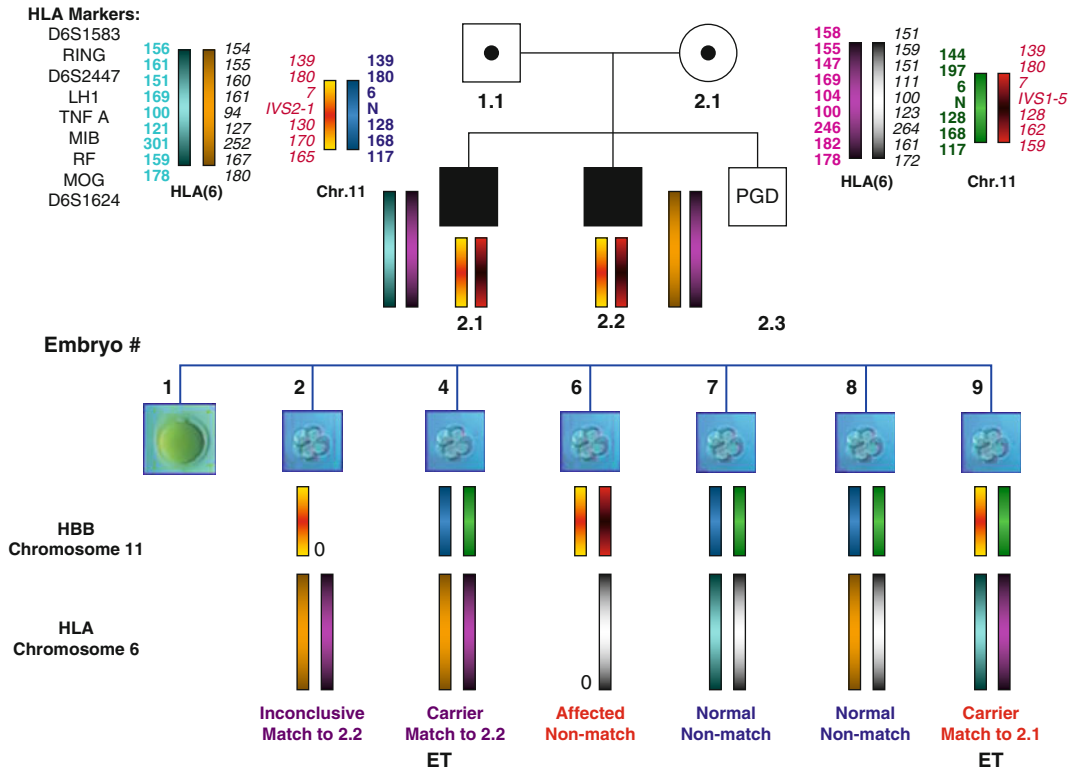



**Fig. 4.3** PGD for HLA typing in a couple with two thalassemic children requiring HLA-matched bone marrow transplantation. (*Upper panel*) Family pedigree with HLA haplotype analysis based on parental (1.1 and 1.2) and affected children's (2.1; 2.2) genomic DNA testing. HLA marker order is presented on the *upper left* for the father and *right* for the mother. Paternal and maternal matching HLA haplotypes to the affected children (2.1; 2.2) are shown in *different colors*. Maternal and paternal mutations and the linked markers are also presented accordingly. (*Lower panel*) HLA typing by short tandem repeats (*STRs*) along with mutation analysis was performed on blastomeres from seven embryos, one of which (#4) was predicted to be a carrier and an HLA match to the affected sibling 2.2, and another (#9) also a carrier and match to the affected sibling 2.1. Three others (embryos #6–8) were non-matched, while embryo #2 was matched but had inconclusive results of mutation testing due to lack of maternal chromosome 11. Both carrier matched embryos were transferred, but singleton pregnancy was obtained with the birth of a thalassemia-free child matched to one of the affected siblings

**Table 4.2** Results of PGD for thalassemia with HLA testing

| Patient/cycle | No. of embryos total/ amplified | No. of normal embryos | | No. of transfers/no. of embryos | Pregnancy/ birth |
|---|---|---|---|---|---|
| | | Non-match | Match | | |
| 52/144 | 907/824 | ***476*** | **126** | 78/123 | 18/13 |
| | 91% | | | 1.57 | 23% |

chromosome 6 (embryo #6). A single aneuploid oocyte suggesting trisomy 22 in the resulting embryo was detected by FISH analysis of PB1 and PB2 (excluded from further analysis of the causative gene and HLA type), in addition to the embryos with monosomy 11 (embryo #2) and monosomy 6 (embryo #6) mentioned. Two unaffected embryos and HLA-matched to each of the affected children were also aneuploidy-free (embryos #4 and #9) and transferred, resulting in a singleton pregnancy and birth of a healthy baby, HLA-matched to one of the affected siblings with thalassemia.

With the current progress in the treatment of hemoglobin disorders, PGD may have an increasing impact on the decision of the well-treated patients to reproduce. In fact, the life expectancy of the patients with hemoglobin disorders has been dramatically improved with the increasing success rate of radical treatment by stem cell transplantation [18]. However, the further impact of this treatment will depend on the availability of HLA-identical donors.

As seen from the above experience, PGD for HLA typing is an efficient tool for couples at risk to ensure having thalassemia-free children HLA-identical to the affected siblings, to serve as potential donors for stem cell transplantation treatment. This currently is available for a wider application in those communities where thalassemia is highly prevalent, and will improve the access to HLA-matched bone marrow transplantation of thalassemia.

For example, in the second largest series of PGD for HLA typing in thalassemia performed in Turkey, 236 PGD cycles were performed resulting in the birth of 70 thalassemia-free children, stem cells of 19 of whom were used for cord blood or bone marrow transplantation, which resulted in successful bone marrow reconstitution in all of them [29, 30].

## 4.3 Immunodeficiencies

Severe congenital immunodeficiencies (SCID) are a large group of conditions requiring PGD for HLA typing, as without compatible bone marrow transplantation the patients with SCID cannot survive. HLA-matched stem cell transplantation improves or completely replenishes the immune system, so PGD is an obvious alternative for inherited forms of SCID, to ensure the birth of unaffected children, who may then also serve as potential stem cell donor progeny for the affected siblings. Our accumulated experience of PGD for SCID is presented below, including PGD for ataxia telangiectasia (AT), Omen syndrome (OMS) (OMIM, 2001), FANCA, hyperimmunoglobulin M syndrome (HIGM), X-linked adrenoleukodystrophy (X-ALD), Wiscott–Aldrich syndrome (WAS), and X-linked hypohidrotic ectodermal displasia with immune deficiency (HED-ID) (Table 4.3) [31].

A total of 23 PGD cycles for 11 couples for producing affected progeny with the above conditions were performed, including 8 cycles for HIGM, 3 for AT, 2 for WAS, 9 for HED-ID, and 1 for OMS (Table 4.3), confirming the usefulness of preimplantation HLA matching as part of PGD, which potentially provides an HLA-matched progeny for treatment of affected siblings.

*Omen syndrome* (OMS) is an extremely rare autosomal-recessive disease with a prevalence of 1 in over 50,000, for which there is still no available cure other than stem cell transplantation. OMS is an early-onset fatal immunodeficiency with the absence of B cells and excess production of highly restricted T lymphocytes, which is caused by mutation in recombinase-activating genes RAG1 and RAG2 located on chromosome 11p, coding the lymphoid-specific proteins responsible for the process of variable, diversity, and joining (V (D) J) segment recombination

**Table 4.3** Results and outcomes of PGD for immunodeficiencies

| Disease/gene/mutations | Patient/cycle | Cell type tested | No. of embryos total/ amplified | No. of normal embryos | No. of abnormal embryos | No. of transfers/ no. of embryos | Pregnancy/ birth |
|---|---|---|---|---|---|---|---|
| HIGM/TNFSF (CD40L) gene/C218X, exon 4 c.437–438 ins A, exon 4 c.397 ins T | 5/8 | Pbs, BL; BL | 46/43 | 31 | 12 | 6/9 | 3/2 |
| OMS/RAG1 gene/ R 396 C; L86 AA del | 1/1 | Pbs, BL | 5/5 | 3 | 2 | 1/2 | 1/2 |
| HED-IP/ IKBKG(NEMO)gene/ D113N; Q348X; L153R | 2/9 | Pbs, BL | 36/35 | 24 | 11 | 6/8 | 2/3 |
| AT/ATM gene/ K1807E; A2262P; Unknown | 1/3 | Pbs, BL | 20/20 | 10 | 10 | 2/4 | 1/1 |
| WAS/WASP gene/ L39P; Nt. 361 (–2) A→G | 2/2 | Pbs, BL: BL | 10/10 | 6 | 4 | 1/2 | 1/1 |
| Total | 11/23 | N/A | 117/113 | 74 | 39 | 16/25 | 8/9 |

required for generation of the T- and B-cell repertoire (MIM 603554). This severe primary immunodeficiency disease is characterized by generalized erithrodermia, protracted diarrhea, repeated infections, hepatomegaly, and leukocytosis with eosinophilia and elevated immunoglobulin E. The large phenotypic variability of patients may be determined by different mutations in RAG1 and RAG2 genes, involving missense and splice mutations or deletions. Despite specific therapy for dermatitis and lymphoadenitis, using immunosuppression or replacement therapy with intravenous immunoglobulins, persistent viral, bacterial infections and chronic diarrhea resulting in inanition may be responsible for death, with the only cure being HLA-identical bone marrow transplantation.

A couple at risk for producing a progeny with OMS had two previous children, including the younger daughter with a severe OMS resulting in death (Fig. 4.4). The child was double heterozygous, with the inherited paternal

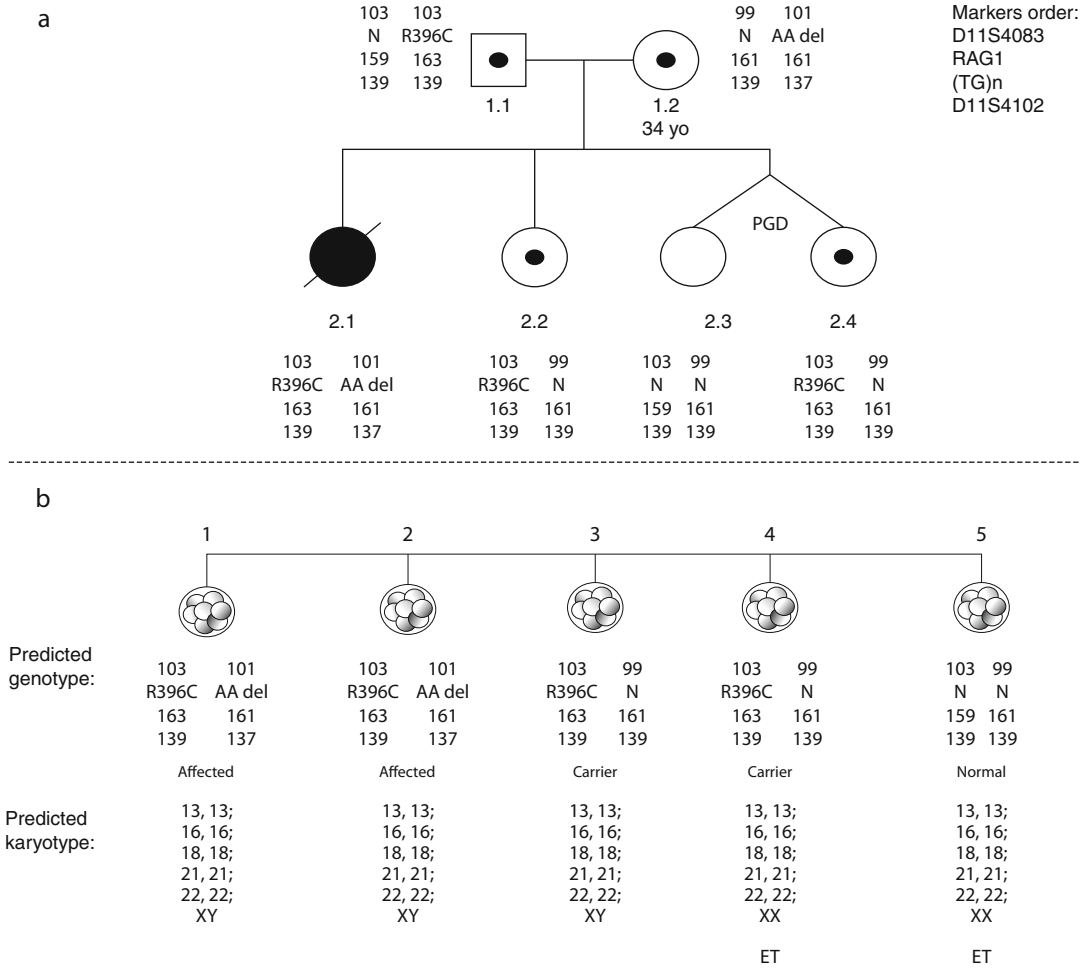



**Fig. 4.4** PGD for OMS with aneuploidy testing. (**a**) Family pedigree with the mutation and haplotype analysis of parents (1.1 and 1.2) and children (2.1 affected, and 2.2 healthy heterozygous carrier of paternal mutation). (**b**) (*Top*) Blastomere analysis involving mutation analysis of five embryos, including two affected (embryos #1 and #2), two carriers of paternal mutation (embryos #3 and #4), and one free of both paternal and maternal mutations (embryo #5). (**b**) (*Bottom*) Blastomere analysis for aneuploidy, showing normal chromosomal sets for all five embryos, two of which were transferred (embryos #4 and #5) resulting in the birth of healthy twins (2.3 normal, and 2.4 heterozygous carrier of the paternal mutation)

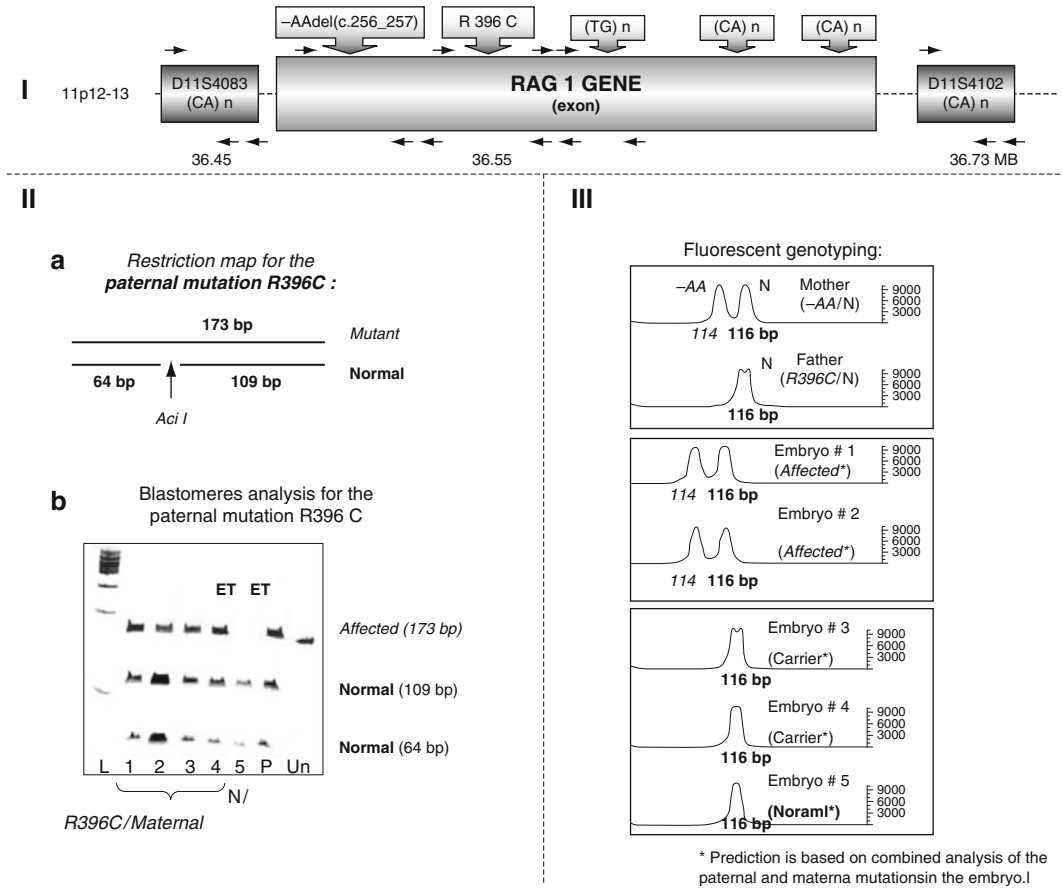



**Fig. 4.5** PGD for OMS by blastomere analysis of paternal and maternal mutations. (**I**) Position of parental mutations and informative linked polymorphic markers used in PGD. (**IIa**) Restriction map of the paternal mutation R396C, following Aci I digestion, creating two fragments of 64 and 109 bp in PCR product of the normal gene. (**IIb**) The polyacrilamide gel electroforegram of the Aci I-digested PCR products of biopsied blastomeres from five embryos for the paternal mutation R396C, showing that only embryo #5 is free of paternal mutation, while the remaining embryos contain the paternal mutation. (**III**) Capillary electroforegram of fluorescently labeled PCR products of RAG1 gene obtained from mother, father, and five embryos, showing normal fragment of 116 bp for the father, two fragments of 114 pp (AA del) and 116 bp for the mother, the same two fragments including AA del for embryos #1 and #2, and one normal 116 bp fragment for embryos #3, #4, and #5, the latter two of which were transferred, resulting in the birth of healthy twins

R396C mutation, representing a sequence change from arginine to cysteine at amino acid position 396, caused by a single C to T sequence change (CGG to TGG) in codon 396 of the RAG1 gene, and maternal mutation c.256_57 del AA within the lysine 86 codon, causing a frameshift mutation that results in a premature termination signal 32 codons downstream. The older heterozygous unaffected daughter inherited only the paternal A396C mutation in the RAG1gene.

The paternal mutation was tested by Aci I digestion, which creates two fragments of 64 and 109 bp in the PCR product of normal RAG1 gene, leaving the mutant allele uncut. The two "A" nucleotide deletions in codon 86 of the maternal allele of RAG1 gene were detected by capillary electroforesis of the fluorescent labeled PCR product, visualizing the 114 bp fragment vs. the 116 bp fragment in the normal allele (Fig. 4.5).

This case represented the world's first PGD for OMS, which was performed using PB1 and

PB2 and blastomere analysis, and resulted in the transfer of two unaffected and aneuploidy-free embryos, yielding the birth of healthy twins. Because in this case the affected sibling died early in childhood, there was no need for HLA typing, but the couples with previous OMS children will definitely be potential candidates for performing PGD with HLA typing to provide also an identical HLA donor progeny for stem cell transplantation.

*Ataxia Telangiectasia* (*AT*) is a progressive, neurodegenerative childhood disease that affects the brain and other body systems (MIM 208900). A weakened immune system makes the patients susceptible to recurrent respiratory infections. The disease presents between 1 and 4 years of age, as a delayed development of motor skills, poor balance, and slurred speech. Telangiectasias appear in the corners of the eyes or on the surface of the ears and cheeks. Patients with AT may develop cancer, such as acute lymphocytic leukemia or lymphoma. Other features may include mild diabetes mellitus, premature graying of the hair, difficulty swallowing, and delayed physical and sexual development. Although the currently used symptomatic and supportive treatment, including high-dose vitamin regimens, physical and occupational therapy, and gammaglobulin injections to supplement a weakened immune system may be helpful, the prognosis is very poor, and patients still die in their teens. More than 500 unique mutations are known in the ataxia telangiectasia-mutated (ATM) gene associated with AT, resulting in the absence of serine–protein kinase coded by the ATM gene located on chromosome 11q22.3 (MIM 607585). Sequence analysis detects as many as 90% mutations, and others may be identified by the linkage analysis, which is extremely accurate, based on testing for intragenic markers presently available.

PGD was performed for the couple at risk for producing a progeny with AT, who had one affected child who died in early infancy and one spontaneous abortion (Fig. 4.6). The mother was a carrier of two ATM sequence changes, involving exon 38 (5419A>G, K1807E) and exon 48 (6784G>C, A2262P). As it is not known which of these two mutations is responsible for AT, both were tested in PB1 and PB2 and blastomeres. As shown in Fig. 4.7, maternal mutation K1807E was identified by Bsm AI digestion, creating two fragments of 96 and 92 bp in the PCR product of the mutant gene. In contrast, maternal mutation A2262P was not cut by Hae III restriction digestion, but created two fragments of 35 and 99 bp in the normal gene.

As the paternal mutation in the ATM gene was not identified, it was traced using four closely linked markers, listed in Fig. 4.6a. So we performed sequential PB1 and PB2 removal following maturation and fertilization of oocytes, to identify the mutation-free oocytes, as shown in Fig. 4.7IIb, and then tested the resulting embryos for paternal mutation by linkage analysis, simultaneously with testing for aneuploidy (Fig. 4.6c). As shown in Fig. 4.6, the transfer of two unaffected carrier embryos resulted in a singleton pregnancy and the birth of a healthy baby boy, confirmed to be an unaffected carrier of maternal mutations.

The case of PGD for AT was reported previously for a Saudi family with three affected children [32]. The disease was caused by a large deletion of more than two-thirds of the AT gene, which was detected by amplification of one of the deleted exons (exon 19). Of three embryos available for biopsy and testing, one was deletion-free and transferred, resulting in an unaffected pregnancy.

*Fanconi anemia complementation group A* (*FANCA*), similar to FANCC, described above in Sect. 4.1, is an autosomal-recessive disorder causing an inherited bone marrow failure with increased predisposition to leukemia. As mentioned, bone marrow transplantation is the only treatment for FA, as it restores hematopoiesis in FANCA patients. However, because any modification of the conditioning is too toxic for these patients, as in FANCC, leading to a high rate of transplant-related mortality, the HLA-identical cord blood transplantation from a sibling is particularly valuable, to avoid late complications due to severe GVH, as mentioned above.

Of 17 couples at risk for producing a progeny with FA (Table 4.1), in addition to two carriers of IVS 4+4A-T mutation in the FANCC gene, three

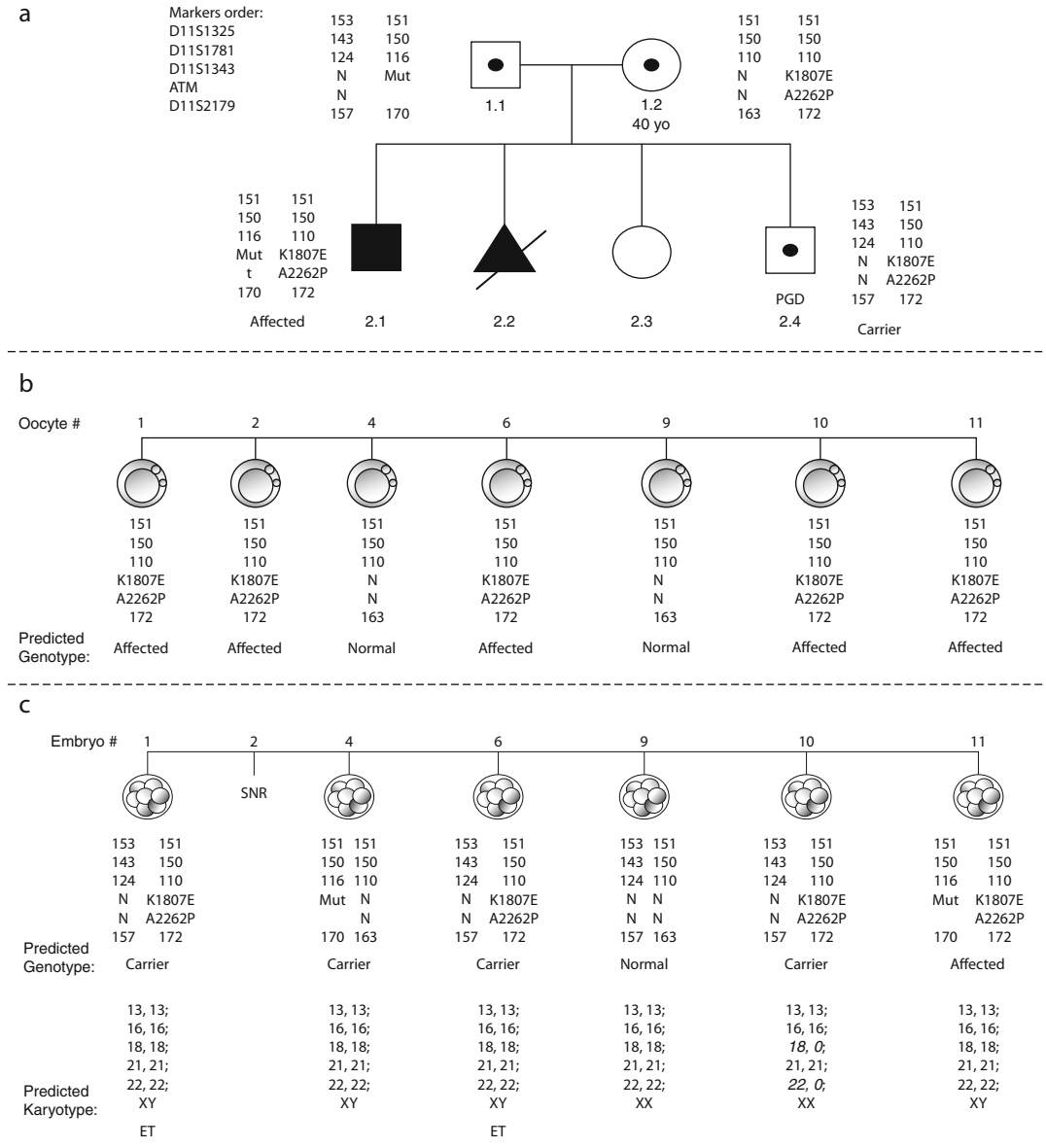



**Fig. 4.6** PGD for AT with aneuploidy testing. (**a**) Family pedigree showing the results of mutation and haplotype analysis in the parents (1.1 and 1.2) and the affected child (2.1). (**b**) Results of PB analysis of seven oocytes, only two of which (oocytes #4 and #9) were free of mutation, based on mutation and marker analysis. The remaining five oocytes were affected, containing both maternal mutations tested. (**c**) (*Upper panel*) Results of mutation and linked marker analysis of six embryos originating from the above oocytes (no sample was available from the embryo originating from oocyte #2). Five of these six embryos were either normal (embryo #9) or carriers (embryos #1, #4, #6, and #10), while the remaining one embryo (embryo #11) was affected, inheriting both maternal and paternal mutations. (**c**) (*Bottom panel*) Results of aneuploidy testing for chromosomes 13,16,18,21,22,X, and Y, showing one double monosomy 18 and 22 in heterozygous unaffected embryo #10

were carriers of FANCD2, FANCF, FANCI, FAMCCJ, and FANCA gene mutations, including one with different maternal and paternal mutations, the maternal one involving A*T*G to A*A*G substitution in exon 1, resulting in methionine to lysine amino acid substitution, and the paternal-14 bp deletion in exon 2, representing a frameshift mutation. The paternal mutation was detected as the

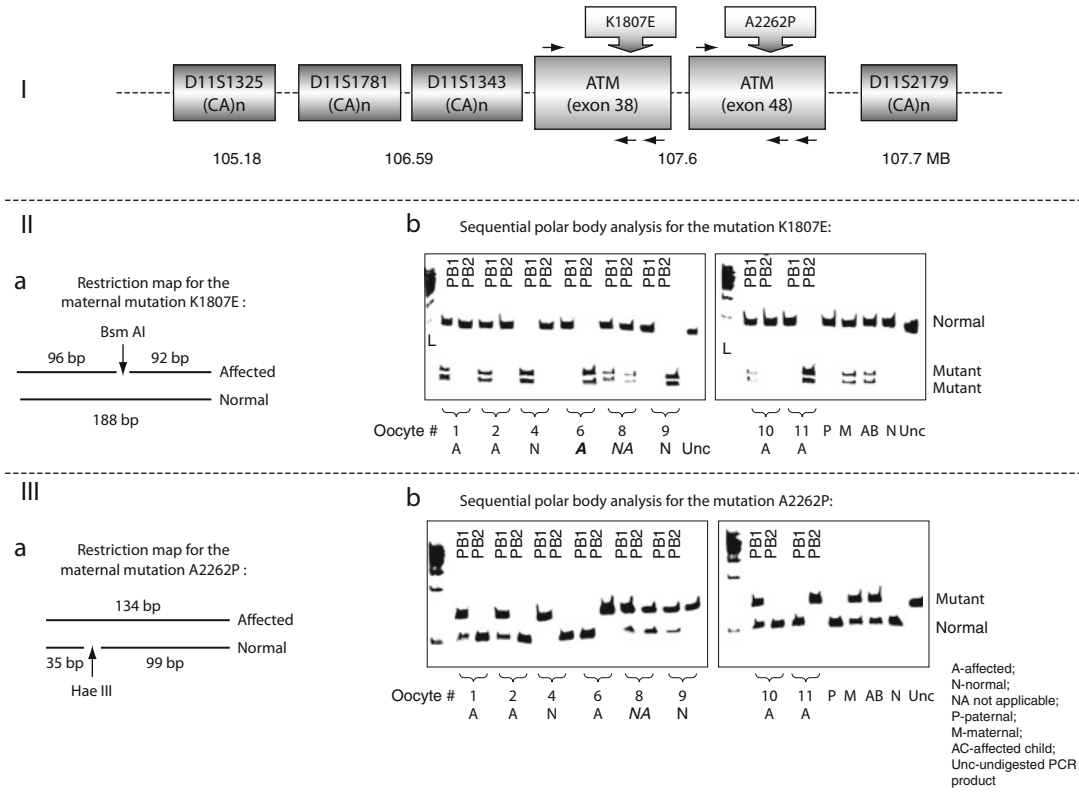



**Fig. 4.7** PGD for AT by sequential PB1 and PB2 analysis of maternal mutations. (**I**) Position of two different mutations and informative linked polymorphic markers used in PGD. (**IIa**) Restriction map of maternal mutation K1807E, following Bsm AI digestion, which creates two fragments in the PCR product of the mutant gene. (**IIb**) The polyacrilamide gel electroforegram of the Bsm AI-digested PCR products of PB1 and PB2 from eight oocytes, of which only two (oocytes #4 and #9) were free of maternal mutation. Five oocytes were mutant, and one, with the heterozygous status of both PB1 and PB2, excluded from further study, due to possible DNA contamination. (**IIIa**) Restriction maps for the maternal mutation A2262P following Hae III restriction digestion, which creates two fragments of 35 and 99 bp in a normal allele, leaving the mutant allele uncut. (**IIIb**) The polyacrilamide gel electrophoregram of the Hae III-digested PCR products of PB1 and PB2 from eight oocytes, of which two (oocytes #4 and #9) were free of maternal mutation, five oocytes (oocytes #1, #2, #6, #10, and #11) were mutant, and one, with the heterozygous status of both PB1 and PB2, excluded from the further study, due to possible DNA contamination, as in the analysis for K1807E mutation

size difference in capillary electroforesis of the PCR product, while the maternal mutation was detected by Nla*II* restriction digestion, which cuts the normal sequence, leaving the mutant sequence uncut. In the other couple, only paternal mutation was known, representing T1131A mutation, due to *A*CT to *G*CT substitution in exon 34, which creates a restriction site for Fsp4H*I*. In addition, another restriction enzyme, TspR*I*, was used, which cuts the normal sequence on the opposite end. When a mutation was not identified, unaffected embryos were chosen by linkage analysis, using five closely linked polymorphic markers. Overall, 52 unaffected HLA-matched embryos were transferred in 34 cycles, resulting in seven unaffected pregnancies and four FA-free and HLA-matched children, as potential donors for their siblings (Table 4.1).

*X-linked adrenoleukodystrophy* (*X-ALD*) affects the nervous system and the adrenal cortex, with three main phenotypes. One of them manifests between ages 4 and 8 as attention deficit disorder, followed by progressive impairment of cognition and behavior, vision, hearing, and motor function, leading to total disability within 2 years. The other phenotype, called adrenomyeloneuropathy, manifests in the late twenties as progressive paraparesis, sphincter disturbances, and hearing loss, while the third presents with primary adrenocortical insufficiency by 7–8 years of age. Regardless of the presence of symptoms,

99% of patients have an elevated concentration of very long chain fatty acids (VLCFA). The disease is caused by mutations of ABCD1 gene, with more than 200 different mutations reported by the present time, which may be detected by PCR and direct sequencing, except for large deletions identified by Southern blot analysis. Carrier screening and prenatal diagnosis is available and the same method may be applied for PGD with simultaneous HLA typing.

PGD was performed for two couples, involving the testing for G343D mutation, representing a sequence change from aspartic acid to glycine at amino acid position 343, caused by a single (G to A) sequence change in the nucleotide 1414 (G1414A) of the ABCD gene. PGD was based on Fok *I* restriction digestion, which creates two fragments in the PCR product of a normal gene, leaving the mutant one uncut. A total of five PGD cycles were performed, which resulted in only one unaffected HLA matched embryo identified for transfer, which however failed to produce a clinical pregnancy (Table 4.1).

*Hyperimmunoglobulin M Syndrome* (HIGM) (Table 4.1) is a rare immunodeficiency characterized by normal or elevated serum IgM levels, with absence of IgG, IgA, and IgE, which results in an increased susceptibility to infections, manifested in the first few years of life, and a high frequency of autoimmune hematologic disorders, accompanied by gingivitis, ulcerative stomatitis, fever, and weight loss. HIGM is caused by mutation in the CD40 ligand gene (CD40LG), located on chromosome Xq26, which leads to a defective CD40 ligand expression resulting in the failure of T cells to induce IgE synthesis in interleukin-4-treated B cells. Although a regular administration of intravenous immunoglobulins may be used for treatment, the best results were obtained by HLA-matched bone marrow transplantation, which makes PGD the method of choice for those who cannot find an HLA match among their relatives.

PGD was performed for five couples with HIGM, (Table 4.1) one with C218X mutation in exon 5 of CD40 ligand gene (CD40LG), three with maternal mutations C218X exon 4 c.437_38 ins A, and one with exon 4 c.397 ins T, using the primers listed in (Table 4.4). The maternal mutations were analyzed by PB1 and PB2, followed by HLA and aneuploidy testing in biopsied blastomeres. CYS218STOP mutation in exon 5 was detected by restriction digestion, which eliminates the restriction site for *Cac 81*, creating two fragments in PCR product from the normal gene, leaving the mutant gene product uncut. For higher accuracy, another restriction enzyme (*Mnl I*) was applied, which creates three fragments in the mutant PCR product, compared to two fragments in the normal gene (Fig. 4.8).

Figure 4.9 presents the case of PGD and HLA typing for a couple at risk for producing offspring with HIGM. Of 15 oocytes tested by PB1 and PB2, 5 of 11 oocytes with conclusive results

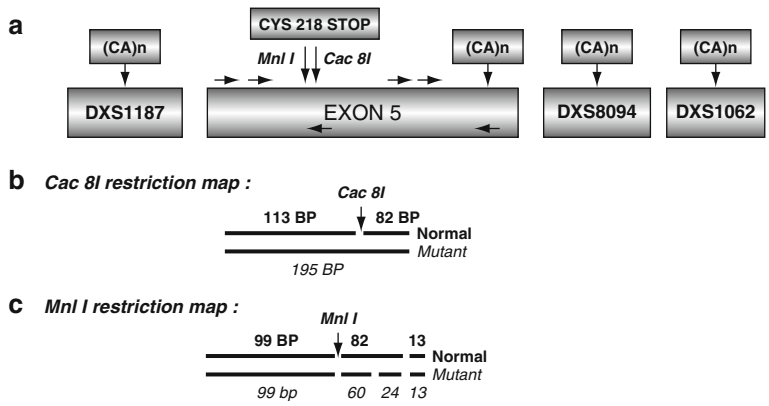



**Fig. 4.8** PGD design for HIGM in combination with HLA typing (see also Fig. 4.5). (**a**) Position of the C218X mutation in exon 5 of CD40 ligand gene (Xq26.3) and tightly linked dinucleotide polymorphic markers inside the gene (exon 5) and outside the gene (DXS1187, DXS8094, DXS1062). Horizontal arrows represent primer positions. Vertical arrows indicate the location of *Mnl*I and *Cac*8I restriction sites, and the positions of the dinucleotide polymorphic markers. (**b**) Restriction map of the *Cac*I restriction digestion. (**c**) Restriction map for the Mnl I digestion

**Table 4.4** Primers and reaction conditions for PGD of immunodeficiencies

| Gene/polymorphism | Accession no. | Heterozy gosity index | No. of alleles | Upper primer | Lower primer | Annealing $T_m$ (°C) |
|---|---|---|---|---|---|---|
| ATM K 1807 E (Heminested) *Bsm AI* cuts mutant sequence | AH004875 | NA | NA | Outside: 5′ AGTTTTTAGAAGTACCCA GATTTGA 3′ | 5 TAGATAAACAGGTCATAA ACAAGGA 3′ | 62–45 |
| | | | | Inside: 5′ AGTTTTTAGAAGTACCCA GATTTGA 3′ | 5′ TCTTCTTACTTCACACAT TGGCT 3′ | 55 |
| ATM A 2262 P (Heminested) *Hae III* cuts normal sequence | AH004875 | NA | NA | Outside: 5′ AAGGAAATGGACAACTC ACAAAG 3′ | 5′ CCCTCAGGCTTTCTGT TTTTTA 3′ | 62–45 |
| | | | | Inside: 5′ TCTCACCAAACACCTTGTA GAACTC 3′ | 5′ CCCTCAGGCTTTCTGT TTTTTA 3′ | 55 |
| D11S1325 (Heminested) | Z23828 | 0.52 | 3 | Outside: 5′ AACATCAAATGGTTCTTG CTTC 3′ | 5′ TTTTATCTCTTTTTCAATA CAATGC 3′ | 62–45 |
| | | | | Inside: 5′ AACATCAAATGGTTCTTG CTTC 3′ | 5′ *Fam* GGGATTCTGCTTTTT TCCTTTA 3′ | 55 |
| D11S1781 (Heminested) | Z52108 | 0.34 | 4 | Outside: 5′ GGGGATGAGTAATGATATAAGA CAA 3′ | 5′ ACTTCTACTGTGTATATTT ACGGCA 3′ | 62–45 |
| | | | | Inside: 5′ GGGGATGAGTAATGATATAAGA CAA 3′ | 5′ *Fam* CGGCATATAACATAGTG TTATTTTG 3′ | 55 |
| D11S1343 (Heminested) | Z24175 | 0.56 | 5 | Outside: 5′ CTCCTTCCCAAACAATCCACT 3′ | 5′ CCTGGTTCATGTAGCAG TTCCT 3′ | 62–45 |
| | | | | Inside: 5′ CTCCTTCCCAAACAAT CCACT 3′ | 5′ *Fam* CCCCTACTGTTTTATG ACCCA 3′ | 55 |
| D11S2179 (Heminested) | AF119249 | NA | 8 | Outside: 5′ CTCCTCATTCTAAACAA CAACTG 3′ | 5′ GCTTGCAACATCTACTATA TATTTT 3′ | 62–45 |
| | | | | Inside: 5′ *Fam* TTCTCTTCTATGAATATAAC AGGAG 3′ | 5′ GCTTGCAACATCTACTATA TATTTT 3′ | 55 |
| RAG1 R 396 C (Heminested) *AciI* cuts normal sequence | NT_009237 | NA | NA | Outside: 5′ CCACATCTCAAGTCACAA GGAA 3′ | 5′GCCAGCAGGAACAAG GTCAT 3′ | 62–45 |
| | | | | Inside: 5′ CCACATCTCAAGTCACA AGGAA 3′ | 5′ ACTTCACATCTCCAC CTTCTTCT 3′ | 55 |

| | | | | | | |
|---|---|---|---|---|---|---|
| RAG1 c.256_57 del AA (Nested) | NT_009237 | NA | NA | Outside: 5′ GAAACCCTCTCTGGAGCAATCT 3′ | 5′ GCTCTAAAAGAATTCC CACAGA 3′ | 62–45 |
| | | | | Inside: 5′ ACAAGGCTGATGGTCAGAAG 3′ | 5′ *Fam* TTGGCTTGATGG ATCGCTT 3′ | 55 |
| D11S4083 (Heminested) | Z52164 | 0.87 | 15 | Outside: 5′ GGACTCTTGGAACTCTGGACT 3′ | 5′ TTGGGGATCATGTG TACCC 3′ | 62–45 |
| | | | | Inside: 5′ GGACTCTTGGAACTCTGGACT 3′ | 5′ *Fam* AGGGCAGAGTATTTAC AAAGAAG 3′ | 55 |
| RAG1 (TG)n (Heminested) | NT_009237 | NA | 4 | Outside: 5′ AGAAGTTTGTTGGTTTCATTTA | 5′ GTATCCAGCAGAGT GCCTAGT 3′ | 62–45 |
| | | | | Inside: 5′ *Fam* CCTTGCTTCTTTAGTTGCTTT | 5′ GTATCCAGCAGAG TGCCTAGT 3′ | 55 |
| D11S4102 (Heminested) | Z52543 | 0.77 | 10 | Outside: 5′ ATCCTCACCTTATTCACCCTG 3′ | 5′ AATCCTGGAAAG CCCTGG 3′ | 62–45 |
| | | | | Inside: 5′ ATCCTCACCTTATT CACCCTG 3′ | 5′ *Hex* TAGGGATTTTAGGAGG GATGACT 3′ | 55 |
| WAS Nt.361 A--G (Heminested) *HpyCH 4 V* cuts normal sequence | AF115548, AF115549 | NA | NA | Outside: 5′ AGGAGATGGGAAAGTTGCGG 3′ | 5 CCAACTTCCTTTCCT CCCCTG 3′ | 62–45 |
| | | | | Inside: 5′ AGGAGATGGGAAAGTTGCGG 3′ | 5′ GATTCCTTTTTTGTATC TTCTCCTG 3′ | 55 |
| WAS L 39 P (Heminested) *ScrF I* cuts mutant sequence | AF115548, AF115549 | NA | NA | Outside: 5′ TCAGCAGAACATA CCCTCC 3′ | 5′ AGAGAGAGAAGGAG GAGAGG 3′ | 62–45 |
| | | | | Inside: 5′ TCAGCAGAACATAC CCTCC 3′ | 5′ GAAGAAACGGTGGG GAC 3′ | 55 |
| DXS1003 (Heminested) | Z17201 | 0.8 | 11 | Outside: 5′ AGAAGCCGTTATTGGTGGA CTC 3′ | 5′ ACACTGCTACTCCTTGG GAAATC 3′ | 62–45 |
| | | | | Inside: 5′ AGAAGCCGTTATTGGTGG ACTC 3′ | 5′ *Hex* CATTCCTCACTGGC AAGTTTTA 3′ | 55 |
| GATA160B08 (Heminested) | G10694 | 0.71 | 5 | Outside: 5′ CCAATTGCCTACTGGA TATTACCAA 3′ | 5′ TGGGAACAAAACAGGC AAAGTC 3′ | 62–45 |
| | | | | Inside: 5′ CCAATTGCCTACTGGATA TTACCAA 3′ | 5′ *Fam* TTTGCCCTCATGG AGTGCC 3′ | 55 |

(continued)

**Table 4.4** (continued)

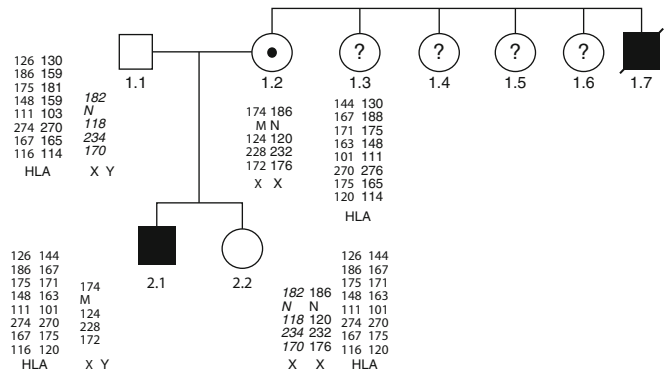
| | | | | | | |
|---|---|---|---|---|---|---|
| DXS1208 (Heminested) | Z23944 | 0.54 | 8 | Outside: 5′ TCTAAAGCCCAGGACCCCG 3′ | 5′ TGGTTAAAGGATTT GGGAGGC 3′ | 62–45 |
| | | | | Inside: 5′ *Hex* TCAGGGCTCCAACTCCAGG 3′ | 5′ TGGTTAAAGGATTTG GGAGGC 3′ | 55 |
| DXS1039 (Heminested) | Z23372 | 0.56 | 9 | Outside: 5′ CCCTCTTCACTTTTCCAGTCAAT 3′ | 5′ GGAAAGGGAAGAA GAATGCC 3′ | 62–45 |
| | | | | Inside: 5′ *Fam* TGTTCCTGGTATGTGACAATGC 3′ | 5′ GGAAAGGGAAGA AGAATGCC 3′ | 55 |
| DXS8023 (Heminested) | Z52342 | 0.57 | 9 | Outside: 5′ GTGCAAACTGTTCCACCTGG 3′ | 5′ CTCAAAGAATGAAG TAGAATAAGGATA 3′ | 62–45 |
| | | | | Inside: 5′ GTGCAAACTGTTCCACCTGG 3′ | 5′ *Fam* TTGATAAAGTAGTCAGG AAAGGCT 3′ | 55 |
| HED-ID (IKBKG gene) Q 348 X *Hpy188 III* cuts normal sequence | NT_025965 | NA | NA | Outside: 5′ GGGAGTACAGCAAACTGAAGGC 3′ | 5′ CCCTAACCCAGAACA CCAGG 3′ | 62–45 |
| | | | | Inside: 5′ GGGAGTACAGCAAACTGAAGGC 3′ | 5′ CCATCCGTCTCCTG TGGTC 3′ | 55 |
| HED-ID (IKBKG gene) D 113 N *Mbo I* cuts normal sequence | NT_025965 | NA | NA | Outside: 5′ AGGAGTTCCTCATGTGCAAGTT 3′ | 5′ CCTTGTGGAACAC TGGCG 3′ | 62–45 |
| | | | | Inside: 5′ AGGAGTTCCTCATGTGCAAGTT 3′ | 5′ GTTTTCAGAACCT GGCCCTG 3′ | 55 |
| TNFSF (CD40 gene) Exon4 C.397 ins T Exon4 C.437_38 ins A (Heminested) | D31796 | NA | NA | Outside: 5′ TTTGGTTCCATTTCAGGTGATC 3′ | 5′ AACATGACTTCGGC ATCCCA 3′ | 62–45 |
| | | | | Inside: 5′ TTTGGTTCCATTTCAGGTGATC 3′ | 5′ *Fam* CGCTCAGATGCTGTGT GACTTAC 3′ | 55 |

appeared to be free of maternal mutation, but only one was a maternal HLA match (embryo #2 in Fig. 4.9). In addition, 3 of 5 oocytes with maternal mutation were HLA-matched (embryos #11, #13, and #15 in Fig. 4.9). However, embryos #13 and #15 were affected and a non-paternal match, while only a maternal mutant chromosome was detected in embryo #11. Only one embryo (embryos #2), predicted to be mutation-free and a maternal match by PB analysis, appeared to be a normal female with also a paternal match. The transfer of this single embryo resulted in a singleton pregnancy, confirmed to be unaffected and HLA-matched by amniocentesis, yielding the birth of a healthy HLA-matched baby girl.

The first transplantation treatment was done using cord blood stem cell obtained from this child, but no engraftment was achieved. So the second transplantation was performed 1 year later, using bone marrow mixed with the remaining portion of the cord blood sample, which resulted in a successful engraftment and reconstitution of the sibling's bone marrow, resulting in a total cure of the patient.

*Wiscott–Aldrich syndrome* (WAS) is a lethal X-linked immune deficiency, in which lymphocyte dysfunction and thrombocytopenia result in severe infections, bleeding episodes, and increased risk of lymphoproliferative malignancies. While supportive therapy may increase survival rate, the only hope for avoiding early mortality is bone

a




b

| Embryo # | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 9 | 10 | 11 | 13 | 15 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| X-chromosome markers: | | | | | | | | | | | | |
| DXS1187 | 186 | *182* 186 | *182* 186 | *182* 174 | *182* 174 | 186 | *182* 186 | 186 | *182* 186 | 174 | 174 | 174 |
| CD40 | N | *N* N | *N* N | *N* M | *N* M | N | *N* N | N | *N* N | M | M | M |
| (CA)n | 120 | *118* 120 | *118* 120 | *118* 124 | *118* 124 | 120 | *118* 120 | 120 | *118* 120 | 124 | 124 | 124 |
| DXS8094 | 232 | *234* 232 | *234* 232 | *234* 228 | *234* 228 | 232 | *234* 232 | 232 | *234* 232 | 228 | 228 | 228 |
| DXS1062 | 176 Y | *170* 176 | *170* 176 | *170* 172 | *170* 172 | Y 176 | *170* 176 | Y 176 | *170* 176 | 172 | Y 172 | Y 172 |
| HLA markers: | 130 130 | 126 144 | 126 130 | 130 130 | 126 130 | 126 130 | 130 144 | 126 130 | 126 130 | 126 144 | 130 144 | 130 144 |
| D6S276 | 159 188 | 186 167 | 186 188 | 159 188 | 186 188 | 186 188 | 159 167 | 186 188 | 186 188 | 186 167 | 159 167 | 159 167 |
| Moga | 181 175 | 175 171 | 175 175 | 181 175 | 175 175 | 175 175 | 181 171 | 175 175 | 175 175 | 175 171 | 181 171 | 181 171 |
| D6s265 | 159 148 | 148 163 | 148 148 | 159 148 | 148 148 | 148 148 | 159 163 | 148 148 | 148 148 | 148 163 | 159 163 | 159 163 |
| D6S510 | 103 111 | 111 101 | 111 111 | 103 111 | 111 111 | 111 111 | 103 101 | 111 111 | 111 111 | 111 101 | 103 101 | 103 101 |
| TNF a | 270 276 | 274 270 | 274 276 | 270 276 | 274 276 | 274 276 | 270 270 | 274 276 | 274 276 | 274 270 | 270 270 | 270 270 |
| D6S273 | 165 165 | 167 175 | 167 165 | 165 165 | 167 165 | 167 165 | 165 175 | 167 165 | 167 165 | 167 175 | 165 175 | 165 175 |
| LHI D6S291 | 114 114 | 116 120 | 116 114 | 114 114 | 116 114 | 116 114 | 114 120 | 116 114 | 116 114 | 116 120 | 114 120 | 114 120 |
| Predicted genotype | Normal male Non-match | Normal female Match ET | Normal female Non-match | Carrier Non-match | Carrier Non-match | Normal male Non-match | Normal female Non-match | Normal male Non-match | Normal female Non-match | *X 0 Match* | Affected Male Non-matched | Affected Male Non-matched |

**Fig. 4.9** Preimplantation HLA typing combined with PGD for X-linked hyperimmunoglobulin M syndrome (**a**) Family pedigree in three generations. Marker order is located next to maternal haplotypes. Paternal (2.1), maternal (2.2), and the affected sibling (3.1). CD 40 gene haplotype assignment is based on genomic DNA testing. Paternal and maternal matching HLA haplotypes are shown in *bold face*. (**b**) (*Upper panel*) PCR analysis of blastomeres removed from 12 embryos showed that all but three embryos (#11, #13, and #15) were predicted to be unaffected. (**b**) (*Lower panel*) HLA typing was performed simultaneously with mutation analysis of all blastomeres. Embryo #2 was predicted to be a normal female and to have the same HLA profile as the affected sibling (3.1). The transfer of this embryo resulted in pregnancy and the birth of a healthy unaffected HLA-matched baby girl (3.2). Cord blood stem cells were collected at birth for stem cell transplantation

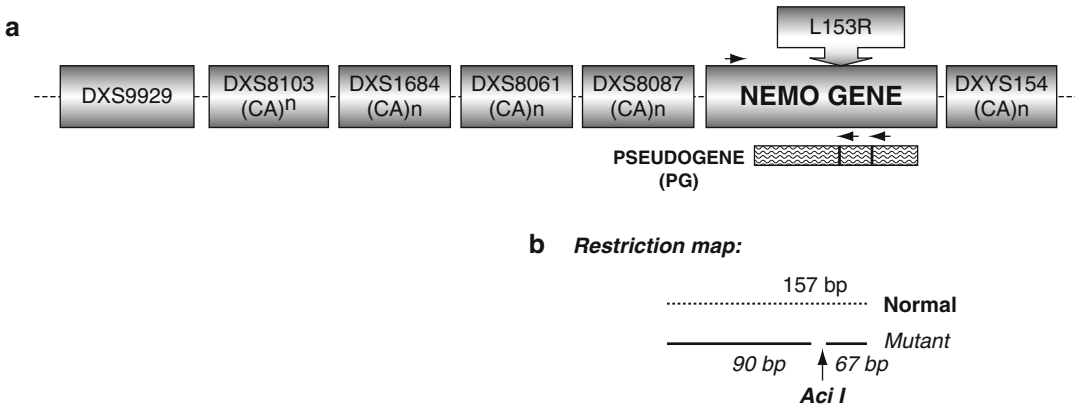



**Fig. 4.10** PGD design for the mutation in NEMO gene in combination with HLA typing (see also Fig. 4.11). (**a**) Position of the L153R mutation in exon 5 of CD40 ligand gene (Xq26.3) and tightly linked dinucleotide polymorphic markers inside the gene (exon 4). (**b**) Restriction maps for *Aci*I restriction enzyme

marrow transplantation. WAS is caused by a mutation in the WAS gene mapped to the Xp11.22–11.23 region, which results in actin polymerization, with T lymphocytes of males exhibiting a severe disturbance of the actin cytoskeleton. The gene has 12 exons that encode a 502 amino acid cytosolic protein, expressed exclusively in hematopoietic cells.

PGD was performed for two couples at risk for producing a progeny with WAS. One of them had two affected sons carrying the missence Leu39Pro mutation in exon 1 of WAS gene, due to a single nucleotide (CTT to CCT) substitution at position 150, which leads to substitution of leucine by proline at position 39. The mutation testing was done using Scr FI restriction digestion, cutting the mutant and leaving the normal gene product intact. A total of four unaffected HLA-matched embryos were detected, resulting in one singleton pregnancy and the birth of an unaffected HLA-matched child, as a potential donor for bone marrow transplantation of her affected sibling (Table 4.1).

*X-linked hypohidrotic ectodermal displasia with immune deficiency* (*HED-ID*) is a congenital disorder of the teeth, hair, and eccrine sweat glands, inherited as an X-linked recessive condition, caused by approximately two dozens of different mutations in the IKK-gamma gene (IKBKG, or NEMO) located in Xq28. The gene consists of ten exons and codes for a scaffold protein that binds IKK-alpha and IKK-beta, being essential for forming a functional IKK complex. The disease is characterized by susceptibility to microbial and streptococcal infections, dys-gamma-globulinemia, poor polysaccharide-specific antibody responses, and depressed antigen-specific lymphocyte proliferation. Intravenous immunoglobulins and prophylactic antibiotics may be useful in improving clinical status, but bone marrow transplantation is required to prevent early mortality.

One of the PGD cycles for HED-ID with HLA typing is presented in Figs. 4.10 and 4.11. The mother was a carrier of a L153R mutation, resulting from T to G change (CTG->CGG) in exon 4 of the NEMO (IKBKG) gene, replacing leucine with arginine at position 153 of the resultant protein. Because of the presence of a closely linked pseudogene with a normal sequence at the position of the mutation, which is co-amplified with the transcribed gene, a special design was developed to avoid misdiagnosis (Fig. 4.10).

A total of 16 embryos were analyzed, of which 6 were derived from oocytes free of maternal mutation, based on PB1 and PB2 testing, but none of these was a maternal HLA match. As seen from Fig. 4.11, of 16 resulting embryos, for which blastomere biopsy results were available both for mutation analysis and HLA typing, 3 were affected males (embryos #17, #20, and #21; only the latter being HLA-matched), 4 female carriers, 2 of which were non-matched (embryos #3 and #4), one HLA-recombinant (embryo #13), and one HLA-matched (embryo #12). The remaining 7 embryos were unaffected, including 2 male non-matched embryos (embryos #16 and #24), the former containing extra maternal X-chromosome, and 5

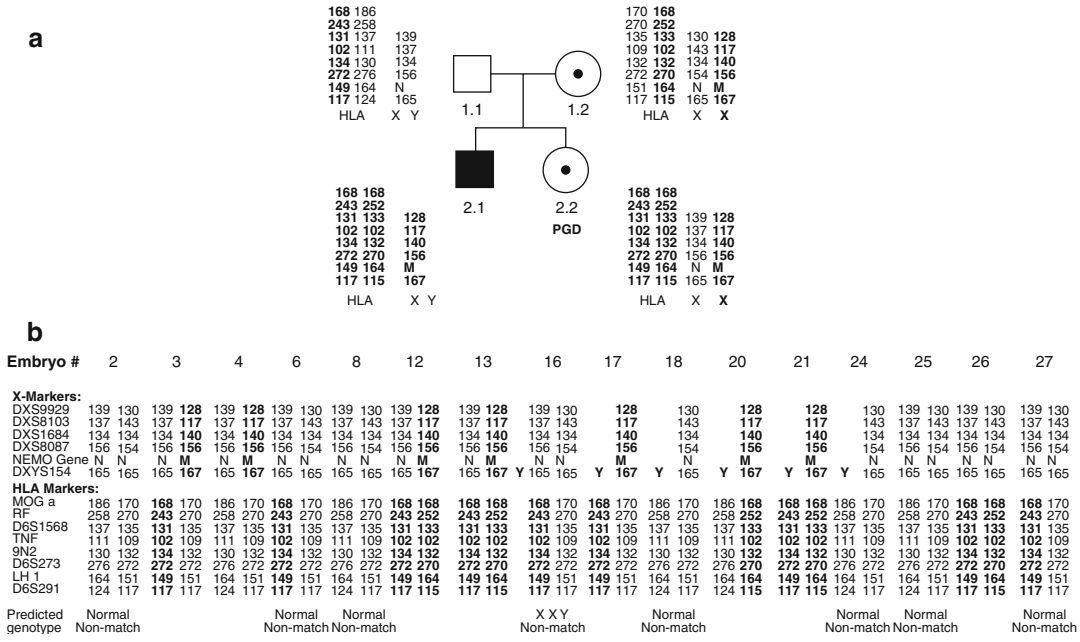



**Fig. 4.11** PGD for the mutation in NEMO gene with preimplantation HLA typing. (**a**) Family pedigree showing maternal and paternal matching HLA haplotypes in *bold face*. Marker order for testing NEMO gene is located next to the maternal haplotypes. Paternal (1.1), maternal (1.2), and affected sibling (2.1) NEMO gene haplotype assignment is based on genomic DNA testing. (**b**) (*Upper panel*) Results of blastomere DNA analysis from 16 embryos showing that 3 embryos were affected (embryos #17, #20, and #21), one with an extra X chromosome, suggesting the XXY genotype, with the remaining being either carriers or unaffected. (**b**) (*Lower panel*) HLA typing was performed simultaneously with mutation analysis of all blastomeres, showing that 2 of the unaffected embryos (#12 and #26) were also HLA-matched to the affected sibling. The transfer of this embryo resulted in pregnancy and the birth of a healthy unaffected HLA-matched baby girl (2.2). Cord blood stem cells were collected at birth for stem cell transplantation

normal female embryos, of which only 1 (embryo #26) was HLA-matched. While the normal embryos, which were not HLA-matched to the affected sibling, were frozen for future use by the couple, embryo #26, together with embryo #12, which was a normal female carrier, was transferred, resulting in a singleton pregnancy and the birth of an unaffected child that was confirmed to be HLA-matched to the affected sibling. Cord blood from this child was collected and transplanted to the affected sibling, resulting in a complete cure.

The presented data show the usefulness of PGD for SCID, as there is no effective treatment for these conditions other than stem cell transplantation. PGD provides the couples at risk with the option to avoid the affected pregnancy and have a progeny free of SCID. If there is already an affected child in the family, PGD with HLA typing makes it also possible to have access to the HLA-identical stem cell transplantation through selection and transfer of those unaffected embryos which are also HLA-matched to the sibling. Because the finding of the HLA-identical stem cell donor is the key for achieving the success in stem cell transplantation [33], a complete cure was observed in both cases of stem cell transplantation in siblings with HIGM and HED-ID.

## 4.4 Preimplantation HLA Matching Without PGD

The first report of experience of preimplantation HLA matching without testing for a causative gene included 13 IVF cycles initiated for 9 couples wishing to have another child who may also be a potential cord blood donor for the affected siblings with leukemia or Diamond–Blackfan anemia (DBA), the conditions requiring bone

**Table 4.5** Preimplantation HLA typing with and without PGD

| Preimplantation testing | Patients | Cycles | No. of embryo transfers | No. of embryos transferred | Pregnancy/ birth |
|---|---|---|---|---|---|
| HLA testing only | 46 | 98 | 65 | 99 | 24/19 |
| HLA + mutation | 85 | 237 | 139 | 219 | 38/32 |
| Total | 131 | 335 | 204 | 318 | 62/51 |

marrow transplantation or cord blood transplantation treatment [34]. Although the latter condition was sporadic and did not require mutation testing, with a sole indication being of HLA typing, mutation analysis may be also required for patients at risk of producing offspring with DBA, caused by mutations in the gene encoding ribosomal protein S19 on chromosome 19 (19q13.2), and other genes mapped to chromosome 8 (8p23.3-p22). However, the majority of DBA are sporadic with no mutation detected, such as in both cases performed in our experience [34].

There was no difference in performing preimplantation HLA testing without PGD, except in limiting the analysis of day 3 or day 5 embryo to only HLA typing with the sibling requiring stem cell transplantation, using a multiplex heminested PCR system (see Chap. 2). A haplotype analysis for the father, mother, and affected child was performed for each family prior to preimplantation HLA typing, using a set of polymorphic STR markers located throughout the HLA region, as shown in Fig. 4.1. This allowed detecting and avoiding misdiagnosis due to preferential amplification and ADO, potential recombination within the HLA region, and a possible aneuploidy or uniparental disomy of chromosome 6, which may also affect the diagnostic accuracy of HLA typing of the embryo.

At the present time, a total of 98 clinical cycles from 46 couples were performed, in which 99 HLA-matched embryos were preselected for transfer (Table 4.5). The proportion of embryos predicted to be HLA-matched to the affected siblings was 21.5%, not significantly different from the expected 25% (Table 4.6). The transfer of 99 HLA-matched embryos in 65 clinical cycles resulted in 24 singleton clinical pregnancies and 19 HLA-matched children born. These results suggest that testing of an available number of embryos per cycle allows preselecting a sufficient number of the HLA-matched embryos for transfer to achieve a clinical pregnancy and birth of an HLA-matched progeny.

**Table 4.6** Chances for detection of disease-free and HLA-matched embryo in preimplantation HLA typing

| |
|---|
| HLA MATCH only – ¼ (25%) |
| Autosomal-recessive or X-linked free + HLA MATCH – ¾ × ¼ = 3/16 (18.75%) |
| Autosomal-dominant free + HLA MATCH – ½ × ¼ = 1/8 (12.5%) |
| Autosomal-recessive or X-linked free + HLA MATCH + ANEUPLOIDY-free – ¾ × ¼ × ½ = 3/32 (9.4%) |
| Autosomal-dominant free + HLA MATCH + ANEUPLOIDY-free – ½ × ¼ × ½ = 1/16 (6.25%) |

The usefulness of detection of recombination within the HLA region is demonstrated in Table 4.7, describing the results of HLA typing of one of the cycles resulting in the birth of an HLA-matched child to a sibling with acute lymphoid leukemia (ALL). Of 10 embryos tested simultaneously for 11 alleles within the HLA region in this family, crossing over between D6S2426 and Ring alleles was observed in embryos #4, #7, and #9. Of the remaining 7 embryos, 3 were fully matched (embryos #2, #6, and #8), while the other 4 were HLA-incompatible to the affected sibling, as seen from the haplotypes of the mother, father, and affected child, presented in Table 4.7.

Recombinations were detected also in both cases of preimplantation HLA typing for DBA presented in Table 4.8 and Fig. 4.12. In one of them, maternal recombination is seen in the embryo #8 results (Table 4.8), while HLA testing of the other six embryos revealed three HLA-matches to the affected sibling and were transferred, resulting in a singleton pregnancy and the

**Table 4.7** Preimplantation HLA typing resulting in the birth of an HLA-matched baby for the affected sibling with acute lymphoid leukemia

| HLA genes and STRs | Embryo no. | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | Father | Mother | Affected baby |
| D6S276* | *130/118* | **144/139** | *130/118* | *130/118* | *130*/**139** | **144/139** | *130/118* | **144/139** | *130*/**139** | *130*/**139** | **144**/*130* | *118*/**139** | **144/139** |
| HLA A | *3/2* | **1/32** | *3/2* | *3/2* | *3*/**32** | **1/32** | *FA* | **1/32** | *3*/**32** | *3*/**32** | **1**/*3* | *2*/**32** | **1/32** |
| D6S510 | *16 167* | **148/163** | *163/167* | *163/167* | *163*/**163** | **148/163** | **148**/*167* | **148/163** | *163*/**163** | *163*/**163** | **148**/*163* | *167*/**163** | **148/163** |
| HLA B | *AD 27* | **27/18** | *ADO/27* | *FA* | **FA** | **27/18** | *ADO/27* | **27/18** | *ADO*/**18** | *ADO*/**18** | **27**/*57* | *27*/**18** | **27/18** |
| MIB | *118/114* | **114/124** | *118/114* | *118/114* | *118*/**124** | **114/124** | *118/114* | **114/124** | *118*/**124** | *118*/**124** | **114**/*118* | *114*/**124** | **114/124** |
| TNF a | *94/102* | **110/102** | *94/102* | *94/102* | *94*/**102** | **110/102** | *94/102* | **110/102** | *94*/**102** | *94*/**102** | **110**/*94* | *102*/**102** | **110/102** |
| D6S273* | *275/273* | **273/271** | *275/273* | *275/273* | *275*/**271** | **273/271** | *275/273* | **273/271** | *275*/**271** | *275*/**271** | **273**/*275* | *273*/**271** | **273/271** |
| HLA-DRB1 | *ADO/1* | **11/10** | *ADO/1* | *ADO/1* | *ADO*/**10** | **11/10** | *ADO/1* | **11/10** | *ADO*/**10** | *FA* | **11**/*7* | *1*/**10** | **11/10** |
| G51152 | *181/208* | **181/191** | *181/208* | *181/208* | *181*/**191** | **181/191** | *181/208* | **181/191** | *181*/**191** | *181*/**191** | **181**/*181* | *208*/**191** | **181/191** |
| Ring 3CA | *162/162* | **160/155** | *162/162* | *162/162* | *162*/**155** | **160/155** | *162/162* | **160/155** | *162*/**155** | *162*/**155** | **160**/*162* | *162*/**155** | **160/155** |
| D6S426 | *144/130* | **140/144** | *144/130* | **140**/*130* | *144*/**144** | **140/144** | *144/130* | **140/144** | *144/130* | *144*/**144** | **140**/*144* | *130*/**144** | **140/144** |
| Predicted genotype | *Non-match* | **Match** | *Non-match* | *Non-match recombinant* | *Non-match* | **Match** | *Non-match recombinant* | **Match** | *Non-match recombinant* | *Non-match* | **NA** | **NA** | **NA** |

*ADO* allele dropout, *FA* failed amplification. *HLA* matched alleles are shown in **bold**

birth of an HLA-matched baby. In the other case, one embryo with a maternal (embryo #8) and another (embryo #16) with both maternal and paternal crossing over (both in Ring allele) were detected in testing of 16 embryos (only 8 embryos shown). There was another embryo with trisomy 6 (embryo #5) with an extra maternal chromosome 6, making this and the other two above also unacceptable for transfer. However, five embryos appeared to be HLA-matched, of which two were transferred back to the patient, resulting in the birth of an HLA-matched baby (Fig. 4.12). The cord blood collected from this baby was transplanted to the affected sibling, resulting in a complete cure.

The relevance of aneuploidy testing for chromosome 6 for accuracy of diagnosis is seen from the results of HLA typing in the other cycle, resulting in the birth of a baby who was HLA-matched to the sibling with ALL (Fig. 4.13). Two of ten embryos tested in one case (of which only eight embryos are shown in Fig. 4.13) appeared to have only maternally derived chromosomes 6, one with only one maternal chromosome (embryo #1), and the other with two maternal chromosomes, representing uniparental maternal disomy of chromosome 6 (embryo #2). In addition, crossing over between D6S291 and class II HLA alleles was evident, making one embryo unacceptable for transfer. Of the remaining embryos, only two were HLA-matched to the affected sibling, which were transferred resulting in the birth of an HLA-matched baby.

Presented data show feasibility of preimplantation HLA matching for families with affected children with bone marrow disorders who may wish to have another child as a potential HLA-matched donor of stem cells for transplantation treatment of the affected sibling. As seen from our data, HLA-matched embryos were preselected and transferred in all cycles, resulting in clinical pregnancies and the birth of HLA-matched children in almost every second transferred cycle.

The results also demonstrate the prospects for the application of this approach to other conditions, requiring an HLA-compatible donor for stem cell transplantation. This provides a realistic option for those couples who would like to have another child anyway, as they may potentially provide an HLA-matched progeny for an affected sibling. In addition to leukemias and sporadic forms of DBA, the method may be applied for any other condition, such as for couples having affected children with different cancers awaiting an HLA-compatible donor with no success for years. These new indications make preimplantation testing a genuine alternative to conventional prenatal diagnosis, providing patients with important prospects not only to avoid an inherited risk without facing termination of pregnancy, but also to establish a pregnancy with particular genetic parameters to benefit the affected member of the family.

## 4.5 Limitations and Future Prospects of PGD for HLA Typing

Presented data demonstrate that PGD for HLA typing may become a practical option, available for a wider application in order to further improve the radical treatment for congenital and acquired bone marrow failures by stem cell transplantation. Despite the high rate of preferential amplification and ADO in PCR analysis of single blastomeres, a potential recombination within the HLA region described in our material, and a high rate of mosaicism for aneuploidies at the cleavage stage (see Sect. 5), the approaches described above appear to be highly accurate in preselecting HLA-matched embryos for transfer. The approaches involve a multiplex PCR analysis involving simultaneous testing for HLA alleles together with STR markers within HLA and flanking regions, allowing avoidance of misdiagnosis due to ADO, aneuploidy, or recombination of HLA alleles, which cannot be detected by other currently used DNA methods of HLA typing.

One of the major limitations of PGD for HLA typing is a relatively high frequency of recombination in the HLA region, with a few possible hot spots, which may affect not only the accuracy of preimplantation HLA typing, but also the outcome of the procedure. In our experience, of

**Table 4.8** Preimplantation HLA typing resulting in the birth of an HLA-matched baby for the affected sibling with Diamond-Blackfan anemia

| HLA genes and STRs | Embryo no. | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 2 | 3 | 7 | 8 | 9 | 10 | 11 | Father | Mother | Affected child |
| D6S461* | **225/227** | **225/227** | *229*/**227** | *229*/**227** | **225/227** | *229/229* | *229/229* | **225**/*229* | *229*/**227** | **225/227** |
| D6S276* | **143/143** | **143/143** | *141*/**143** | *141*/**ADO** | **143/143** | *141/117* | *141/117* | **143**/*141* | *117*/**143** | **143/143** |
| D6S258 | **144/132** | **144/132** | *135*/**132** | *135*/**132** | **144/132** | *135/135* | *135/135* | **144**/*135* | 135/**132** | **144/132** |
| D6S248* | **253/278** | **253/278** | *276*/**278** | *276*/**278** | **253/278** | *276/278* | *276/278* | **253**/*276* | *278*/**278** | **253/278** |
| MOG a | **159/169** | **159/169** | *167*/**169** | *167*/**169** | **159/169** | *167/169* | *167/169* | **159**/*167* | *169*/**169** | **159/169** |
| RF | **275/269** | **275/269** | *257*/**269** | *257*/**269** | **275/269** | *257/263* | *257/263* | **275**/*257* | *263*/**269** | **275/269** |
| 9N-2 | **129/133** | **129/133** | *131*/**133** | *131/127*/**133** | **129/133** | *131/127* | *131/127* | **129**/131 | *127*/**133** | **129/133** |
| D6S273 | **276/270** | **276/270** | *274*/**270** | *274*/**270** | **276/270** | *274/270* | *274/270* | **276**/*274* | *270*/**270** | **276/270** |
| LH 1 | **163/168** | **163/168** | *163*/**168** | *163*/**168** | **163/168** | *163/179* | *163/179* | **163**/*163* | *179*/**168** | **163/168** |
| D6S2447 | **147/159** | **147/159** | *152*/**159** | *152*/**159** | **147/159** | *151/151* | *151/151* | **147**/*151* | *151*/**159** | **147/159** |
| TAP 1 | **205/220** | **205/220** | *205*/**220** | *205*/**220** | **205/220** | *205/207* | *205/207* | **205**/*205* | *207*/**220** | **205/220** |
| Ring 3CA | **159/155** | **159/155** | *155*/**155** | *155*/**155** | **159/155** | *155/159* | *155/159* | **159**/*155* | *159*/**155** | **159/155** |
| D6S439 | **125/125** | **125/125** | *123*/**125** | *123*/**125** | **125/125** | *123/125* | *123/125* | **125**/*123* | *125*/**125** | **125/125** |
| D6S291 | **114/123** | **114/123** | *116*/**123** | *116/114* | **114/123** | *116/114* | *116/114* | **114**/*116* | *114*/**123** | **114/123** |
| D6S426 | **129/129** | **129/129** | *144*/**129** | *FA* | **129/129** | *144/142* | *144/142* | **129**/*144* | *142*/**129** | **129/129** |
| Predicted genotype | **Match** | **Match** | *Non-match* | *Non-match recombinant*[c] | **Match** | *Non-match* | *Non-match* | **NA** | **NA** | **NA** |

*HLA* matched alleles are shown in bold

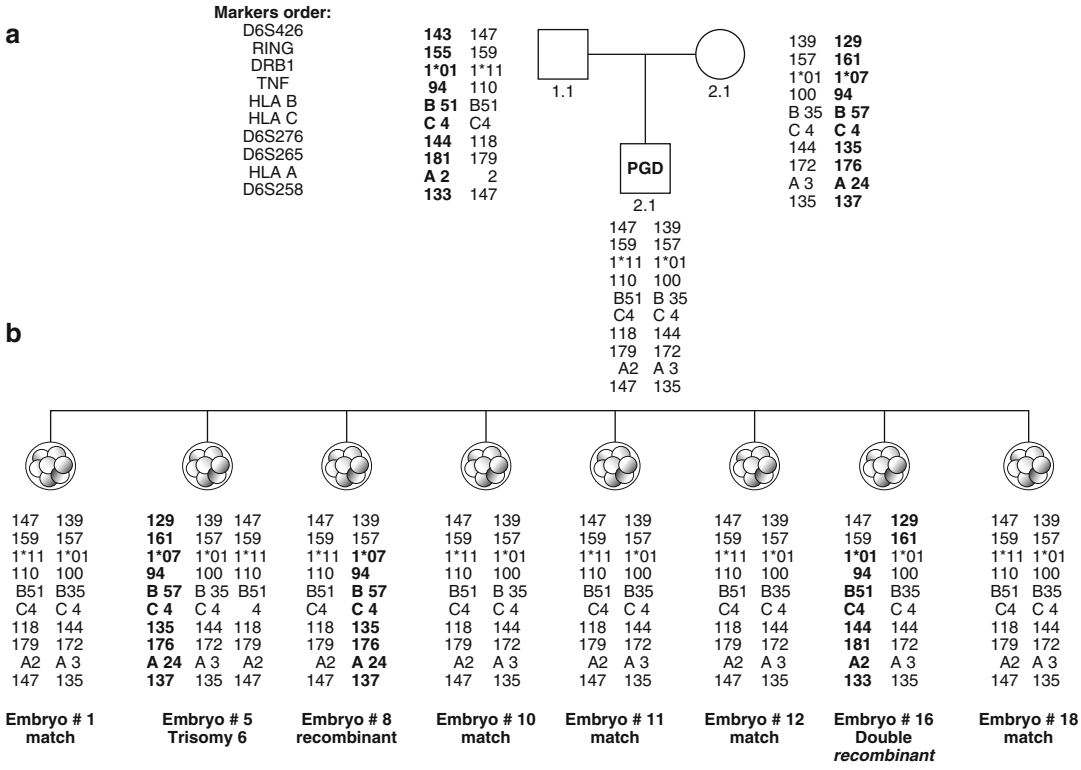



**Fig. 4.12** Preimplantation HLA typing for Diamond–Blackfan anemia, resulting in the birth of an HLA-matched child. (**a**) Family pedigree with marker order and haplotypes of the mother, father, and affected child. HLA matching haplotypes are shown in non-bold face. (**b**) Results of HLA typing of biopsied blastomeres from eight embryos (other eight embryos which were also tested are not shown). Embryos #1, #10, #11, #12, and #18 are HLA-matched to the affected sibling (see **a** *panel*). Embryo #8 is a maternal non-match due to maternal recombination in the Ring allele, as well as embryo #16, which is both a paternal and maternal non-match, due to double recombination in the paternal and maternal Ring alleles. Embryo #5 is also a non-match due to an extra maternal chromosome, suggesting trisomy 6

1,713 embryos tested for HLA, 1,634 (95.5%) were non-recombinant, 52 (3%) with maternal, and 27 (1.5%) with paternal recombination. The prevalence was even higher based on family evaluation, performed prior to PGD in a series of 114 families: in 104 of these families tested, recombination in siblings requiring HLA-compatible bone marrow transplantation was identified in 7 (6.1%), suggesting that preimplantation HLA typing may never be able to identify the HLA match for these siblings. Therefore, haplotype analysis prior to initiation of the actual cycle is strongly required, so that the couples may be informed about their possible options. For example, in one of our cases performed for thalassemia, the fact that the child was recombinant became obvious only during PB1 analysis, without which maternal haplotypes cannot be established. While paternal haplotypes may be identified through sperm typing, the testing for maternal haplotypes requires maternal somatic cell haploidization, which may be performed by somatic cell nuclei transfer and fusion with matured oocytes, as described in Chap. 2 [35]. As shown in Fig. 4.14, the preparatory testing identified the sibling with maternal recombination, so it could have been unrealistic to identify the exact match, and therefore the couples should be informed that only relatively close matches may be possible, which may be discussed with the pediatric hematologist in the preselection process of the embryos for transfer.

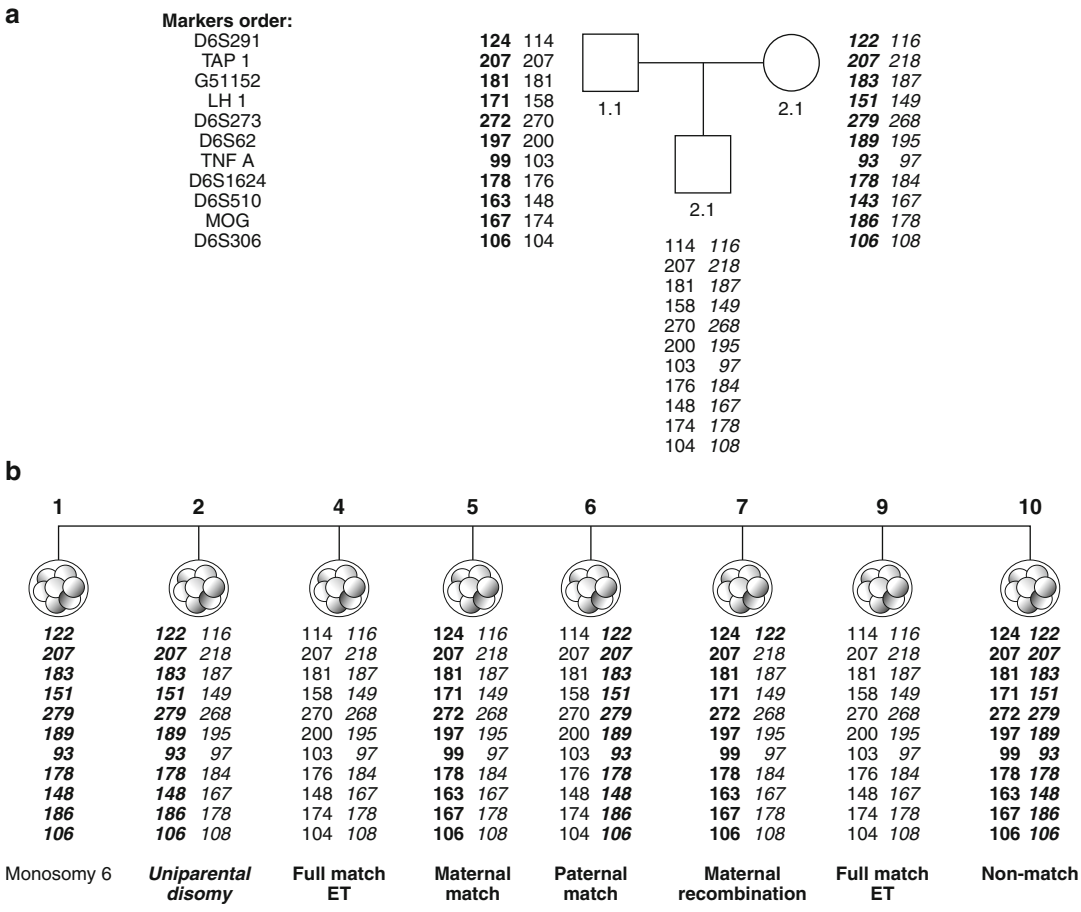



**Fig. 4.13** Preimplantation HLA typing for acute lymphoid leukemia (*ALL*), resulting in the birth of an HLA baby. (**a**) Family pedigree with marker order and haplotypes of the mother, father, and affected child. Matching maternal and paternal haplotypes are shown in *non-bold face*. (**b**) Results of HLA typing of biopsied blastomeres from eight embryos. Embryos #4 and #9 are HLA-matched to the affected sibling (see **a** *panel*). Embryo #1 is a maternal non-match with no paternal chromosome present (monosomy 6). Embryo #2 is also a non-match due to only maternal chromosomes present (uniparental disomy). Embryo #7 is both a paternal and maternal non-match, the latter being due to maternal recombination in the Ring allele. The other two embryos are a non-match, embryo #6 being a paternal non-match and embryo #10 both a maternal and paternal non-match

Despite the need for further improvement of the technique as mentioned, the presented results show that the couples with affected children requiring HLA-compatible stem cell transplantation have a realistic option to undergo IVF and PGD with a combined preimplantation HLA typing, so as to have an unaffected HLA-matched child as a potential donor of compatible stem cells for the sibling.

The other important limitation is that most patients requesting preimplantation HLA typing are of advanced reproductive age, so the outcome of the procedure has not yet been sufficiently high, and many patients still undergo two or more attempts before they become pregnant and deliver an HLA-identical offspring. So testing for age-related aneuploidy may appear useful for improving the reproductive outcome of preimplantation HLA typing, which will also minimize the risk of delivering a child with chromosomal disorders, providing reassurance for patients who are usually concerned about their pregnancy outcomes.

Aneuploidy testing is currently offered as an integral component of preimplantation HLA typing to the patients of advanced reproductive age,

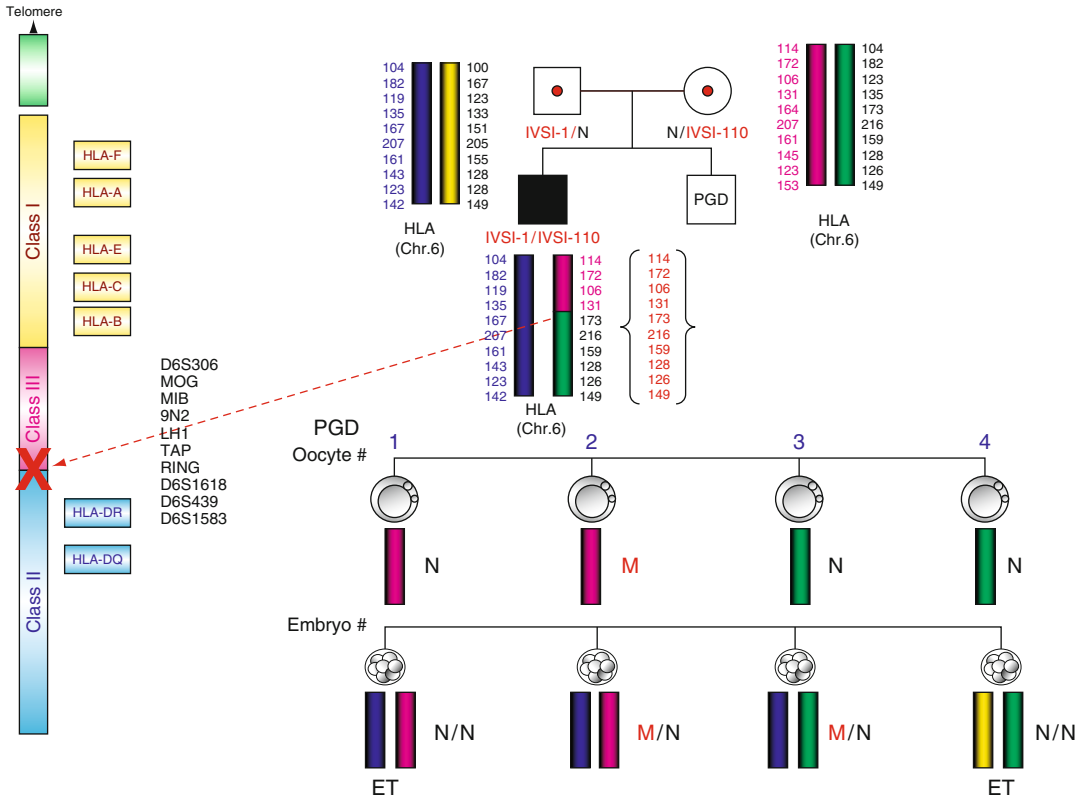



**Fig. 4.14** Maternal recombination detected in thalassemia major sibling in preimplantation HLA typing combined with PGD. (*Top panel*) Family pedigree with HLA haplotype analysis based on parental and affected child's genomic DNA testing. HLA marker order is presented on the *upper left* for the father, who was a heterozygous carrier of thalassemia gene IVS 1–1, and on the *upper right* for the mother, a heterozygous carrier of thalassemia mutation IVS1–110. Paternal and maternal HLA haplotypes are shown in different colors: paternal in *blue/yellow*, and maternal in red/green. As seen from the HLA haplotypes of the affected child in need for transplantation, for whom HLA matching is performed, the maternal HLA contribution is recombinant (*red and green* instead of the expected *red or green*) between HLA-DR and HLA-B genes, shown schematically on chromosome 6 (on the *far left*). (*Middle panel*) Sequential multiplex polar body analysis for maternal mutation, linked polymorphic markers, and HLA haplotypes, showing that the oocyte #2 is affected (IVS1–110), while oocytes #1, #3, and #4 are normal, with no recombination in the HLA cluster. (*Bottom panel*) Blastomere results revealed two heterozygous carrier embryos (embryos #2 carries maternal mutation IVS1–110 and embryo #3 carries paternal mutation IVS1–1) and two homozygous normal ones (#1 and #4). HLA typing (presented by respective colors) shows that neither of these embryos is fully HLA-matched to the sick sibling. Two embryos, #1 and #4, predicted to be homozygous normal, and partially HLA-matched, were transferred back to the mother, yielding a singleton pregnancy and the birth of a thalassemia-free baby, who may still be considered for possible bone marrow transplantation, as there is no probability of producing a completely HLA-matched offspring for the affected sibling with recombinant HLA haplotypes

performed in an increasing number of preimplantation HLA typing cycles combined with or without PGD. Although the chances of preselecting unaffected HLA-matched embryos that could be also euploid are very low (see Table 4.6), our preliminary results of the reproductive outcome comparison between the groups of combined PGD/HLA with and without aneuploidy testing showed a significant difference (Table 4.9). Despite transferring a lower number of embryos, the pregnancy rate was higher in the aneuploidy testing group, suggesting the potential utility of aneuploidy testing in preimplantation HLA typing, allowing the avoidance of transfer of those HLA-identical embryos that are chromosomally abnormal which are destined to be lost anyway

**Table 4.9** Outcome of preimplantation HLA typing with and without aneuploidy testing

| | HLA | HLA plus Aneuploidy testing | Total |
|---|---|---|---|
| Patient/cycle | 11/25 | 14/27 | 25/52 |
| Total embryos | 224 | 204 | 428 |
| Matched embryos | 48 | 21 (36) | 69 (84) |
| Non-matchedembryos | 176 | 168 | 344 |
| Transfers | 21 (84%) | 13 (48%) | 34 |
| No. of embryos transferred | 33 (1.6) | 19 (1.4) | 52 |
| Pregnancy | 6 (28.5%) | 7 (53.8%) | 13 (38%) |
| Birth | 6 | 6 | 12 |

either before or after implantation. Alternatively, incidental transfer of aneuploid embryos in the absence of chromosomal testing should lead to implantation and pregnancy failures in preimplantation HLA typing cycles, or may compromise the pregnancy outcome through spontaneous abortions.

Although more data are still needed to further prove the impact of aneuploidy testing on the outcomes of preimplantation HLA typing, the presented data suggest that approximately half of the aneuploidy-free embryo transfers, following preimplantation HLA matching, resulted in pregnancy and the birth of HLA-matched children, compared to the 28.5% pregnancy rate following the transfer of HLA-matched embryos not tested for aneuploidy. In a comparable number of cycles performed with or without aneuploidy testing, despite unavailability of aneuploidy-free embryos for transfer in over half of the cycles, compared to only 26% of cycles without aneuploidy testing, comparable numbers of pregnancies and births of HLA-matched children were observed, indicating a possible clinical relevance of avoiding chromosomally abnormal embryos from transfer in preimplantation HLA typing.

As mentioned, the addition of aneuploidy testing expected to identify at least 50% of chromosomally abnormal embryos in patients of advanced reproductive age, will be also lowering the probability of detecting the embryos for transfer by half. In fact, the mean number of embryos for transfer was approximately 1.0 on the average, which also reflects the lower probability of identification of HLA-matched unaffected embryos free of aneuploidy, taking into consideration the average number of available embryos with results, which is usually much lower in women of advanced reproductive age (under ten embryos on the average in our experience). With one in two embryos expected to be aneuploid, one in four HLA-matched, and three in four unaffected in autosomal-recessive conditions, the overall probability of finding a suitable embryo for transfer could not be expected to be higher than one in ten embryos (see also Table 4.6). So with the availability of only under ten embryos on the average with conclusive results in our material, only one HLA-matched unaffected euploid embryo may have been expected to be available for transfer, assuming also that not all embryos develop to the status acceptable for transfer, which is of course below the optimal number of embryos to be replaced to ensure a clinical pregnancy and birth outcome. However, with present tendency of limiting the transfer to only one blastocyst, to avoid multiple pregnancies, the availability of a single euploid embryo for transfer is quite sufficient to obtain a clinical pregnancy and birth of an HLA-identical progeny for stem cell transplantation for the affected siblings.

The usefulness of aneuploidy testing is also obvious for the diagnostic accuracy improvement, as the error in the copy number of chromosomes may lead to misdiagnosis in testing for the causative gene and HLA typing. For example, our data further confirm an approximately 6% aneuploidy rate for chromosome 6 (see Chap. 5), which could affect the HLA typing results. Comparable aneuploidy rates for other chromosomes on which causative genes tested are located, such as beta-globin gene on chromosome 11, may also affect PGD results [25]. Thus, in addition to avoiding chromosomally abnormal embryos from transfer, testing for the copy number of chromosomes may become an important

requirement for achieving the accuracy of PGD and preimplantation HLA typing. In fact, the follow-up analysis of the mutant oocytes and embryos and the pregnancy outcomes in our experience did not find any misdiagnosis, suggesting an extremely high specificity and sensitivity in the presently used molecular genetic analysis.

Therefore, patients should be fully aware of the limits of the expected successful outcome of the above testing, which was shown to result in preselection and transfer of the HLA-matched unaffected embryos in only 13.7% of the embryos tested, which is even a bit lower than may have been predicted. Despite such a relatively moderate success rate, the number of PGD requests in combination with HLA typing has been increasing overall, with the recent emergence of a considerable proportion of cases involving preimplantation HLA typing without PGD.

## 4.6 Practical Implications of PGD for HLA Typing

Preimplantation HLA typing opens an important possibility of PGD application for stem cell therapy. Because of limited availability of the HLA-matched donors even among family members, this approach appeared to be attractive for couples with children requiring HLA-matched bone marrow transplantation.

It is well known that to achieve an acceptable engraftment and survival in stem cell therapy requires the finding of an HLA-identical stem cell transplant. However, there remain a large number of patients for whom no HLA-matched family member exists, so the search is extended to haplotype-matched unrelated donors. This has allowed successful application of stem cell transplantation to some individuals without a matched related donor, despite resulting in severe complications in more than half of the patients [36].

The experience of bone marrow transplantation for hemoglobinopathies presently comprises thousands of patients, showing 68% probability of cure in the world's largest center [18, 33]. The success rate is reported as 87%, 85%, and 80% for Class 1 (patients with regular iron chelation therapy who have neither hepatomegaly nor liver fibrosis), Class 2 (with regular/irregular chelation, borderline hepatomegaly, and fibrosis), and Class 3 (with irregular chelation, hepatomegaly and fibrosis) patients under age 17, respectively, suggesting that this may have wider implication for congenital bone marrow failures, depending primarily on the availability of HLA-matched donors.

Due to the small number of children per family, only one-third of patients are able to find an HLA-identical sibling, which may further be improved by 3% using an extended family search for a matched related donor with one or two identical ancestral haplotypes [37]. In the remaining patients, the only resort is the identification of a matched unrelated donor, which might be maximized by establishing national registries. These registries allow overcoming to some extent the genetic heterogeneity in the populations, which may affect the frequency of unique haplotypes, thus improving the donor selection process.

Stem cell transplantation obtained from umbilical cord blood provides the potential for further expanding the donor pool to patients without a suitable family match [38], which have presently been performed for thousands of children and adults, allowing for a greater degree of HLA disparity in choosing donor cord blood units, thus increasing the likelihood that a suitable unit can be identified for any particular patient. However, the advantages of this alternative source of stem cells can be more fully realized in the setting of the availability of matched related donor cord blood units, such as from siblings, providing the advantages of earlier transplantation, lower risk of complications, and lower treatment-related mortality.

HLA-identical sibling donors of cord blood stem cells are the only solution for patients affected with FA, allowing successful treatment in up to 85% of cases. So, over two decades, these families were offered the option of prenatal diagnosis combined with HLA typing on cells derived from chorionic villus sampling or amniocentesis

[39]. Of more than 80 pregnancies conceived during this time, one resulted in the first successful use of cord blood transplant in 1988 [3], opening the era of an alternative to bone marrow transplantation. However, because the probability of having an unaffected child who may be also an HLA match for an affected sibling is only one in five, these families often went through multiple cycles of pregnancy before conceiving an unaffected HLA match. So PGD provides a much more attractive approach, because a sufficient number of embryos may be tested at a time, increasing the chances to identify an appropriate match. In addition, PGD allows identification of the match before pregnancy, obviating the risk for termination of pregnancy for the HLA type alone, which cannot be acceptable on ethical grounds.

Presented data show that couples undergoing preimplantation HLA typing may be expected to require a repeated cycle to be able to preselect and transfer HLA-matched embryos. Even with the probability of selecting only one HLA-matched embryo from five tested, an acceptable pregnancy rate was observed, despite transferring only one or two embryos on the average, suggesting the usefulness of preimplantation HLA matching as part of PGD. The data provide a realistic option for the couples desiring to establish a pregnancy potentially providing an HLA match progeny for the treatment of the affected family member(s). However, preimplantation HLA typing raises important ethical, legal, and social issues, which are discussed in detail in Chap. 8.

Despite ethical issues involved in preimplantation HLA typing, there is an increase in the attractiveness of this option for couples with affected children requiring HLA-compatible stem cell transplantation, providing a practical option for those couples who would like to have another child anyway. This and other new indications above make preimplantation testing a genuine alternative to conventional prenatal diagnosis, providing patients with important prospect not only to avoid an inherited risk without facing termination of pregnancy, but also to establish a pregnancy with particular genetic parameters, which may also benefit the affected member of the family.

The present experience of PGD for HLA typing includes over 1,000 cases, resulting in the birth of more than 200 HLA-matched children, whose HLA-identical stem cells have already been used for a successful transplantation therapy in up to 100 affected siblings [27, 29, 30, 40]. The world's largest two experiences include preimplantation HLA typing in over 700 cases, with the accuracy rate per transfer of 99.4% [27, 29, 30]. The majority of cases were performed in combination with PGD for various genetic disorders, including thalassemia, sickle cell disease, FA, WAS, X-ALD, HIGM1, HED-ID, Krabbe disease, inherited form of DBA, and X-Linked chronic granulomatous disease (CGD), involving the preselection of unaffected children who were also HLA-identical to the affected sibling. With the introduction of aneuploidy testing, this may also expand the practical application of preimplantation HLA typing to patients of advanced reproductive age, allowing an improvement of their chances to become pregnant and deliver an HLA-matched progeny for stem cell transplantation in the affected siblings. This also makes it possible to apply this approach to HLA-compatible stem cell transplantation for older affected siblings, which has already been performed in our experience for the 14-year-old sibling with thalassemia, resulting in 10% of donor cell engraftment with neither acute nor chronic GVHD (unpublished data).

In conclusion, despite ethical issues involved in preimplantation HLA typing [41–43], the presented results show the increasing attractiveness of this option for couples with affected children requiring HLA-compatible stem cell transplantation. It is also important that no embryo is discarded based on the results of preimplantation HLA typing, as all unaffected embryos are frozen for future use by the couple. So the couples at risk of having children with congenital bone marrow disorders have to be informed about presently available options not only of avoiding the birth of an affected child, but also of selecting a suitable stem cell donor for their affected siblings, which may presently be the only hope for treating siblings with congenital bone marrow failures.

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# Preimplantation Diagnosis for Chromosomal Disorders

5

It is well known that chromosomal abnormalities originate predominantly from female meiosis. As demonstrated by DNA polymorphism studies performed in families with aneuploid spontaneous abortions or liveborn babies with trisomy syndromes, these abnormalities derive mainly from meiosis I [1–3]. It was suggested that the age-related increase of common trisomies is probably determined by the age-related reduction of meiotic recombination, resulting in premature separation of bivalents and chromosomal nondisjunction. Meiosis II errors were also postulated to derive from meiosis I, as a result of the increased meiotic recombination rate, which may lead to a separation failure of bivalents [4].

With the advent of PGD for aneuploidies, it has become possible to directly test the outcome of the first and second meiotic divisions, using PB1 and PB2, as described in Chap. 2. PB1 is extruded following maturation of oocytes, representing a by-product of meiosis I, while PB2 is a by-product of meiosis II and is extruded following the exposure of oocytes to sperm or ICSI. As will be described below, the frequency and types of chromosomal errors detected by this approach are different from what was described in the traditional studies of meiotic chromosomes in metaphase II (MII) oocytes, according to which chromosomal anomalies in oocytes originate mainly from the errors of whole bivalents as a result of chromosomal nondisjunctions [5]. In contrast, direct testing of meiotic outcome using PB1 and PB2 analysis showed not only a higher prevalence of meiotic errors, but also significant contribution of chromatid, rather than chromosomal, errors. The discrepancy may be due to the poor quality of meiotic chromosome preparations in earlier studies, and also the lack of testing of the corresponding chromosome set extruded in PB1, without which the resulting oocyte karyotype could not be reliably evaluated, particularly in the cases of missing chromosomes or chromatids. This was demonstrated in the study of simultaneous testing of MII oocytes with their corresponding PB1, which showed that the normal chromosome pattern is represented by paired fluorescent signals for each chromosome, while the lack or addition of one or both signals in either oocyte or PB1 reflects an exactly opposite pattern in the corresponding MII oocytes or PB1, suggesting a high accuracy of PB1 testing for prediction of the oocyte genotype [6–10].

Based on the above data, PB1 testing was applied clinically for the preselection of aneuploidy-free oocytes, which has demonstrated the practical relevance of PB1 testing for IVF patients of advanced reproductive age [11–16]. The data also demonstrated that the genotype of the resulting zygotes could not be accurately predicted without information about the outcome of the second meiotic division, which may be inferred from PB2 testing. The present experience includes FISH analysis of over 20,000 oocytes presented below, demonstrating the accuracy of the evaluation of oocyte karyotype by testing PB1 and PB2, and also providing an attractive approach for the study of the origin of human aneuploidies [17]. This has recently been confirmed by the

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introduction of microarray technology for testing PB1 and PB2 in PGD for aneuploidy of all 24 chromosomes (see below).

Aneuploidy testing of PB1 and PB2 provides the least noninvasive approach to a possible preselection of oocytes and embryos with the highest developmental potential, currently based on morophological criteria, which is of an obvious interest for improving the efficiency of assisted reproduction technology (ART). However, morphological parameters have no sufficient value for excluding aneuploid embryos from transfer, which requires chromosomal studies of oocytes and embryos, still limited to only some of the IVF centers [18–22].

In addition to aneuploidy testing, different approaches have been tested for possible prediction of the developmental potential of oocytes, such as pronuclear morphology scoring and microtubule and microfilament organization assessment [23–25] and PB1 grading [26, 27]. As will be described below, approximately 42% of oocytes were shown to have PB1 aneuploidies, which also allows predicting, approximately a half of the second meiotic division errors detected by PB2 testing, thus, making it possible to exclude almost two-thirds of aneuploid oocytes from ICSI.

## 5.1 First Polar Body Morphological Grading as Possible Potential Means for Preselecting Viable Oocytes

Because chromosomal studies are still not readily available and require specialized expertise, the possibility of PB1 morphological grading, as a potential means for preselection of viable oocytes and embryos for transfer may seem to be an attractive approach, since it could be easily adopted in any IVF practice. Preliminary reports on the utility of PB1 morphology have been controversial, suggesting a positive correlation between well-shaped, round PB1 within a cohort and fertilization rates, embryo quality and implantation rates [26, 27], or no correlation with fertilization but a positive correlation between implantation rates and the presence of fragmented PB1 within a cohort [27]. Furthermore, these were retrospective studies in which chromosomal status of the oocytes and embryos was not investigated.

So a prospective study of PB1 morphology in relation to fertilization, chromosomal status, development potential of the resulting embryos, and outcome of embryo transfer was carried out to investigate if such testing could be used for predicting developmental potential and chromosomal status of oocytes and embryos without cytogenetic analysis.

Oocytes were obtained from 90 patients in 91 IVF cycles randomly for different indications, using a standard IVF protocol. PB1 morphology was assessed in 831 mature oocytes prior to ICSI (day 0) and at fertilization assessment (day 1), and placed into three major categories: Grade-1 representing a round or oval shape which may sometimes be flattened; grade-2 representing a nonfragmented PB1 of irregular shape; and grade-3 representing a partially or totally fragmented PB1 (Fig. 5.1). PB1 observation was performed using an inverted microscope (Diaphot, Nikon, Garden City, NY, USA) with Hoffman modulation contrast optics and a magnification of 200X. Prior to ICSI, oocytes were rotated so that both the side view and top view of PB1 was observed. Oocytes were then followed up after ICSI from fertilization throughout preimplantation development and embryo transfer. Data on PB1 grading for each oocyte was correlated to fertilization rate, day 3 embryo quality as to cell number and degree of fragmentation present, development capability to the blastocyst stage and embryo transfer outcome.

Due to the differences in patient response to hormonal stimulation, patients were divided into two groups, consisting of 42 poor responders (50 cycles; Group I) in which the number of retrieved oocytes did not exceed 10, and 48 good responders (50 cycles; Group II) with greater than 10 available oocytes.

Aneuploidy testing was requested by 49 patients in 50 cycles in which 395 oocytes and embryos were tested on day 1 (PB analysis), day

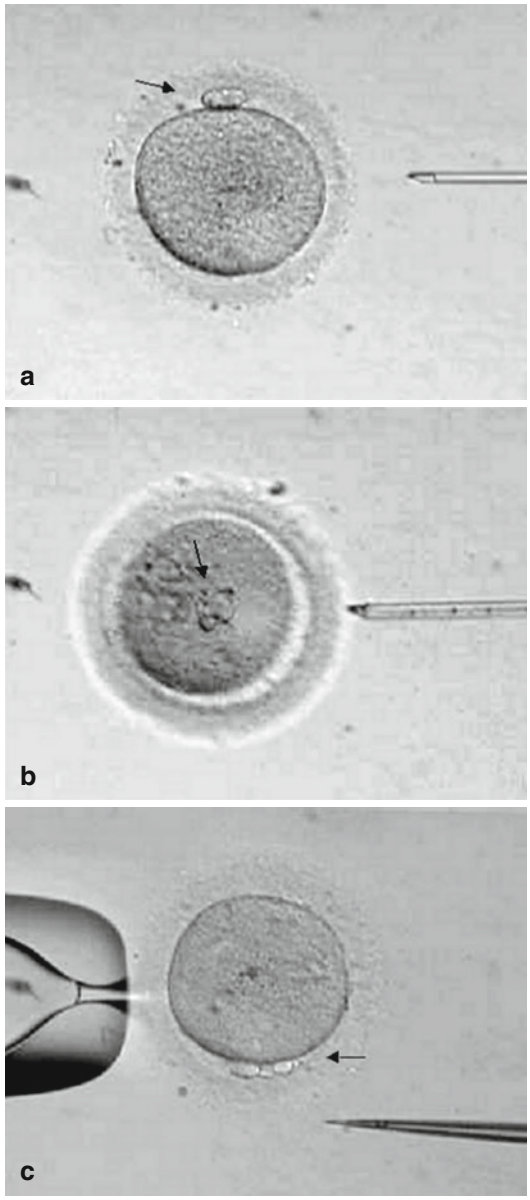



**Fig. 5.1** *Arrows* are significant as they show different first polar body grading based on morphology. (**a**) Grade 1: intact, round or oval (*side view*); (**b**) Grade 2: nonfragmented irregularly shaped (*top view*; magnification does not present actual working magnification of ×200); (**c**) Grade 3: fragmented

3 (blastomere analysis) or both, using micromanipulation techniques and FISH analysis as previously described with the application of a five-color probe specific for chromosomes 13, 16, 18, 21 and 22 (Vysis, Downers Grove, IL). Chi-square analysis was performed on all data.

The overall distribution of oocytes according to PB1 grades on day 0 and day 1 in Group I and Group II is presented in Table 5.1. As seen from this table, the number of oocytes with PB1 of different grades on day 0 was similar and distributed equally in both patient groups. PB1 grading on day 1 revealed the changes of PB1 morphology in both patient groups for each grade category except grade 3. Overall, the grade changes were observed in 331 (36.2%) of 831 PB1, and were similar in both Groups I and II. Significant differences for such changes were observed only for the oocytes with grade 2 PB1, 79.1% and 66.3% of which, in Group I and II respectively, became grade 3 on day 1 ($P<0.001$).

Analysis of fertilization rates suggested lower rates for oocytes with grade 1 PB1 (69.5% in Group I and 73.6% in Group II), compared to grade 2 (85.5%) in Group I ($P<0.05$), and to grade 3 (83.6%) in Group II ($P<0.001$). Table 5.2 presents the cleavage rate of embryos, deriving from oocytes with different PB1 grades, evaluated by the number of blastomeres present on day 3. The rates were similar in each PB1 category for each patient group, evidenced by the number of embryos with 5–10 cells: 68.3%, 68.5%, and 73.9% in grade 1, 2, 3, respectively, in Group I, and 67.7%, 70.9%, 71.9% in Group II. The morphological quality of the embryos with respect to the degree of fragmentation observed on day 3 in relation to the oocytes with different PB1 grading showed no difference in the three PB1 grades or patient groups. The proportion of grade 1–1.5 embryos (little or no fragmentation present), resulting from oocytes with PB1 grades 1, 2, and 3 were 68.3%, 67.1%, and 79.7% in Group I, and 64.6%, 65.1%, and 61.9% in Group II, respectively.

Similar results were observed in the embryos potential to reach the blastocyst stage on days 5 and 6. Of 109 Group I embryos, 35 (32%) reached the blastocyst stage of development with 24 (22%) at a gradable expanded stage, with no significant difference in any of the PB1 grade groups. The same was true for 555 Group II embryos, of which 249 (44.9%) reached the blastocyst stage with 148 (26.7%) at a gradable expanded stage, again with no significant difference in any of the PB1 grade groups.

**Table 5.1** Morphological characteristics of the first polar body of human oocytes before ICSI and after 16 h in culture

| PB1 grade | Characteristics of PB1 | No. of oocytes (%) | |
|---|---|---|---|
| | | Total[a] | With changed grade after 16 h |
| *Patients with number of oocytes ≤10* | | | |
| 1 | Regular shape | 77 | 36 (46.8)* |
| 2 | Irregular shape | 67 | 53 (79.1)* |
| 3 | Fragmented | 68 | 0 |
| Total | | 212 | 89 (42.0) |
| *Patients with number of oocytes >10* | | | |
| 1 | Regular shape | 200 | 95 (47.5)* |
| 2 | Irregular shape | 208 | 147 (70.7)* |
| 3 | Fragmented | 211 | 0 |
| Total | | 619 | 242 (39.1) |

*$P<0.001$
[a]PB grade on day 1 was not known for all fertilized oocytes

**Table 5.2** Embryo cell # on day 3 in relation to PB1 grades

| PB1 grade | Total no. of zygotes[a] | Group I[b] | | | Group II[c] | | |
|---|---|---|---|---|---|---|---|
| | | Zygotes | 2–4 cell | 5–10 cell | Zygotes | 2–4 cell | 5–10 cell |
| 1 | 276 | 82 | 26 (31.7) | 56 (68.3) | 194[d] | 62 (32.0) | 130[d] (67.0) |
| 2 | 277 | 71[d] | 22 (31.0) | 48 (67.6) | 206 | 60 (29.1) | 146[d] (70.9) |
| 3 | 280 | 69 | 18 (26.1) | 51 (73.9) | 211[d] | 59 (28.0) | 151[d] (71.5) |
| Total | 833 | 222 | 66 (29.7) | 155 (69.8) | 611 | 181 (29.6) | 427 (69.9) |

[a]Some zygotes were cryopreserved
[b]Patients with number of oocytes ≤ 10
[c]Patients with number of oocytes >10
[d]Some zygotes did not develop [4]
No significant differences between Grades 1, 2 & 3

**Table 5.3** Implantation rates in relation to PB1 grades

| PB1 grade | # embryos transferred | # implanted |
|---|---|---|
| 1 | 30 | 13[a] (43.3) |
| 2 | 25 | 9[a] (36) |
| 3 | 31 | 11[a] (35.5) |
| 1+2 | 63 | 12[a] (19) |
| 2+3 | 44 | 12[a] (27.3) |
| 1+3 | 66 | 19[a] (28.8) |
| 1+2+3 | 58 | 5 (8.6) |
| Total | 317 | 81 (25.6) |

[a]No significant difference

Table 5.3 presents implantation rates of 317 embryos, 124 from Group I, 122 from Group II, and 71 from the patients, in whom aneuploidy testing was performed. Embryos were grouped according to the oocytes corresponding PB1 grade in which they were derived. No difference was seen between PB1 grades 1, 2, and 3 as well as embryo transfers consisting of a combination of embryos in which there were two different PB1 grades. The overall implantation rate for Group I, Group II, and the group of PGD patients was 20.2%, 32.8% and 22.5%, respectively.

**Table 5.4** Oocyte and embryo chromosome abnormalities in relation to PB1 grades

| | | | | Chromosome | | | | |
|---|---|---|---|---|---|---|---|---|
| PB1 grade | # of embryos | Normal | Abnormal | 13 | 16 | 18 | 21 | 22 |
| 1 | 137 | 33[a] (24.1) | 104 (75.9) | 29 (20.7) | 29 (20.7) | 24 (17.1) | 31 (22.1) | 26 (18.6) |
| 2 | 118 | 41[a] (34.7) | 77 (65.3) | 17 (14.4) | 20 (16.9) | 13 (11.0) | 22 (18.6) | 26 (22.0) |
| 3 | 140 | 48[a] (33.6) | 93 (66.4) | 22 (15.6) | 17 (12.1) | 23 (16.3) | 30 (21.3) | 24 (17.0) |
| Total | 395 | 121 (30.6) | 276 (69.4) | 68 (17.0) | 66 (16.5) | 60 (15.0) | 83 (20.8) | 76 (19.0) |

Mean age of patients 37 ±3.6 years
[a]No significant difference

Results of aneuploidy testing in 395 embryos, resulting from oocytes with different PB1 grades obtained from 49 patients (50 cycles) of the mean age of 37 ±3.6 years, are summarized in Table 5.4. The data show no difference in aneuploidy rates in any PB1 grade category, which were 75.9%, 65.3% and 66.4% for PB 1, grade 1, 2 and 3, respectively. The data also failed to reveal any difference in the frequency of error for each chromosome tested in relation to the PB1 grade (Figs. 5.2 and 5.3). The higher prevalence of aneuploidy for chromosomes 21 and 22 for all PB1 grades is in agreement with our findings for women of advanced reproductive age (see below).

The data show that PB1 morphology may not be a useful predictor of developmental potential or chromosomal normalcy of the resulting embryos, either in good or poor responders. Because PB1 morphology was graded in sequence before and after fertilization, it was possible to detect the grading changes we observed in greater than one-third of the oocytes in both patient groups. These changes were significant for the grade 2, intact, irregular-shaped PB1, in which the majority on day 0 became grade 3 by day 1. Although this may be representative of postextrusion changes, they seem to have no practical relevance, since all the clinical parameters studied showed no correlation with any of PB1 grades. The relevance of the observed higher fertilization rate in oocytes with the irregular PB1 shape, compared to those with the regular shape PB1 in poor responders, as well as to those with the fragmented PB1 in good responders is not clear, since these findings conflict with other data. Previous retrospective studies suggested either no correlation, or even the possibility for positive relationship of PB1 morphology with higher fertilization rate [25–27]. This conflicting data may be attributed to the timing of the ICSI procedure in relation to the cytoplasmic maturation of the oocytes.

On the other hand, in contrast to previous reports, we found no relationship of PB1 morphology to the embryo quality and cleavage rate, which makes PB1 grading questionable for reliable preselection of the cleavage-stage embryos for transfer. Furthermore, in contrast to the earlier findings of positive relationship of PB1 morphology to blastocyst formation potential [25], our data showed no relationship either in good or poor responders. No relationship was observed either between PB1 grading and the outcome of embryo transfer, which was similar in different patient groups, based on PB1 morphology. This is not in agreement with previous data, some of which suggested higher implantation and pregnancy rates for embryos derived from oocytes with intact, round versus fragmented PB1 [25, 26], nor does it agree with another report which describes an association between higher implantation and pregnancy rates and cohorts of oocytes in which a greater percentage of fragmented PB1 are present [27].

It is of interest that the aneuploidy testing of embryos resulting from oocytes with different PB1 grading failed to reveal any relationship between PB1 morphology and karyotype, suggesting that PB1 morphology is not useful for testing of chromosomal aneuplodies in preimplantation development. Thus, the data provide no evidence for any relationship of PB1 morphology with chromosomal normalcy, embryo quality and developmental potential, and outcome of embryos transfer, suggesting that PB1 grading is of no prognostic value for the developmental potential of embryos to be used in preselection of embryos for transfer.

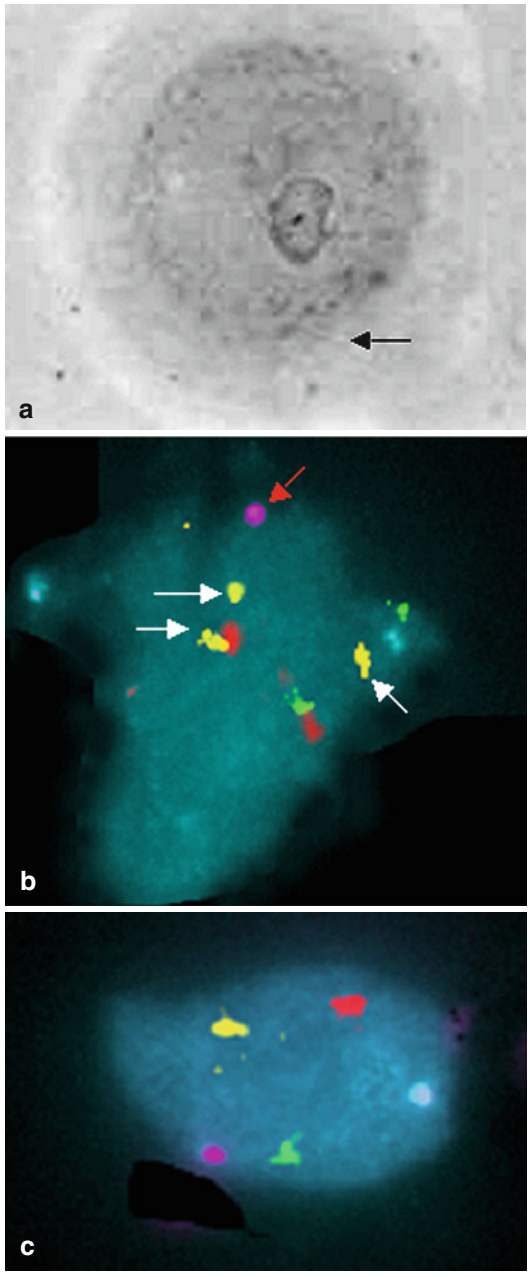



**Fig. 5.2** Monosomy 18 and trisomy 22 in Grade 2 first polar body. (*Top*) First polar body with a slightly irregular shape, Grade 2 morphology on day 0 (*Middle*) Corresponding FISH image of chromosomes 13, 16, 18, 21, and 22 (day 1) showing a missing *violet-blue* signal (chromatid) for chromosome 18 and an extra *gold* signal (chromatid) for chromosome 22. (*Bottom*) Corresponding FISH image of the second polar body (day 1) showing a normal number of signals for each of the 5 chromosomes tested

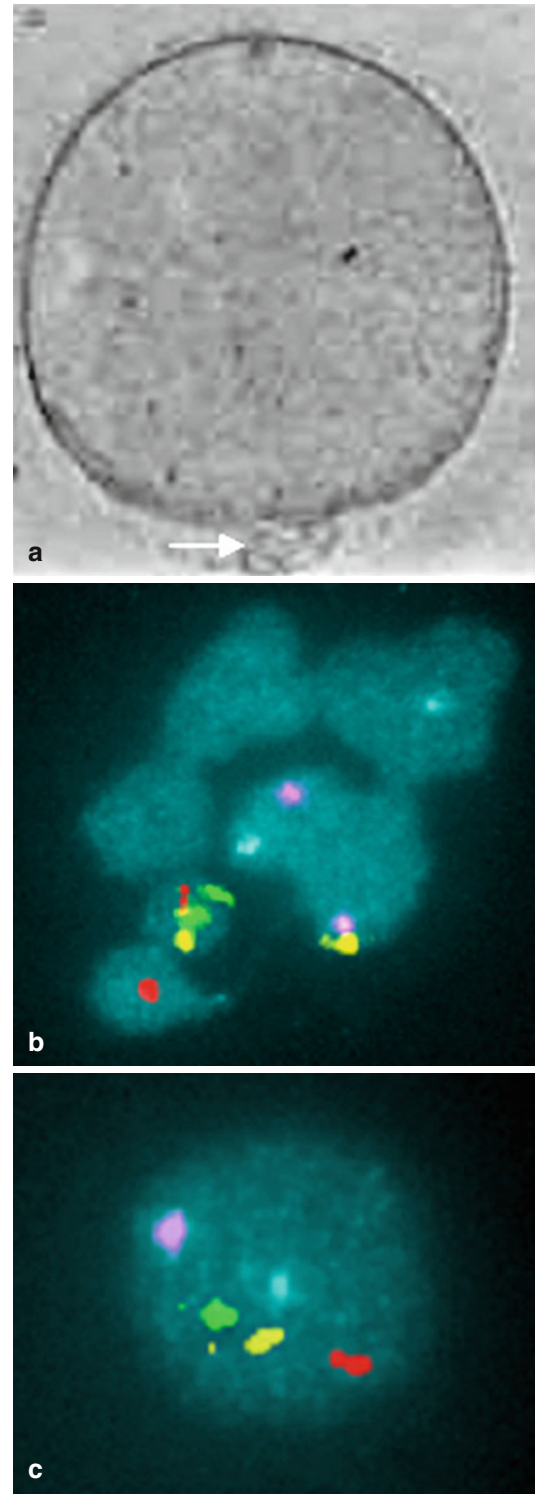



**Fig. 5.3** Normal karyotype of fragmented (Garade 3) first polar body. (*Top*) Fragmented first polar body. (*Middle*) FISH image of chromosomes 13, 16, 18, 21, and 22 (day 1) in the first polar body showing normal pattern. (*Bottom*) Normal FISH image for the second polar body

## 5.2 Aneuploidy in Human Oocytes

As mentioned, the direct testing of the meiosis errors has become possible with the introduction of PGD for chromosomal disorders, based on the use of PB1 and PB2 sampling, which are removed simultaneously following fertilization and fixed and analyzed by FISH on the same slide, as described in Chap. 2. As mentioned, because PB1 and PB2 are extruded from oocytes as a normal process of maturation and fertilization, their removal is not expected to have any biological effect on the embryo development, which is obvious from the outcomes of hundreds of pregnancies resulting from PGD [28]. The biopsied and fixed PB1 and PB2 were studied using fluorescent probes specific for chromosomes 13, 16, 18, 21, and 22 (Abbott, Downers Grove, IL), and also currently for 24 chromosomes by microarray technology. The results of such studies are presently available for 20,986 oocytes, overall, obtained from 3,953 PGD cycles performed for indication of aneuploidy testing for 2,830 IVF patients of an average age of 38.8 years [17].

### 5.2.1 Testing for Both Meiosis I and Meiosis II Errors Required for PGD of Aneuploidies

As shown in Table 5.5, a total of 9,812 (46.81%) of these oocytes were with aneuploidies, of which 2,921 (29.8%) had errors in both PB1 and PB2, 2,983 (30.4%) in only PB1 and 3,908 (39.8%) in only PB2 (Table 5.6). As expected, the aneuploidy rates rose with increasing maternal age, from 20% in patients 35 years of age, to over 40% in patients of 40 years of age (Fig. 5.4).

So, approximately half of the oocytes from IVF patients of advanced reproductive age (greater than 38 years) are abnormal, with a higher risk of meiotic errors with increasing reproductive age. This is in agreement with the data from the recent report of PB testing in 684 cycles from infertility patients, in which 55% of oocytes were found to be aneuploid after PB1 and PB2 testing [29]. The proportion of oocytes with meiosis I errors was 39% in younger than 38 years and 58% in 44-year-old patients [30]. It is not clear to what extent the reported high prevalence of abnormalities is related to IVF treatment, involving aggressive hormonal stimulation, but preliminary data on testing of donated oocytes from young fertile women suggest that the actual prevalence may be much lower, although clearly more data is needed [31].

Table 5.7 shows comparable proportions of detectable aneuploidies originating from meiosis I (31%) and meiosis II (34%), which is in contrast to the well-established concept of female meiosis I origin of chromosomal abnormalities [2, 32], suggesting that the observed aneuploidies originate equally from both meiosis I and II. As mentioned, almost one-third of the chromosomally abnormal oocytes were outcomes of sequential meiosis I and meiosis II errors, so almost one-third of meiosis II errors were associated with the preceding meiosis I errors (Table 5.6). This may be explored in light of previous considerations on the possible relationship of meiosis II errors with the increased meiotic recombination rate [33]. However, half of meiosis II errors are still observed as independent from meiosis I errors, emphasizing their clinical significance, as the genotype of the resulting zygote cannot be predicted without testing the outcomes of both meiotic divisions, inferred from PB1 and PB2. The biological significance of both meiotic errors may also be obvious from the age dependence of isolated errors of meiosis I and meiosis II, as well as sequential meiosis I and meiosis II errors (Fig. 5.5) [34]. The data shows the strongest age dependence of aneuploidies originating from the sequential meiosis I and

**Table 5.5** Frequency of chromosomal abnormalities in human oocytes detected by FISH analysis using specific probes for chromosomes 13, 16, 18, 21, and 22

| Couples | Cycles | Oocytes with FISH results | Normal oocytes | Abnormal oocytes |
|---|---|---|---|---|
| 2,830 | 3,953 | 20,986 | 11,174 (53.2%) | 9,812 (46.8%) |

**Table 5.6** Types of abnormal oocytes by polar body analysis

| Types | Number | % |
|---|---|---|
| I PB + II PB | 2,921 | 29.8 |
| I PB | 2,983 | 30.4 |
| II PB | 3,908 | 39.8 |
| Total abnormal | 9,812 | 100 |

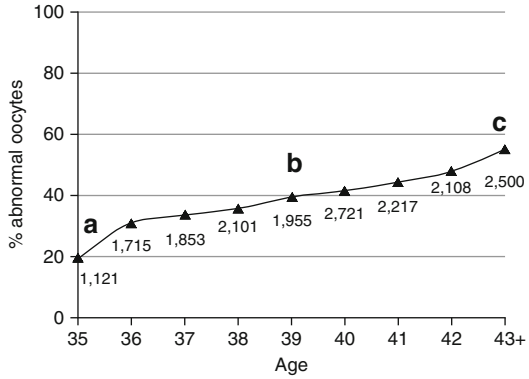



**Fig. 5.4** Aneuploid oocytes in relation to maternal age (testing for chromosomes 13, 16, 18, 21 and 22). Number of oocytes tested for each age group are shown under the curve evidencing the increase of the overall frequency from 20% in the age group of 35 to close to 60% in the age group of 43 and over

**Table 5.7** Frequency and types of meiosis I and meiosis II errors

| | Meiosis I (PB1) | | Meiosis II (PB2) | |
|---|---|---|---|---|
| FISH data | No. | % | No. | % |
| *Normal* | 13,097 | 69.0 | 13,635 | 66.0 |
| Abnormal | | | | |
| Disomy | 1,514 | 26.0 | 2,721 | 39.0 |
| Nullisomy | 3,136 | 53.0 | 2,875 | 41.0 |
| Complex | 1,271 | 21.0 | 1,342 | 20.0 |
| Total abnormal | 5,921 | 31.0 | 6,938 | 34.0 |
| Total | 19,018 | 100 | 20,573 | 100 |

meiosis II errors, which more than doubles in 40-year-old patients when compared to those of 35 years.

The schematic representation of the types of errors observed in PB1 and PB2 are presented in Figs. 5.6 and 5.7, showing at least two times higher frequency for missing signals (nullisomy) compared to extra signals (disomy) in PB1

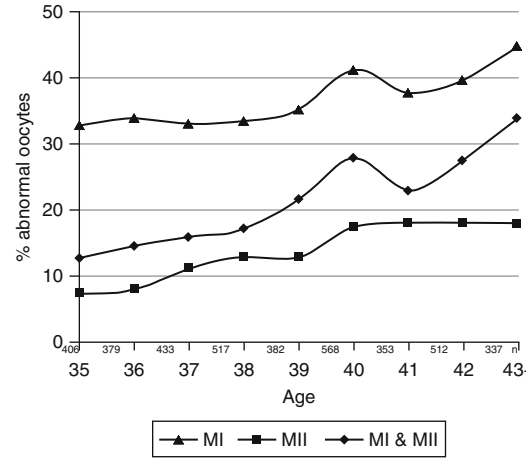



**Fig. 5.5** Relationship of different meiotic errors to maternal age, based on the analysis of 822 cycles, in which testing was performed for chromosomes 13, 16, 18, 21, and 22. *Upper curve* includes oocytes with PB1 errors irrespective of having or not having sequential abnormality of PB2. *Middle curve* includes errors originating in MI with sequential meiosis II errors, which does not include isolated MI errors. *Lower curve* includes errors originating only in MII

(approximately 2:1 ratio), in contrast to a comparable distribution of missing and extra signals in PB2. PB1 data also showed a 72.3% chromatid error rate (46.7% missing and 25.5% extra chromatids), compared to 6.3% chromosome error rate (5.2% missing and 1.1% extra chromosomes) (Fig. 5.6). Therefore, as in chromatid errors, missing chromosomes were more frequent than extra chromosomes (5.2% and 1.1%, respectively), suggesting a possible maintenance of the extra chromatid or chromosome material in MII oocytes, which is in agreement with a higher frequency of trisomies over monosomies in post-implantation embryos, detected by testing of spontaneous abortions. Only 26% of PB1 abnormalities were disomies, compared to 53% nullisomies, with the remaining being of complex origin (Table 5.7). Although the observed excess of missing signals in PB1 may be also attributable to technical errors, such as hybridization failure, it is also possible that a certain meiosis I mechanism exists, preventing an extra chromosome material extrusion into PB1 if meiotic errors occur during the oocyte maturation process. Another proof of the biological nature of this

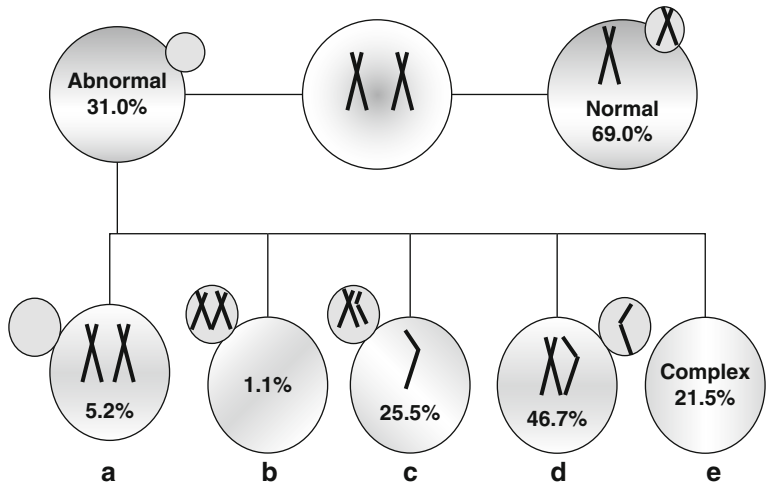



**Fig. 5.6** Chromosome (chromatid) segregation errors in Meiosis I. *Upper panel*, *center*: Primary oocyte containing diploid set of chromosomes with the doubled amount of chromatin (4n) prior to maturation. *Upper panel*, *right*: Normal segregation of homologues in the first meiotic division, resulting in the extrusion of the first polar body (PB1) (*smaller circle*) containing one of the homologues. Accordingly, the resulting secondary (metaphase II) oocyte contains the remaining homologue with two chromatids. *Upper panel*, *left*: Meiotic errors leading to the extrusion of PB1 containing abnormal set of chromosomes. *Lower panel*, *a*: Chromosomal non-disjunction, leading to segregation of both homologues to MII oocyte, so that the extruded PB1 will not contain any material, resulting in a disomic oocyte. *Lower panel*, *b*: Chromosomal non-disjunction, leading to segregation of both homologues to PB1(*smaller circle*), which will result in a nullisomic oocyte. *Lower panel*, *c*: Chromatid malsegregation, leading to an extra chromatid extrusion with PB1, which results in the lack of one chromatid in MII oocyte. *Lower panel*, *d*: Chromatid malsegregation, leading to a single chromatid extrusion with PB1, which results in the extra chromatid material in MII oocyte. *Lower panel*, *e*: Chromatid or chromosome malsegregation involving different chromosomes, resulting in complex errors, involving different types of errors of different chromatids or chromosomes in MII oocyte

phenomenon could be the age dependence of missing chromatids (Fig. 5.8), and missing chromosomes (Fig. 5.9), suggesting that the observed anomalies may be due to the overall disturbances of the meiosis process with advanced reproductive age.

The data show that testing only for five chromosomes has revealed as high as 46.8% aneuploidy rate. Although some overestimate attributable to limitations of FISH technique cannot be excluded, the majority of PB1 and PB2 abnormalities were true errors as were confirmed by the follow-up studies of the embryos resulting from oocytes with meiosis I and meiosis II errors [12], and also showed a strong age dependence of errors in both PB1 and PB2 (Fig. 5.5). In contrast to the well-established concept of female meiosis I origin of chromosomal abnormalities, our results show that the observed errors originate from both meiosis I and II as per the expected patterns of segregation illustrated in Figs. 5.6 and 5.7. The results are of clinical significance, suggesting that the genotype of the resulting zygote cannot be predicted without testing of the outcomes of both meiotic divisions, inferred from PB1 and PB2. For example, testing of meiosis I errors alone should reduce aneuploidy rate in the resulting embryos at least by two-thirds. Despite the fact that, over one-third of these oocytes will be still aneuploid following the second meiotic division, PB1 testing still could sufficiently improve the implantation and pregnancy rates in poor prognosis IVF patients, or ICSI patients, by applying ICSI selectively to the oocytes with aneuploidy-free PB1. On the other hand, only close to a half of the abnormalities deriving from the second meiotic division may be detected by PB1 analysis as complex errors, therefore, to avoid the transfer of all the embryos resulting from aneuploid oocytes, testing of both PB1 and PB2 is still required. As PB1 and PB2 are extruded in a normal process of oocyte maturation and

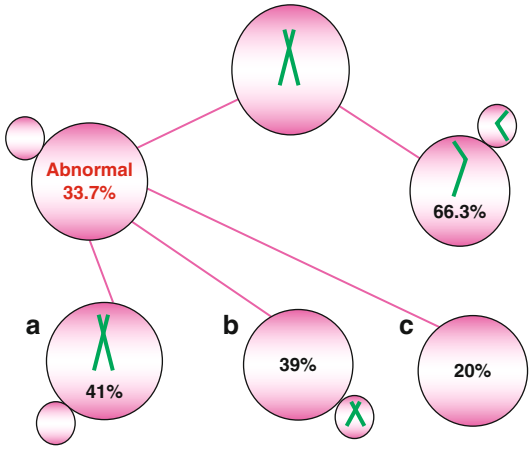



**Fig. 5.7** Meiosis II errors, based on the PB2 FISH analysis. *Upper panel* (*center*): Secondary (metaphase II) oocyte containing haploid set of chromosomes (2n) prior to fertilization. *Upper panel* (*Right*): Normal segregation of chromatids in the second meiotic division (66.3%), resulting in the extrusion of the second polar body (PB2) (*smaller circle*) containing one of the chromatids. Accordingly, the resulting maternal contribution to zygote contains the remaining sestrid chromatid. *Upper panel* (*Light*): *Abn*ormal segregation of chromatids in the second meiotic division (33.7%), involving the abnormal segregation of chromatids, showed in the lower panel. *Low panel* (*a*): Chromotid nondisjunction leading to the extrusion of PB containing no chromatid material, so both chromatids will be left in oocyte, resulting in disomic oocyte (41%). *Low panel* (*b*) Similarly, chromatid nondisjunction leading to the extrusion of PB containing both chromatids, which results in nullisomy of this chromosome in maternal pronucleus (39%). *Low panel* (*c*): *Complex c*hromatid malsegregation, leading to different errors of different chromatids (20%)

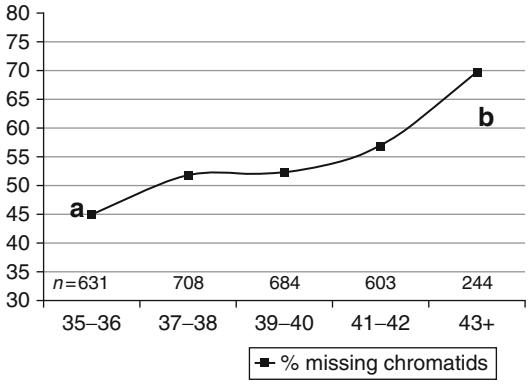



**Fig. 5.8** Prevalence of missing chromatids 13, 16, 18, 21 and 22 in PB1 in relation to maternal age. Numbers of oocytes tested for each age groups are shown under curve, evidencing the increase of the prevalence from 45% in the age group of 35–70% in the age group of 43 years and older

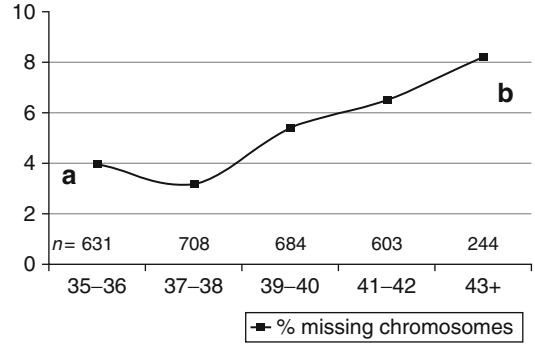



**Fig. 5.9** Prevalence of missing chromosomes 13, 16, 18, 21, and 22 in PB1 in relation to maternal age. Numbers of oocytes tested for each age groups are shown under curve, evidencing the increase of the prevalence from 4% in the age group of 35–8% in the age group of 43 years and older

fertilization, having no biological significance in pre- and postimplantation development, their removal and testing may become a useful tool in assisted reproduction practices to identify the oocytes without nuclear abnormalities, which should help in the preselection of oocytes with the highest potential for establishing a viable pregnancy, improving significantly the IVF efficiency.

As seen from Fig. 5.6, the majority of abnormalities in meiosis I are represented by chromatids errors, in contrast to the expected chromosomal nondisjunction, suggested by previous traditional studies mentioned. However, chromosomal errors are still observed in 6.3% of oocytes, which does not support the other extreme claim that all abnormalities in MII oocytes are of chromatid origin [35]. Although both chromatid and chromosomal errors are involved in producing MII abnormalities, the frequency of chromatid errors are much higher that chromosomal ones (chromatid/chromosome error ratio 10:1). So there is no doubt that both of these meiosis I errors lead to aneuploidy in the resulting embryos, as demonstrated by the follow-up study of the embryos resulting from these oocytes, the transfer of which were avoided. However, differences in the effect of chromatid and chromosomal errors on the pre- and postimplantation development cannot be excluded.

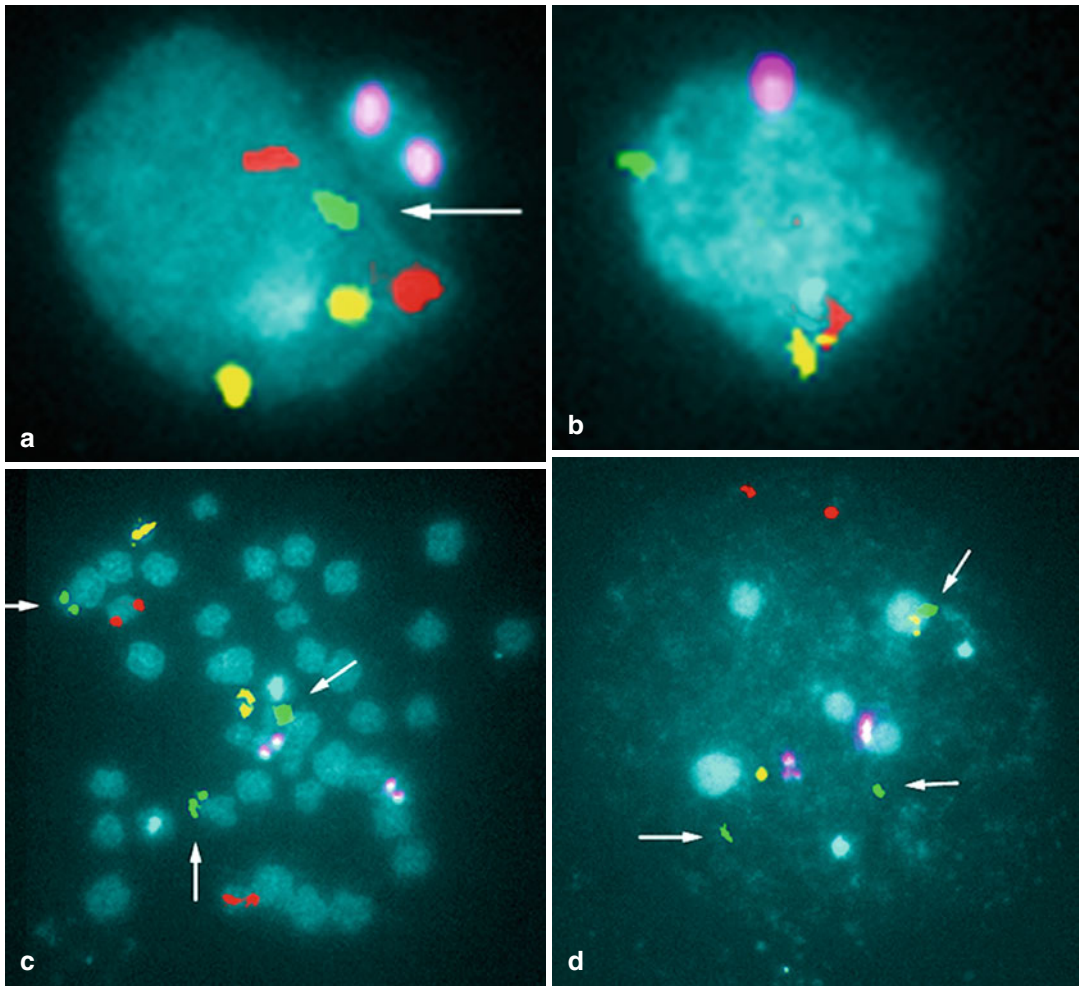



**Fig. 5.10** Meiosis I error resulting in trisomy 21. PB1 and PB2 were simultaneously removed on day 1 at the pronuclear stage of development following fertilization assessment. (*Top left*) FISH image of PB1 after a 3 h hybridization with MultiVysion PB panel probe for chromosomes 13 (*red*), 16 (*aqua*), 18 (*violet blue*), 21 (*green*), and 22 (*gold*), showing one chromosome 21 signal (*white arrow*) instead two, indicating an extra chromatid 21 was retained in the oocyte. (*Top right*) FISH image of PB2 showing a normal number of signals for each of the chromosomes tested. Information obtained by the PB testing indicate trisomy 21 is present in the embryo due to the chromatid error in meiosis I. (*Bottom left*) FISH image of metaphase chromosomes obtained after embryo biopsy and fixation on day 3, confirming the presence of three chromosomes 21 (*white arrows*). Some double dot (*paired*) signals are seen representing each chromatid of these metaphase stage chromosomes. (*Bottom right*) FISH image of an interphase nucleus isolated from a second cell from the corresponding embryo, also showing three signals for chromosome 21 (*white arrows*)

As for diagnostic significance, it is obvious that both PB1 and PB2 should be tested. As much as 60.2% of abnormal oocytes deriving from meiosis I errors may be detected by testing of PB1 (Table 5.6, Fig. 5.10), which also allows predicting 42.8% (2921 of 6,829 oocytes with PB2 aneuploidies) of oocytes with the second meiotic division errors. However, the remaining 3,908 (57%), which represents over one-third of the overall number of abnormal oocytes, became abnormal only following the second meiotic division, which could not be predicted by PB1 results and missed, if testing were limited to PB1 (Fig. 5.11). This may suggest that in order to

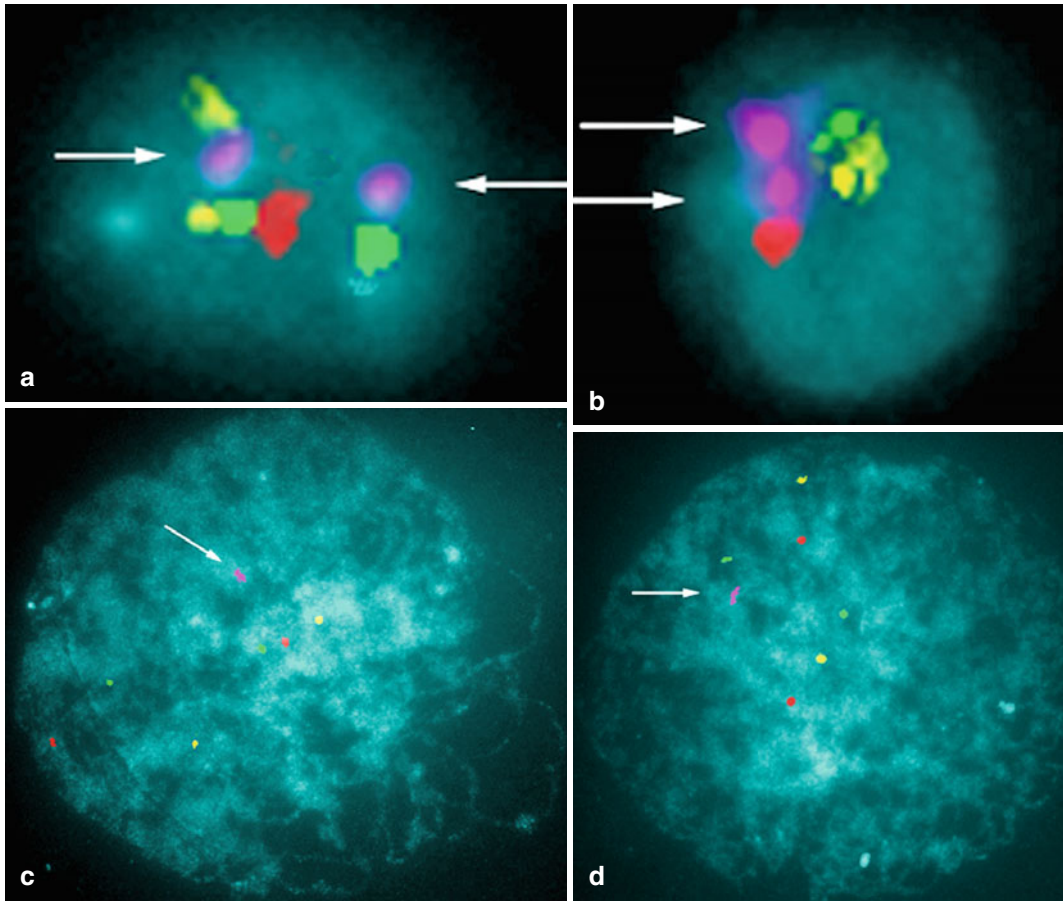



**Fig. 5.11** Meiosis II error resulting in monosomy 18. (*Top left*) FISH image of PB1 after a 3 h hybridization with MultiVysion PB panel probe for chromosomes 13 (*red*), 16 (*aqua*), 18 (*violet blue*), 21 (*green*), and 22 (*gold*), showing two chromosomes 18 signal (*white arrows*), indicating a normal pattern of signals. (*Top right*) FISH image of PB2 showing a normal number of signals for each of the chromosomes tested, except for chromosome 18, which contain 2 instead of one signal (*two white arrows*). Information obtained by the PB testing indicates that monosomy 18 is present in the embryo due to the chromatid error in meiosis II. (*Bottom*) FISH images of two blastomeres of the resulting embryo, confirming the presence of single chromosome 18 (*white arrows*)

identify all oocytes with chromosomal abnormalities, the outcome of both the first and second meiotic divisions should be studied, using PB1 and PB2.

In contrast to meiosis I errors, there was no difference in the frequency of missing or extra chromatid errors following the second meiotic division (Fig. 5.7). Overall, 6,938 oocytes (33.7%) of 20,573 oocytes tested had meiosis II errors, of which 39% were with extra chromatid, 41% with missing chromatid, and 20% with complex errors. The data also show that the direct testing of meiosis I and meiosis II errors allows avoiding from the transfer of at least 50% of embryos resulting from aneuploid oocytes, which should clearly contribute to the pregnancy outcome of the IVF patients, participating in this study, as will be demonstrated below.

Of course, the study may be limited due to testing of only five chromosomes, but these are the most frequent ones involved in human aneuploidies. The testing for 24 chromosomes was attempted by conventional CGH [36, 37], but this

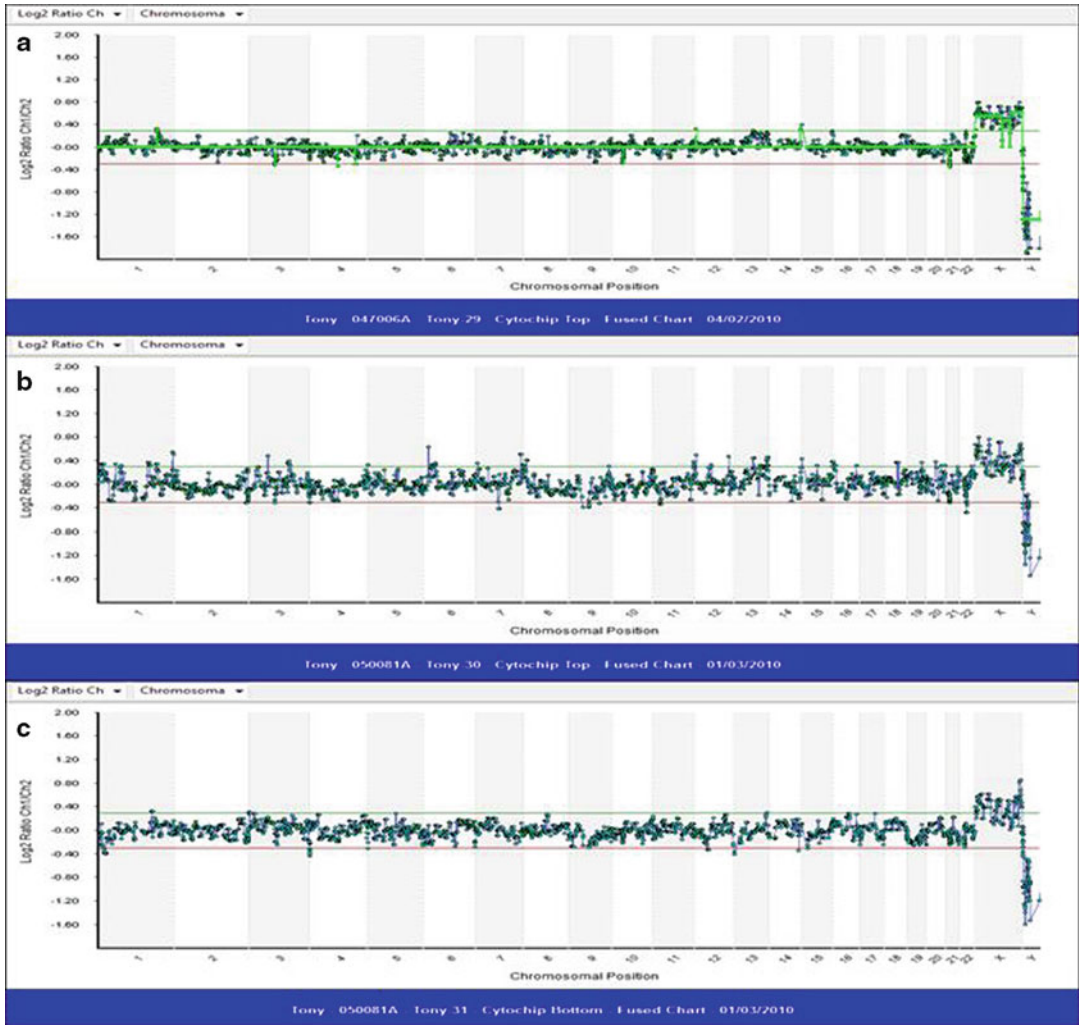
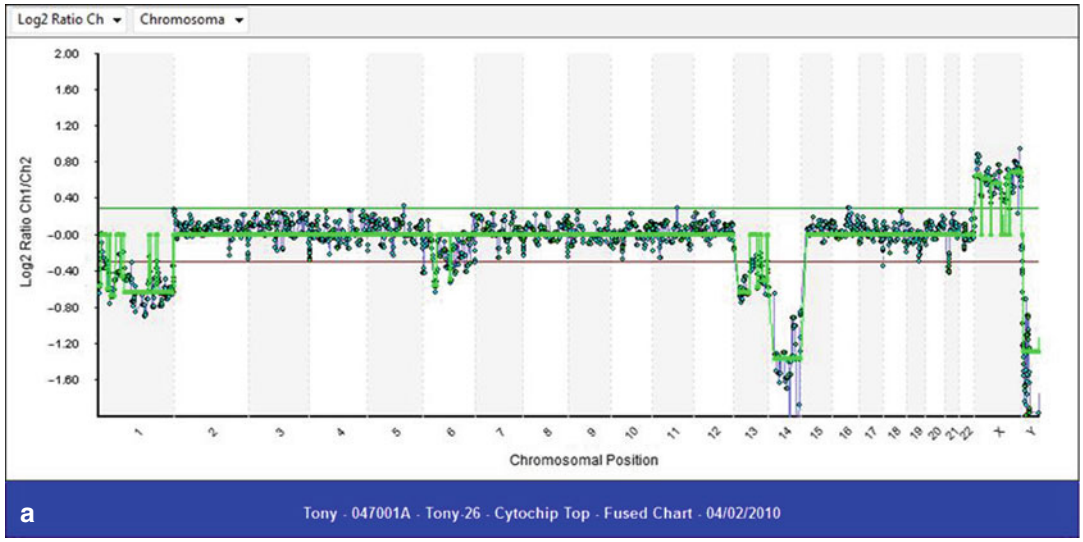
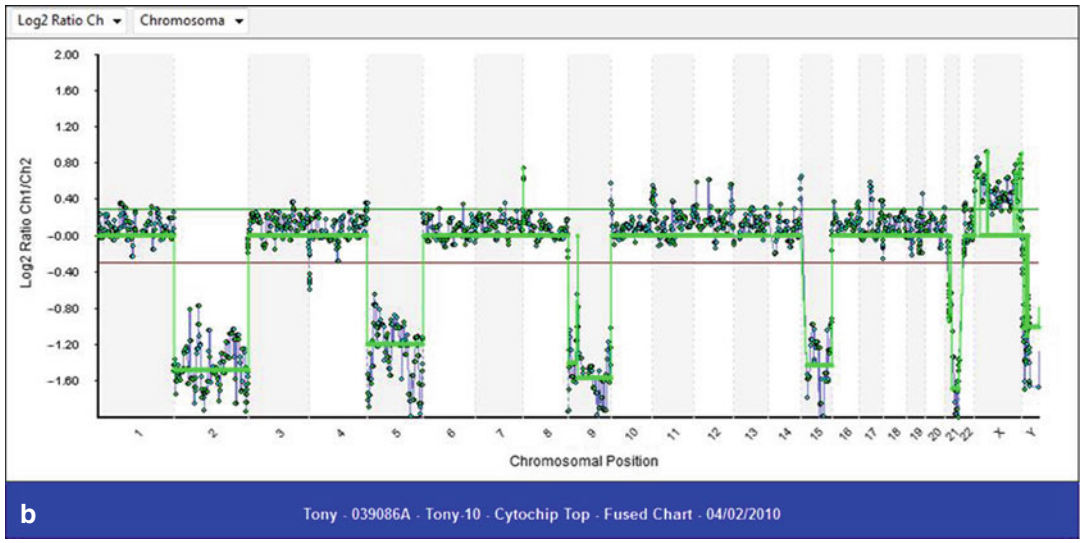



**Fig. 5.12** Array-CGH analysis of PB1, PB2, and resulting oocyte showing normal chromosome set for all 23 chromosomes (performed in collaboration with Antony Gordon, Bluegnome, Cambridge, UK). (**a**) PB1 analysis shows normal results. (**b**) PB2 analysis shows normal results (**c**) Normal set of the resulting oocyte

appeared to have significant limitations in detecting chromatid errors, which are the major source of embryo chromosomal abnormalities. The latter has recently been overcome by the application of array-CGH, as shown in the application of array-CGH for 24-chromosome testing in PB1, PB2 and resulting oocytes in cases of haploidy and triploidy, confirming the above observations by FISH analysis. Figure 5.12 shows the normal array-CGH pattern for 23 chromosomes in PB1, PB2 and resulting normal oocyte, while the example of abnormal oocyte with loss of chromatid 16 was presented in Fig. 2.20 (Chap. 2), showing feasibility of detecting of chromatid errors by array-CGH technology. The abnormal oocytes resulting from meiosis I and meiosis II errors detected by array-CGH analysis of PB1 and PB2 is presented in Fig. 5.13, showing multiple errors of different chromosomes in PB1 and PB2. The relevance of array-CGH for testing PB1 and PB2 has also been confirmed by other recent reports [38–40].




**PB1**




**PB2**

**Fig. 5.13** 24 chromosome aneuploidy testing by array-CGH analysis of PB1&PB2 (performed in collaboration with Antony Gordon, Bluegnome, Cambridge, UK). (*PB1*) Aneuploidy detected in PB1, involving missing chromatids 1, 6, and 13, and missing chromosome 14. (*PB2*) Aneuploidy detected in PB2, involving missing chromatids 2, 5, 9, 15, and 21

### 5.2.2 Inconsistency Between Aneuploidy Types Predicted by PB1 and Detected by Cleavage-Stage Testing

As mentioned, the analysis of the types of errors showed a significantly higher frequency for missing (monosomy/nullisomy) chromosome/chromatids (53%), compared to extra chromosome/chromatid errors (disomy) in PB1 (26%), in contrast to a comparable distribution of missing (41%) and extra (39%) chromatids in PB2. Similarly, missing chromosomes were more frequent compared to extra chromosomes, with an overall observation of the two times higher prevalence of PB1s with chromosome/chromatid losses

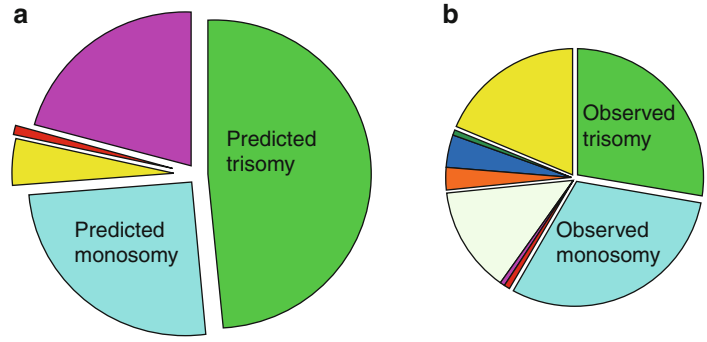



**Fig. 5.14** Inconsistency of predicted and observed aneuploidies in oocytes and embryos. (**a**) Distribution of different types of aneuploidies predicted by testing PB1 and PB2, showing predominance of predicted trisomies (*green sector in the circle*). (**b**) Distribution of different types of aneuploidies by blastomere testing from the day-3 embryos, showing predominance of monosomies (*blue sector in the circle*), opposite to prediction by testing PB1 and PB2

than chromosome/chromatid gains. The higher frequency of chromatid over chromosome errors (10:1 ratio) is in agreement with the other relevant report mentioned [29], and was also previously reported in traditional studies of meiotic chromosomes in MII oocytes [5, 10, 35], and also observed in a mouse model [41], as one of the important mechanisms of aneuploidy. This is also confirmed in the application of array-CGH for PB testing, as mentioned above.

A nonrandom distribution of missing and extra chromatids and chromosomes (2:1 ratio, respectively) is also in agreement with the above-mentioned report [29], suggesting that this might be an important biological mechanism, preventing the extrusion of extra chromosome material into the PB1 in the event of meiosis I errors. As shown in Figs. 5.8 and 5.9, there is also an age dependence of both missing chromatids and missing chromosomes, increasing from 45% to 70% for missing chromatids, and from 4% to 8% for missing chromosomes, between the age groups 35 and 43 [34]. Although the possible relationship of missing signals due to the hybridization failure cannot be completely excluded, the age dependence of this category of abnormalities may suggest that this is a real phenomenon.

The data are in agreement with the types of chromosomal abnormalities in spontaneous abortions, as the only autosomal monosomy observed in postimplantation development is monosomy 21. However, the predicted embryo trisomy predominance is in clear conflict with the observed monosomy predominance at the cleavage-stage [42]. For example, analysis of aneuploidy types in our series of 1,252 embryos

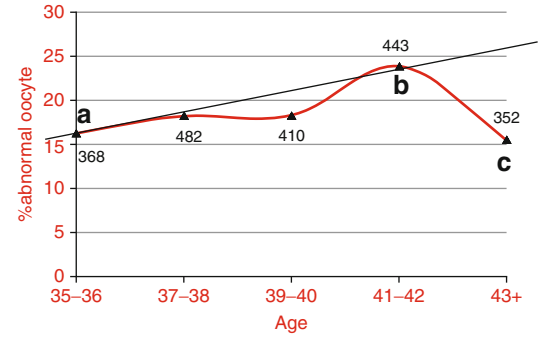



**Fig. 5.15** Prevalence of monosomies 13, 16, 18, 21, and 22 detected at the cleavage stage by blastomere analysis in relation to maternal age. Numbers of embryos with monosomies for each age groups are shown under curve, evidencing the lack of age dependence

tested for aneuploidy by blastomere biopsy, revealed 702 embryos with aneuploidy, of which 30.5% were monosomies, 27.8% trisomies, with the remaining represented by ploidy abnormalities, complex, chaotic or others types of abnormalities. The inconsistency between predicted and observed types of aneuploidy in oocytes and embryos is presented in Fig. 5.14. It is of interest that no age dependence was revealed for these monosomies observed in embryos (Fig. 5.15). Predominance of monosomies detected by the cleavage-stage PGD was also confirmed by PCR-based aneuploidy testing (see below).

The possible explanation for this discordance is that the majority of monosomies detected in embryos may derive from mitotic errors, provided that the technical causes can be excluded. In fact, a significant proportion of the

cleavage-stage monosomies appeared to be euploid after their reanalysis with different probes [43, 44]. The fact that some of the cleavage-stage monosomies are not detected at the blastocyst stage may also suggest that some of the monosomies are either eliminated before implantation or have no biological significance, reflecting the poor viability of the monosomic embryos and their degenerative changes (see below). However, the majority of prezygotically derived monosomies, as well as some of the postzygotic ones may still survive the blastocyst stage, and, therefore, lead to implantation failure or fetal loss [45].

The above inconsistency may also be due to a high prevalence of mosaicism at the cleavage stage, the exact prevalence and the origin of which has not been fully understood. The fact that the overall mosaicism prevalence does not show a relationship with maternal age [45] may suggest that a significant proportion of mosaicism may be either artifactual and of no clinical relevance, or simply transitional without affecting the embryo viability, which may be the consequence of degenerative processes in the embryos prior to embryo arrest. On the other hand, a certain fraction of mosaicism is still dependant on maternal age [46], probably deriving from the aneuploid zygotes, such as trisomics, some of which may result in disomic embryos, due to selective disadvantage of abnormal cells, with also a chance of forming uniparental disomies in one-third of them. Such cases were incidentally detected in PGD for single gene disorders, as well as in the process of haplotyping for preimplantation HLA typing (see below).

The data may explain the recent controversy on the clinical impact of PGD for aneuploidies, as majority of centers perform aneuploidy testing at the cleavage stage, which may not be an ideal choice for aneuploidy detection, unless it can be coupled with additional analysis, such as PB analysis or blastocyst biopsy. So to further clarify the utility of each of these approaches, further studies based on sequential PB and embryo biopsy may be useful to investigate the relative impact of each of these tests in improving the accuracy on detection of aneuploidy-free embryos for transfer.

### 5.2.3 Complex Errors and Aneuloidy Rescue in Female Meiosis

Approximately one-fifth of abnormalities in PB1 and PB2 (21% and 20%, respectively) were of complex origin, represented by different types of errors, errors involving more than one chromosome, or errors in both PB1 and PB2 of same or different chromosomes (Tables 5.7 and 5.8). Of the overall 3,881 oocytes (40%) with complex errors, 2,438 (63%) involved simultaneously the errors of different chromosomes (Fig. 5.16), and 1,507 (37%) the same chromosome(s) errors in both PB1 and PB2. Of 2,742 (71%) with the complex errors involving two or more chromosomes, 2,067 (75%) involved two chromosomes and 675 (25%) involved three or more chromosomes. Of 2,921 (17.0%) oocytes with both PB1 and PB2 abnormal, 1,314 (45%) zygotes appeared to be balanced following these sequential errors (Table 5.8). This is in agreement with the other reported data [29] and may represent a phenomenon of aneuploidy rescue, similar to the well-

**Table 5.8** Oocytes with both PB1 and PB2 abnormal

| | | |
|---|---|---|
| Chromosome 13 only | 155 | 5.3% |
| Chromosome 16 only | 248 | 8.5% |
| Chromosome 18 only | 127 | 4.4% |
| Chromosome 21 only | 418 | 14.3% |
| Chromosome 22 only | 436 | 14.9% |
| Different chromosomes[a] | 1,414 | 48.4% |
| Abnormal for >1 same chromosomes | 123 | 4.2% |
| Total | 2,921 | 100% |
| Total balanced | 1,314 | 45.0% |

[a]The same chromosome can be involved in both meiosis I and II when 2 or more chromosomes are involved

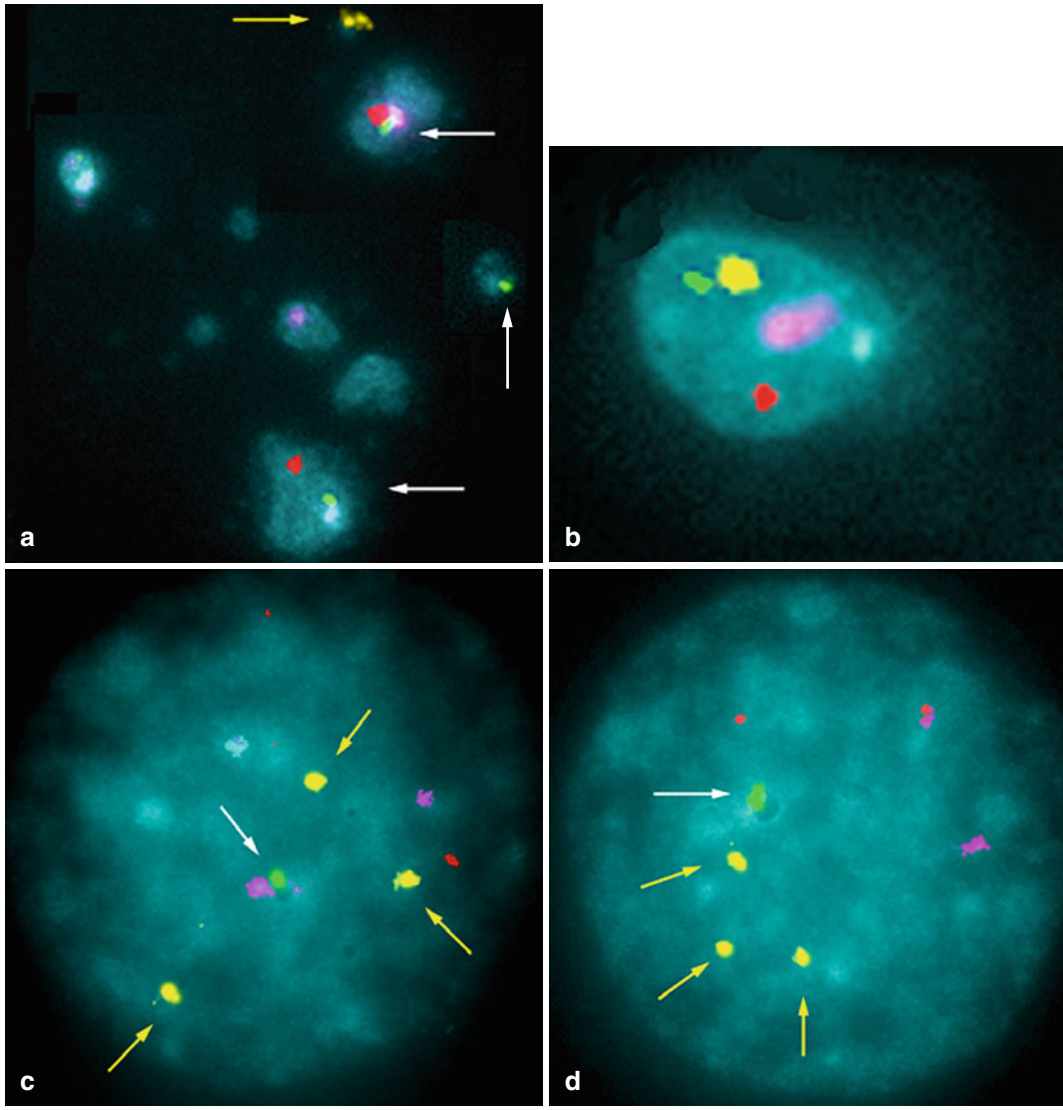



**Fig. 5.16** Complex errors in meiosis I resulting in monosomy 21 and trisomy 22. (*Upper panel*) FISH image of PB1 and PB2 after hybridization with the MultiVysion PB panel probe, revealing a double error in PB1, represented by three (*instead of two*) chromosome 21 signals (*white arrows*) and only one split chromosome 22 signal (*yellow arrow*) (*Upper left*). A normal number of signals (one each) are present in PB2 (*Upper right*). (*Lower panel*) FISH images of nuclei obtained from the resulting embryo, showing three chromosome 22 signals and one chromosome-21 signal confirming the predicted double aneuploidy

known trisomy rescue mechanism, also observed in our array-CGH studies. The example of the resulting balanced chromosome set of the oocyte after complimentary errors in meiosis I and meiosis II is shown in Fig. 5.17. The mechanism of the observed formation of such balanced zygotes is not yet understood and the fate of the embryos resulting from such balanced oocytes is not clear and may also result in abnormal (mosaic) status, uniparental disomy and imprinting disorders. As will be described below, the observed aneuploidy rescue mechanism in female meiosis cannot ensure the chromosomal normalcy of the resulting embryos to be useful for embryos transfer.

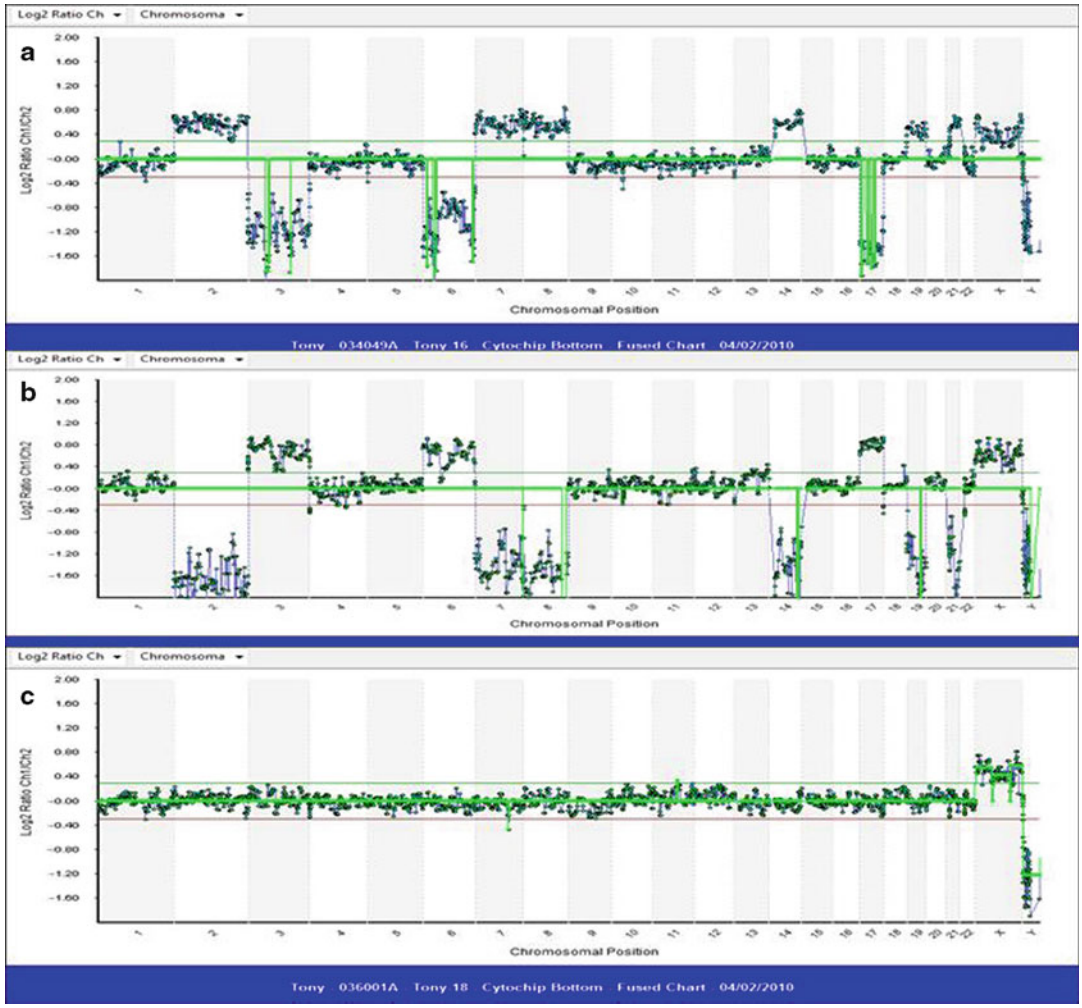



**Fig. 5.17** Array-CGH analysis of PB1, PB2, and resulting oocyte showing abnormal chromosome sets in PB1 and PB2, resulting in a normal chromosome status in the resulting oocyte (performed in collaboration with Antony Gordon, Bluegnome, Cambridge, UK). (**a**) PB1 analysis shows extra chromatids 2, 7, 8, 14, 19, and 21, and missing chromosomes 3, 6 and 17. (**b**) PB2 analysis shows extra chromatids, 3, 6, and 17, and missing chromatids 2, 7, 8, 14, 19, and 21. (**c**) Normal set of the resulting oocyte, which suggests the complete balancing of the karyotype in the resulting oocyte

In addition, although the aneuploidy rate is expected to be higher with the testing for additional chromosomes, available data indicate the increase of the complex abnormalities rather than the overall aneuploidy rate [15]. The fact that the meiotic error of one chromosome may affect the segregation of other chromosomes was demonstrated also in XO female mice [41], and this was also observed in our data on the follow up of meiosis I errors through meiosis II and cleavage of the resulting embryos (see below).

So a high prevalence of complex errors may suggest that by testing for even a few most prevalent chromosome abnormalities, the errors of other chromosomes may simultaneously be detected, together with different types of errors of the same chromosome. This may indicate generalized disturbances in the meiosis process, which may be due to the age-related effect on the recombination frequency, spindle formation errors, also reported to increase with age, loss of chromosome cohesion and mitochondrial and

**Table 5.9** Origin of chromosome 13, 16, 18, 21, and 22 aneuploidies (based on information from both polar bodies of 8,602 oocytes)

| Chromosome | Total abnormal (%) | Meiosis I origin | Meiosis II origin | Meiosis I and meiosis II origin |
|---|---|---|---|---|
| 13 | 1,086 (12.6) | 436 (40.1)[a] | 394 (36.3)[a] | 256 (23.6%)[a] |
| 16 | 1,531 (17.8) | 490 (32.0) | 679 (44.4) | 362 (23.6) |
| | | $P=0.000$ | $P=0.000$ | NS |
| 18 | 1,098(12.8) | 530 (48.3) | 380 (34.6) | 188 (17.1) |
| | | $P=0.000$ | NS | $P=0.000$ |
| 21 | 2,151(25.0) | 891 (41.4) | 790 (36.7) | 470 (21.9) |
| | | NS | NS | NS |
| 22 | 2,736(31.8) | 939 (34.3) | 1,135(41.5) | 662 (24.2) |
| | | $P=0.001$ | $P=0.003$ | NS |

[a]Chi-square analysis, comparison to a, statistically significant $P$ value <0.05

organelle dysfunction [4, 32, 33, 47–51]. So with the testing for additional chromosomes, the prevalence of complex errors may be expected to further increase, not obligatorily significantly affecting the overall aneuploidy prevalence.

### 5.2.4 Chromosome-Specific Meiotic Error Origin and Its Impact on Embryo Viability

The analysis of the chromosome-specific pattern showed that chromosomes 22 and 21 were much more frequently involved in female meiosis errors (31.8% and 25.0%, respectively) than chromosomes 16, 18 and 13 (17.8%, 12.8% and 12.6%, respectively) (Table 5.9), which is in agreement with the data obtained in aneuploidy testing at the cleavage stage [45]. It was also previously demonstrated that despite the differences in chromosome-specific aneuploidy rates, the age dependence was observed for each of these chromosome errors, almost doubling between the age 35 and 43 for chromosomes 16, 21 and 22 (Fig. 5.18), again suggesting the overall disturbance of the meiosis process with advanced reproductive age [34].

Chromosome-specific origin of errors was also not similar: chromosome 16 and 22 errors originated more frequently in meiosis II (44.4% and 41.5% meiosis II errors vs. 32.0% and 34.3% meiosis I errors, respectively), and chromosome 13, 18, and 21 errors more frequently

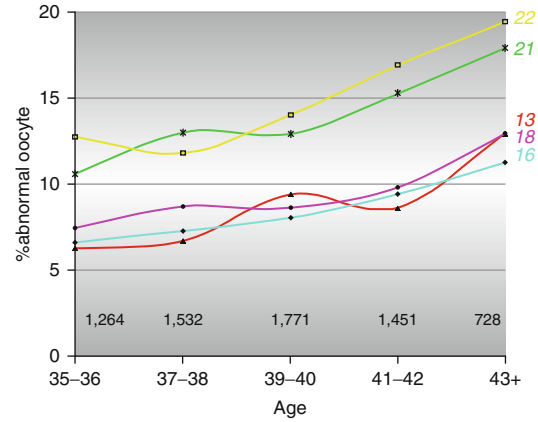



**Fig. 5.18** Frequency of each chromosome specific error in relation to maternal age. Numbers of oocytes tested for each chromosome are shown under the curves, all of which shows the increase with age, particularly high for chromosomes 21 and 22

from meiosis I (40.1%, 48.3%, and 41.4% in meiosis I vs. 36.3%, 34.6%, and 36.7% in meiosis II, respectively), although the differences are not significant for chromosome 13 errors. It is of note that the proportion of oocytes with errors of both meiosis I and meiosis II origin, were not significantly different for errors of different chromosomes, except for chromosome 18 errors (Table 5.9 and Fig. 5.19).

This data is opposite to that observed in spontaneous abortions and live-born children [32, 47] and may indicate poor viability of embryos resulting from the oocytes with the chromosome 16 and 22 errors of the second

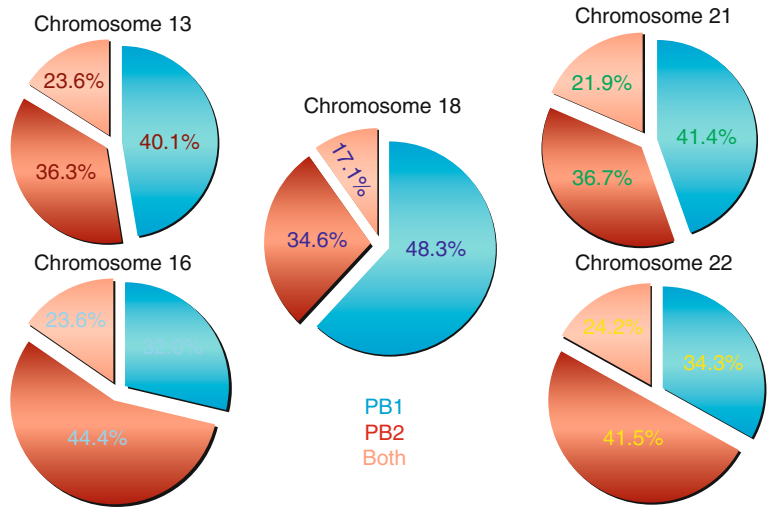



**Fig. 5.19** Error origins for different chromosome aneuploidies. Errors of chromosomes 16 and 22 originate predominantly from meiosis II (*showed in orange*), while errors of chromosome 18 originate predominantly from meiosis I (*showed in blue*). No significant differences were observed in proportion of oocytes with errors of both meiosis I and meiosis II origin (*showed in light orange*), for different chromosome errors, except for chromosome 18 errors

meiotic division, which may be incompatible with implantation and postimplantation development. Presently, there is no explanation of possible biological differences of aneuploidies depending on the meiotic origin, except for a loss of heterozygosity or higher homozygosity of the embryos originating from meiosis II errors for the genes located in these chromosomes, which may lead to imprinting of paternal or maternal genes of the chromosomes 16 or 22. Although there is no proof of the established imprinting genes in these chromosomes, there were case reports of a possible imprinting on chromosome 16, affecting fetal development or associated with cancer [52–55].

The other discrepancy is related to the meiotic origin of chromosome 18 errors, which predominantly originates from meiosis I in our material (Table 5.9 and Fig. 5.19), opposite to that in live-born children [56]. Whatever explanation may be for the above phenomenon, this data provides the first evidence for possible viability differences dependent upon not only the chromosome involved but the meiotic origin of the error. However, this may not apply to other chromosomes, as the origin of chromosome 13 and 21 error patterns were in agreement with that observed in spontaneous abortions and live-born children [32, 47], although differences in the origin of chromosome 13 error patterns are not significant in the current data.

### 5.2.5 Mitotic Errors in Cleaving Embryos in Relation to Meiosis Errors

As shown above, approximately half of meiosis II errors are observed in the oocytes with prior errors in meiosis I. As a result of such sequential errors, one-third of the resulting zygotes may have been considered normal (euplolid), provided that the preceding errors in meiosis I and meiosis II have no effect on the further preimplantation developments of the corresponding embryos. To investigate if these meiosis errors could affect the sequential mitotic divisions in the resulting zygotes and if these apparently euploid zygotes may develop into the chromosomally normal embryos acceptable for embryos transfer in PGD cycles, the follow-up testing of these embryos was carried out at the cleavage stage. As seen from Table 5.10, of 100 embryos tested overall, only 18%, deriving from the apparently balanced zygotes were euploid for all the five chromosomes analyzed, while the remaining majority were with chromosomal abnormalities [57, 58].

All of the chromosomally normal (euploid for five chromosomes tested) embryos appeared to result from zygotes with only one chromosomal error rescue, with none resulting from the zygotes balanced for two chromosomes. The fact that only a few resulting embryos (11%) were abnormal for

**Table 5.10** Chromosome 13, 16, 18, 21 & 22 testing in day-3 embryos originating from oocytes with meiosis I and II errors resulting in balanced chromosome set

| Balanced oocytes | # | Resulting embryos | # |
|---|---|---|---|
| 1 chromosome | 70 | Abnormal for the same chromosome | 8 |
| | | Abnormal for 1 different chromosome | 9 |
| | | Complex abnormality | 35 |
| | | Normal for 5 chromosomes | 18 |
| 2 chromosomes | 10 | Complex abnormality | 10 |
| 1 balanced & 1 unbalanced | 20 | Complex abnormality | 20 |
| Total | 100 | | |
| Total normal (%) | 18 | | |

the same chromosome, for which sequential meiosis I and meiosis II led to the balanced set, may suggest that the observed sequential errors in female meiosis may be attributable to the meiotic apparatus abnormality overall, rather than to a single chromosome segregation defect, which may further lead to a general defect of mitotic apparatus of the resulting embryos. This seems to be also in agreement with the observed types of aneuploidies detected in the resulting embryos, which in 79.3% of cases were represented by complex errors, including mosaicism (Fig. 5.20), known to be highly prevalent at the cleavage stage [18, 19, 42–46].

As the average reproductive age of the patients from whom the oocytes were obtained was approximately 38.5 years, the observed genomic instability in mitotic divisions of the apparently balanced zygotes following meiosis II rescue may be age related. Although the mechanism by which the age factor may lead to these changes is not known, the underlying mechanisms of the aging process involve increasing errors in the mitotic machinery of dividing cells and chromosomal abnormalities. It was also suggested, that deviations in the cytoplasmic organization, such as mitochondrial distribution, may reduce meiotic competence of oocytes and predispose the embryos to common cleavage abnormalities [23, 59–61]. The relationship between these cytoplasmic changes and the nuclear organization during maturation and fertilization of oocytes may determine an abnormal development and mitotic errors at the cleavage stage, as suggested in prospective analysis of pronuclear zygote morphology in relation to chromosomal abnormalities detected in PGD for poor prognosis IVF patients [62, 63].

According to the data on PGD for aneuploidies performed at the cleavage stage, at least 60% of embryos tested were with chromosomal abnormalities [18–20]. Although the reported types of aneuploidies may differ in different studies, there seems to be no doubt that approximately half of these abnormalities are represented by mosaicism. As there was no information about the initial chromosomal set of the zygotes from which the mosaic embryos originated in any of these studies, the nature of mosaicism in preimplantation embryos is not known, despite its high prevalence and the potential clinical relevance. There were, however, some indirect observations, suggesting that the observed mosaicism at the cleavage stage may be of different nature, with some of mosaic types increasing with maternal age [64], and therefore, probably stemming from the female meiosis errors, and the others possibly attributable to immaturity of centrosome structures in sperm, expected to be active from the first mitotic divisions of zygote, suggested for the cases of TESE patients [65].

It may be also suggested that a significant proportion of mosaic embryos originates from the oocytes that are aneuploid from the onset, through a process of "trisomy rescue." A possible high rate of further mitotic errors in cleaving embryos, deriving from the oocytes with the complex aneuploidies, may also explain the phenomenon of chaotic embryos, which makes up

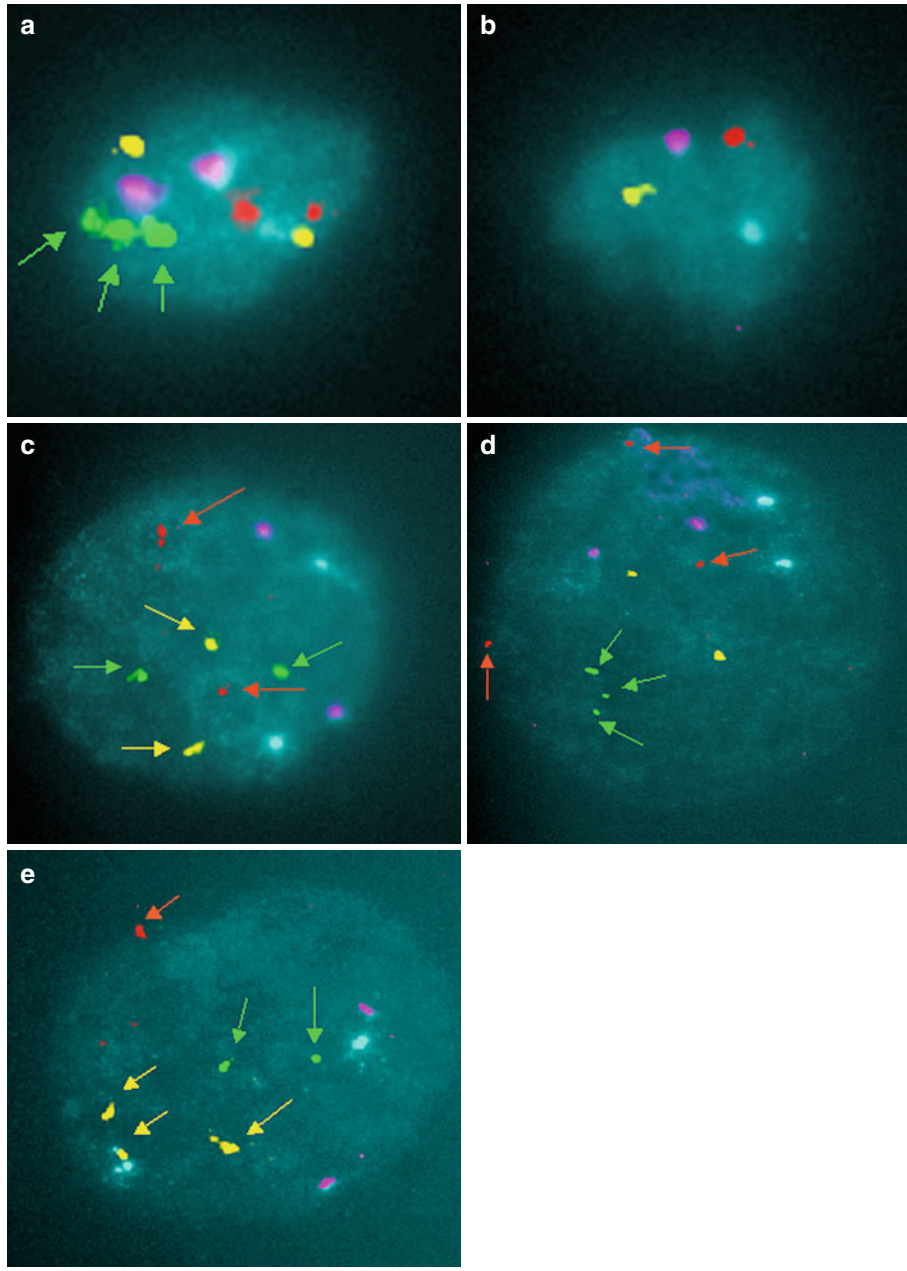



**Fig. 5.20** Sequential chromosome 21 errors in meiosis I and II resulting in an abnormal mosaic embryo. (**a**) FISH image of PB1 after hybridization with the MultiVysion PB panel probe, showing a normal number of signals for each chromosome (*double dots*) in PB1 with the exception of three signals for chromosome 21 (*green arrows*). (**b**) Four instead of five signals are detected in PB2, with a missing green signal for chromosome 21, suggesting the normal number for all five chromosomes in the resulting oocyte. (**c**) FISH image of an interphase nucleus from the resulting embryo , one with normal number of signals, including chromosome 21 (*green arrows*). (**d**) FISH image of a another nucleus from the same embryo, in which three signals for chromosome 13 (*red arrows*) and chromosome 21 (*green arrows*) are present with a normal number of signals for chromosomes 16, 18 and 22. (**e**) FISH image of the third interphase nucleus from the same embryo, in which three signals for chromosome 22 (*yellow arrows*) and only one signal for chromosome 13 (*red arrow*) are present, together with two signals for chromosomes 16, 18 and 21. The observed mosaicism may be associated with the sequential errors of chromosome 21 in meiosis I, and meiosis II

**Table 5.11** Chromosome specific aneuploidy rates in oocytes and cleavage stage embryos[a] (chromosomes 13, 16, 18, 21 and 22)

| Chromosome | Total oocytes studied | Total abnormal | Embryos studied | Total abnormal |
|---|---|---|---|---|
| 13 | 5,907 | 354 (6.0%) | 882 | 21 (2.4%) |
| 16 | 4,583 | 294 (6.4%) | 520 | 27 (5.2) |
| 18 | 6,648 | 455 (6.8%) | 999 | 17 (1.7%) |
| 21 | 6,648 | 725 (10.9%) | 882 | 38 (4.3%) |
| 22 | 4,583 | 539 (11.8%) | 302 | 17 (5.6%) |

[a]Data for Embryos from Cleavage Stage Taken from [19]

almost a half of the embryos with mosaicism. A comparable prevalence of aneuploidies in oocytes and embryos, with the differences of the types of chromosomal anomalies, mainly attributable to a high frequency of mosaicism at the cleavage stage embryos, may also support a prezygotic origin of the majority of the embryo chromosome abnormalities, including mosacism.

The comparison of the chromosome-specific aneuploidy rates in oocytes and embryos may be also of relevance to understanding the relationship between oocyte and embryo abnormalities (Table 5.11). As can be seen from these data, there is almost two times higher rates for each chromosome error, except chromosome 16, in oocytes compared to that in embryos, which may indicate a possible correction of some of the aneuploidies through the mechanism of "trisomy rescue," probably resulting in a certain proportion of mosaic embryos following the first three cleavage divisions. In fact, the exact data on the mosaicism rate in preimplantation development is not known, because only a limited number of the preimplantation embryos were fully studied, with the majority available from PGD for aneuploidies performed through a single biopsied blastomere, which may not be representative of the whole embryo. Although the possibility of the postzygotic mitotic errors in the cleavage stage embryos euploid from the onset cannot be excluded, the proportion of the aneuploidy and mosaicism stemming from these errors is not known, as well as the impact of these postzygotic errors on the pre- and postimplantation embryo development.

There was, however, disagreement between specific types of aneuploidies detected by PB and blastomere testing. As mentioned, PB testing predicted 2:1 disomy/nullisomy ratio in oocytes following meiosis I, which is in agreement with predominance of trisomies over monosomies in spontaneous abortions. With sole exception of monosomy 21, autosomal monosomies are not compatible with postimplantation development and have never been detected in recognized pregnancies or at birth. On the other hand, significantly higher prevalence of autosomal monosomies over trisomies in cleaving embryos, mentioned above, may indicate to their possible postzygotic origin through mitotic nondisjunction or anaphase lag in the first cleavages. To investigate if this is an overestimate of monosomies due to a hybridization failure in FISH analysis, we compared this to the data obtained by PCR-based testing of blastomeres from women of advanced reproductive age, the results of which are presented below.

### 5.2.6 PCR-Based Aneuploidy Testing in Cleaving Embryos

To improve the accuracy of preimplantation genetic diagnosis, it is becoming a current practice to test for single gene disorders together with chromosomal abnormalities, such as in cases of advanced reproductive age, so the same single cell is tested for both genetic and chromosomal disorders. One of possible approaches for PCR-based testing for chromosomal aneuploidies is a DNA fingerprinting, which is based on the patterns of alleles that uniquely identify an individual, relying on a multiplex fluorescent PCR of low template DNA (see Chap. 2). Using STRs with a high heterozygosity, we performed single cell DNA fingerprinting for detection of aneuploidies for chromosomes 2, 3, 4, 5, 6, 7,

8, 9, 11, 12, 13, 15, 16, 17, 18, 19, 21 and 22, in PGD for single gene mutations mapped in these chromosomes, preimplantation HLA matching or testing for aneuploidies. Overall, 2,074 blastomeres were tested, with the number of blastomeres tested for each chromosome ranging from a few for chromosomes 3 and 9 to over 100 for chromosomes 6, 7, 11, 13, 16, 18, 19, 21 and 22, so the individual aneuploidy prevalence was evaluated only for these chromosomes, which was 11%, 5%, 7%, 10.1%, 6.7%, 10.2%, 7%, 16.9%, and 7.6%, respectively. Overall, 42% aneuploidy prevalence was evaluated based in the series of 276 blastomeres (Fig. 5.21), in which copy number of chromosomes 13, 16, 18, 21, and 22 was detected in the same blastomeres. No significant monosomy/trisomy ratio differences were observed, opposite to findings of FISH analysis at the cleavage stage, despite monosomy/trisomy ratio variations for individual chromosomes (Figs. 5.21 and 5.22). As can be seen from these data, there is no significant excess of monosomies over trisomies for any of chromosomes, which is also evident from Table 5.12 showing the comparison of prevalence of trisomy and monosomy for each chromosome, obtained by different methods. Although the numbers of blastomeres tested are not yet comparable, preliminary data fail to confirm the higher prevalence of monosomies in preimplantation embryos. The data also showed the errors of two or more chromosomes observed in one-third of aneuploid blastomeres, in agreement with previous FISH data in oocytes and blatomeres. Further data collection will be required to exclude a possible monosomy overestimate due to allele drop out in single blastomere PCR, as well as trisomy underestimate due a detection failure of extra maternal or paternal chromosomes because of indistinguishable homologs sharing the same polymorphic markers. The data show that, in addition to PGD for single gene disorders, simultaneous DNA fingerprinting for copy number of chromosomes allows avoiding the transfer of chromosomally abnormal embryos in couples of advanced reproductive age.

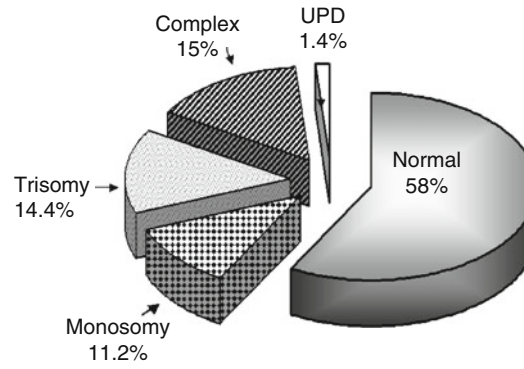



**Fig. 5.21** PCR-based aneuploidy rate for chromosome 13,16,18,21, and 22 in preimplantation embryos. Pie chart showing relative distribution and different types of aneuploid embryos: there is comparable prevalence of trisomies and monosomies, with more than one third of aneuploidies represented by complex errors, and 1.4% were represented by uniparental disomies (UPD)

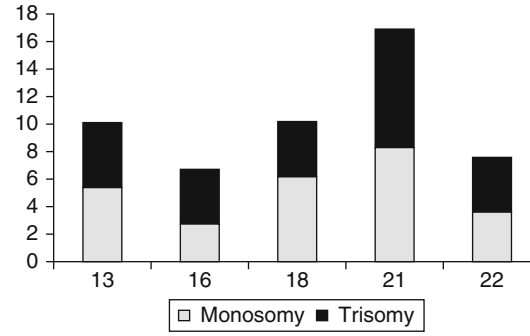



**Fig. 5.22** Relative distribution of trisomies and monosomies for different chromosomes (chromosomes 13,16, 18,21, and 22) detected by PCR-based aneuploidy testing of 276 preimplantation embryos. Bar graph demonstrating the observed distribution of each chromosome aneuploidy with relative proportion of trisomies (*dark*) and monosomies (*white*) showing no significant differences between trisomies and monosomies, although there is a tendency of predominance of trisomy over monosomy for all chromosomes except chromosome 18

### 5.2.7 Practical Relevance of Autosomal Monosomy Detection

Discordance of trisomy/monosomy ratio detected in oocytes and embryos may be also explained by the fact that a certain proportion of monosomies may not be true monosomies, but represent mosaic embryos, which will actually form euploid embryos in the process of pre- or postimplantation

**Table 5.12** Comparison of FISH and PCR-based aneuploidy prevalences

| | # embryos tested | | Monosomic | | Trisomic | | Total | | Total (%) | |
|---|---|---|---|---|---|---|---|---|---|---|
| Chromosome | FISH[a] | PCR[b] | FISH | PCR | FISH | PCR | FISH | PCR | FISH | PCR |
| XY | 1,741 | | 8 | | 13 | | 21 | | 1.2 | |
| 1 | 559 | | 8 | | 6 | | 14 | | 2.5 | |
| 4 | 327 | 42 | 4 | 1 | 3 | | 7 | 1 | 2.1 | 2.3 |
| 6 | 194 | 625 | 2 | 53 | 1 | 16 | 3 | 69 | 1.5 | 1.1 |
| 7 | 244 | 278 | 4 | 13 | 3 | 1 | 7 | 14 | 2.9 | 5 |
| 13 | 1,801 | 276 | 35 | 15 | 18 | 13 | 53 | 28 | 2.9 | 1 |
| 14 | 280 | | 1 | | 2 | | 3 | | 1.1 | |
| 15 | 1,066 | 59 | 31 | 6 | 19 | 4 | 50 | 10 | 4.7 | 16 |
| 16 | 1,665 | 396 | 49 | 11 | 37 | 13 | 86 | 24 | 5.2 | 6.5 |
| 17 | 609 | 65 | 9 | 2 | 7 | 0 | 16 | 2 | 2.6 | 3 |
| 18 | 2,058 | 276 | 23 | 17 | 24 | 11 | 47 | 28 | 2.3 | 10 |
| 21 | 2,011 | 276 | 56 | 23 | 38 | 24 | 94 | 47 | 4.7 | 17 |
| 22 | 1,274 | 312 | 50 | 10 | 34 | 14 | 84 | 24 | 6.6 | 7 |

[a]Munne et al. [42]
[b]Rechitsky et al. unpublished data

development. To investigate the fate of monosomies detected pre- and postzygotically, these embryos were followed up to the blastocyst stage, and the resulting blastocysts were reanalyzed for the chromosomal status by FISH analysis using commercial probes specific for five chromosomes, including chromosomes 13,16, 18, 21 and 22. A total of 3,140 nuclei were analyzed from oocytes and embryos, which were obtained from women of average age of 38.6 (±3.6) years. A total of 134 monosomic embryos were followed up overall, 51 (38%) of which were detected by PB testing (monosomy 13,3; monosomy 16,10; monosomy 18,5; monosomy 21,14; monosomy 22,17; and complex monosomies 2), and 83 (62%) by blastomere analysis (monosomy 13,10; monosomy 16,11; monosomy 18,10; monosomy 21,15; monosomy 22,21; and complex monosomies 16). The proportion of monosomy confirmation in the resulting blastocysts for prezygotic monosomics was 88.1%, which is significantly higher, compared to postzygotic monosomies, confirmed only in 59.6% of cases, the remaining showing the normal karyotype or mosaicism (Table 5.13). It is also of note that 47 of 83 postzygotic monosomics developed to blastocyst stage (56.6%), compared to 41 of 51 prezygotic monosomics (80.4%), suggesting

**Table 5.13** Follow-up of monosomies to blastocyst stage

| Origin of monosomies | Total # | Blastocyst | Confirmation | |
|---|---|---|---|---|
| | | | # | % |
| Prezygotic | 51 | 41 | 37 | 88.1 |
| Postzygotic | 83 | 47 | 28 | 59.6 |

that although the proportion of monosomies that achieve balstocyst depend on the monosomy origin, autosomal monosomies may be compatible with preimplantation development, irrespective of the origin, probably being lost during implantation (Fig. 5.23).

Based on the above data, it may be suggested that the most accurate preselection of embryos for transfer in PGD for aneuploidies may be performed by a sequential testing of meiosis I, meiosis II and mitotic errors, through sequential PB1, PB2, and blastomere sampling. This may allow the avoidance of the transfer of embryos with prezygotic chromosomal errors, which seem to be the major source of chromosomal abnormalities in the embryo, and also the detection of possible mitotic errors in embryos resulting from the euploid zygotes, the proportion of which cannot be evaluated at the present time. The accumulated data on such sequential sampling will help to

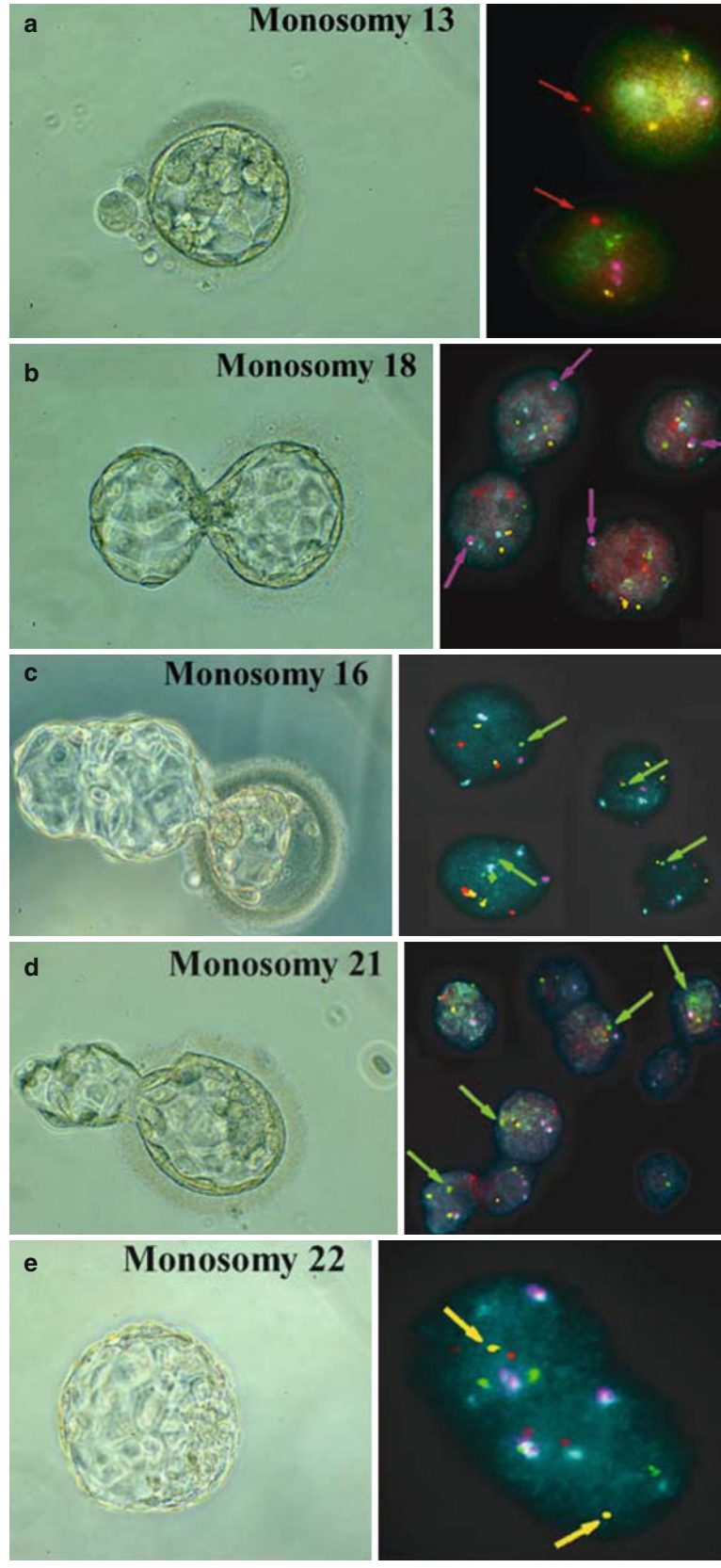



**Fig. 5.23** Development of embryos with monosomy for different chromosomes to blastocyst stage. (**a**) Image of poor quality cavitating morula, resulting from monosomy 13 embryo originating from meiosis II error (a single signal for chromosome 13 in blastomere is shown by *red arrow*). (**b**) Image of hatching blastocyst derived from monosomy 18 embryo, originating from meiosis I error (a single signal for chromsome 18 in blastocyst cells is shown by *violet blue arrow*). (**c**) Image of a hatching blastocyst through the artificial opening creating during biopsy procedure with a compact inner cell mass derived from monosomy 16 embryo originating from meiosis II error (a single chromsome 16 signal in nuclei isolated from this embryo is shown by *green arrow*). (**d**) Day-5 image of the developing embryo with trisomy 21, originating from meiosis I error (a single signal for chromosome 21 in blastocyst cell is shown by *green arrow*). (**e**) Image of fully hatched blastocyst deriving from embryo with monosomy 22, detected by blastomere biopsy (a single signal for chromosome 22 in nuclei isolated from this embryo is shown by *yellow arrow*)

evaluate possible differences in viability of the embryos with chromosomal abnormalities of meiotic and mitotic origin.

### 5.2.8 Uniparental Disomies

The introduction of meiosis error testing as a possible integral component of IVF may be also useful in avoiding some of the imprinting disorders, which have recently been reported to be associated with IVF procedures [66–70]. More than two dozens of cases of imprinting disorders, including Beckwith-Wiedemann and Algelman Syndromes, have been described in association with ART, which may be caused by epigenetic errors, uniparental disomies or other unknown factors. While epigenetic factors have been extensively reviewed [69, 71], the role and contribution of uniparental disomies have not been sufficiently understood. According to recent reviews, uniparental disomies of a few chromosomes was summarized as potentially leading to imprinting disorders, including chromosomes 6, 7, 11, 15, 19 and 20 [71]. There is not sufficient data on the prevalence of uniparental disomies in preimplantation development either, as they cannot be distinguished from normal set of chromosomes by the presently used FISH technique. Uniparental disomy may be suspected in euploid embryos predicted to be trisomic by PB1 and PB2 testing, because one-third of the embryos originating from trisomic zygotes will be with uniparental disomy as a result of "trisomy rescue." Also, uniparental dysomy may be accurately detected using DNA fingerprinting, as demonstrated in Fig. 5.24 (see also Fig. 4.13), showing uniparental disomy 6 detected in preimplantation HLA typing. Preliminary data on DNA fingerprinting for only five chromosomes, including chromosomes 13, 16, 18, 21 and 22 suggest 1.4% prevalence of uniparental disomies (see Fig. 5.21). However, based on the prevalence of different types of aneuploidies, presented above, one of two oocytes (50%) obtained from women of advanced reproductive age may be expected to be aneuploid, one of three trisomic (approximately 35%, overall), one of three trisomy rescued (approximately 12%), resulting in at least one of three to be uniparental disomies, suggesting as high as 4% expected uniparental disomies of maternal origin. On the other hand, despite low prevalence of paternally derived autosomal

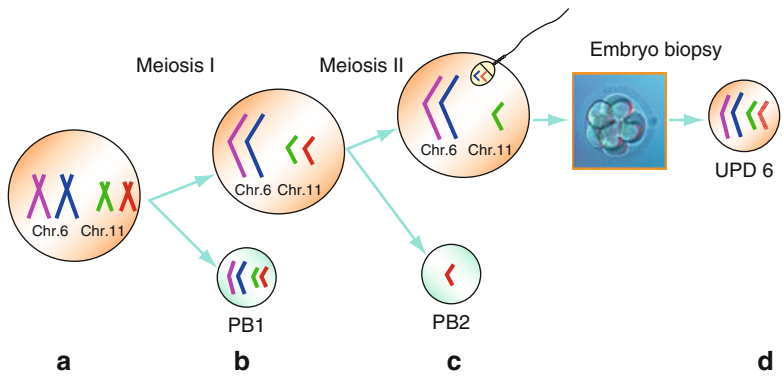



**Fig. 5.24** Uniparental disomy (UPD) for chromosome 6 detected through HLA matching of oocytes. (**a**) A primary oocyte with normal number of chromosome 6 and 11 before maturation. (**b**) Testing of the first polar body (*a smaller circle*) for thalassemia mutation and HLA shows the normal copies number of both chromosomes, suggesting the normal result for metaphase II oocyte (*larger circle*). (**c**) Sequential testing of the second polar body (*smaller circle*) revealed no chromosome 6 material while the normal (*single copy*) of chromosome is present, suggesting an extra chromosome material left in oocyte (*larger circle* showing also penetrated sperm material with a of paternal copy of chromosome 6 and 11 to zygote). (**d**) Blastomere testing from day 3 embryo, revealing normal copy number of chromosomes 6 and 11, however both chromosome 6 copies are from maternal origin, with no paternal contribution, suggesting uniparental disomy 6 of maternal origin, originating from trisomy 6 zygote, as a result of trisomy rescue with loss of paternal chromosome 6

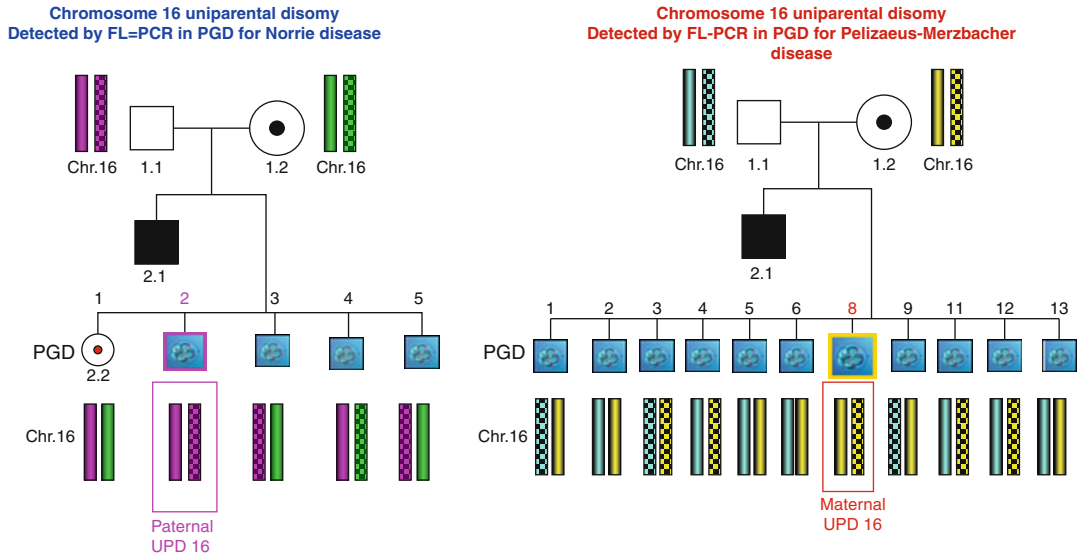



**Fig. 5.25** Chromosome 16 uniparental disomy of Paternal and Maternal Origin. *Left panel*: Chromosome 16 uniparental disomy detected by FL-PCR in PGD for Norrie disease, in which PCR based aneuploidy testing was performed because of advanced reproductive age. No maternal chromosome 16 was present being lost in the process of trisomy rescue of paternal origin. *Right panel*: Chromosome 16 uniparental disomy detected by FL-PCR in PGD for Pelizaeus-Merzbacher. Disease, in which PCR based aneuploidy testing was performed because of advanced reproductive age. No paternal chromosome 16 was present being lost in the process of trisomy rescue of maternal origin

aneuploidies, the available data indicate the occurrence of paternal uniparental disomies as well (Fig. 5.25).

The fact that more than half of the IVF patients are 35 years and older, and that more than half of their oocytes may be with aneuploidies, avoiding the transfer of the embryos resulting from these oocytes through PGD for aneuploidies should be clinically useful, in addition to potentially improving implantation and pregnancy rates, and avoiding the transfer of embryos with uniparental disomies, as possible contributors to the imprinting disorders. Although the biological significance of uniparental disomies in preimplantation development is not known, it is possible that the detection and avoidance of uniparental disomies may also contribute in improvement of implantation and pregnancy rates.

### 5.2.9 Impact of PB Testing in Detection and Avoidance of Aneuploid Embryos for Transfer

The above data indicate the practical relevance of PGD for poor prognosis patients, as more than half of the tested oocytes or embryos are with aneuploidies, which may clearly affect the developmental competence and the embryo potential to implant, if not removed from transfer. In contrast to the data obtained in traditional meiotic studies, the direct testing of the meiotic outcomes in patients of advanced reproductive age shows that chromosomal abnormalities originate comparably from meiosis I and meiosis II and are predominantly of chromatid origin. Although isolated errors in meiosis I and meiosis II were also observed, significant proportion of oocytes with meiosis I errors, overall, had also sequential meiosis II errors, resulting in apparently balanced zygotes in over one-third of cases, called in the previous section a phenomenon of aneuploidy rescue in female meiosis. However, the resulting embryos from such balanced zygotes were predominantly aneuploid, suggesting the inherent predisposition of these zygotes to the postzygotic chromosomal errors, following sequential errors in meiosis I and meiosis II. The chromosome-specific patterns of errors in meiosis I and meiosis II were different for each chromosome tested, and these patterns were not in agreement with the previously reported data based on DNA polymorphism data in liveborn trisomies or spontaneous

abortions. Comparison of the types of chromosomal aneuploidies and the prevalence of each chromosome-specific error in oocytes and embryos detected by FISH and DNA fingerprinting suggest that the majority of chromosomal aneuploidies in embryos originate from female meiosis, predisposing to further sequential potzygotic errors, which may explain the high rate of mosaicism in preimplantation embryos. The introduction of both PB and blastomere testing and the technique of DNA fingerprinting also demonstrated the occurrence of uniparental disomies, the possible impact of which is still to be documented. This may also indicate to the requirements for both the oocyte and embryos testing in PGD for aneuploidies, to exclude the transfer of embryos with aneuploidies orignating from meiosis and mitotic errors.

Because PB1 and PB2 have no biological significance in pre- and postimplantation development and are extruded in a normal process of oocyte maturation and fertilization, their removal and testing may become a useful tool in assisted reproduction practices to identify the aneuploidy-free oocytes. This testing, in conjunction with embryo biopsy for additional chromosomes aneuploidy testing, should help in the preselection of oocytes with the highest potential for establishing a viable ongoing pregnancy, significantly improving IVF efficiency. For example, a significant improvement in the implantation rate was reported even by PB1 preselection of oocytes in 553 infertile patients [30]. So the further improvement could have been achieved by detection of the remaining meiosis II errors contributing to at least one-third of the overall number of aneuploidies in oocytes. This is in agreement with our previously published clinical outcome data, which demonstrated the utility of PB1 and PB2 testing in detecting and avoiding the transfer of aneuploid embryos [72]. The overall impact was particularly obvious from the reduction of spontaneous abortion rate, which was much lower than observed in patients of comparable reproductive age without aneuploidy testing (see below).

In summary, the presented results of aneuploidy testing of the world's largest series of oocytes provide evidence for the usefulness of PB-based aneuploidy testing as part of accurate preselection of aneuploidy-free embryos for transfer, which should be performed by removal and analysis of both PB1 and PB2. The observed predominance of predicted trisomic embryos is in conflict with predominance of monosomies described at the cleavage stage which may be due to postzygotic events, some of which might not be of biological significance, and not representative of the chromosomal status of the embryos tested. Finally, for the first time, evidence is presented for a possible relationship between embryo viability and meiotic origin of chromosomal errors, affecting their clinical impact on preimplantation and postimplantation development. These observations were further explored by the application of 24 chromosome testing by array-CGH analysis, which is currently confirming the observations by FISH results.

## 5.3 Chromosomal Rearrangements

Although PGD for chromosomal translocations was introduced only in 1996, it is currently one of the most practical applications of PGD, which appeared to have a major impact on the clinical outcome of balanced translocation carriers. Initially, PGD for translocations was done for maternally derived translocations and performed by using PB1 [73], based on the fact that PB1 represents metaphase chromosomes. However, because PB1 alone not always provide the complete information, it is currently combined with PB2 analysis, or performed by testing single cells obtained from preimplantation embryos, using, in majority of cases, the interphase FISH analysis, which allows chromosome enumeration on the interphase cell nuclei. However, the number of chromosomes studied by FISH is limited to the number of chromosome-specific probes available. Even with the presently available methods for rehybridization of interphase nuclei for the second and the third time, the complete karyotyping was not realistic until introduction of microarray technology (see below). There are also limitations for the detection of some translocations, due to unavailability of certain segment-specific probes, making it highly important to develop the methods for visualization of chromosomes in single cells, including PBs and individual blastomeres, which may clearly improve the

accuracy of PGD for chromosomal disorders. However, this may soon be replaced by microarray technology, although more data will be required on validation of this method and its accuracy.

### 5.3.1 Polar Body Approach

As mentioned, this approach was originally introduced to PGD of translocations, based on the fact that PB1 never forms an interphase nucleus and consists of metaphase chromosomes. It has been shown that PB1 chromosomes are recognizable when isolated 2–3 h after in vitro culture, with degeneration beginning 6–7 h after extrusion [74]. Therefore, centromeric and whole chromosome painting to determine the number of chromatids or the chromosome segment specific probes were applied for testing of maternally derived chromosomal translocations in PB1 [73]. Although the method resulted in a significant reduction of spontaneous abortions in the patients carrying translocations, yielding unaffected pregnancies and births of healthy children, it has shown to be sensitive to malsegregation and/or recombination between chromatids, requiring a further follow-up analysis of PB2 in order to accurately predict the meiotic outcome following the second meiotic division [75, 76]. Then rehybridization with subtelomeric probes is performed to identify a possible chromatid exchange, as shown in Fig. 5.26.

However, despite the progress in transforming PB2 into metaphase chromosomes via electrofusion of PB2 nucleus with foreign one-cell human embryo, the proportion of metaphase plates did not exceed 64% even after enucleation of the recipient one-cell stage mouse embryo, to be useful in clinical practice [77, 78]. Of a total of 475

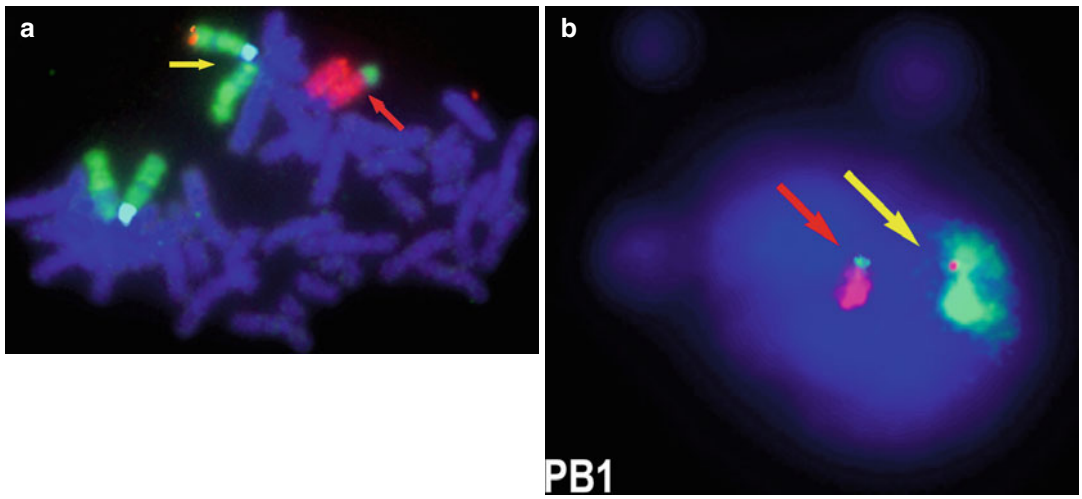



**Fig. 5.26** PGD for a maternally derived reciprocal translocation, [46,XX, t(1;15)(q32;q26)] by polar body analysis. (**a**) FISH analysis of metaphase chromosomes of a peripheral blood lymphocyte from the carrier. Chromosome 1 is identified with whole chromosome paint (WCP) in *green* in conjunction with a centromeric enumeration probe (CEP) 1 in *aqua*. Chromosome 15 is identified with WCP in *orange*, (visualization through a *red* single bandpass filter), in conjunction with a sub-telomeric (Tel) 15q in *orange* since the translocated segment of chromosome 15 onto chromosome 1 is small. (**b**) FISH image of metaphase chromosomes from PB1 in which CE is observed. Chromosome 1 (chromatids) is identified by WCP in *green*. However; only one chromatid carries the signal for Tel 15q (derivative chromatid – *yellow arrow*) while the other chromatid does not (normal chromatid). Chromosome 15 (orange) is visualized in red with one chromatid seen as only *red* and one chromatid seen as *red* and *green* (der(15) – *red arrow*). (**c**) Rehybridization of PB1 metaphase chromosomes with Tel 15q. Two signals are seen for Tel 15q indicating that both normal and derivative chromatids are present for each of the chromosomes of interest. (**d**) Hybridization of PB2 with CEP 1 (*aqua*), Tel 1q (*orange*) and CEP 15 (*green*) shows a balanced/normal number of signals, indicating that the oocyte carries either a normal or balanced chromosome complement. (**e**) Embryo follow up analysis by embryo biopsy and blastomere nucleus conversion to metaphase chromosomes confirms the prediction by PB analysis showing a normal chromosome complement. Normal chromosomes 1 (*white arrows*) and chromosomes 15 (*yellow arrows*) are present

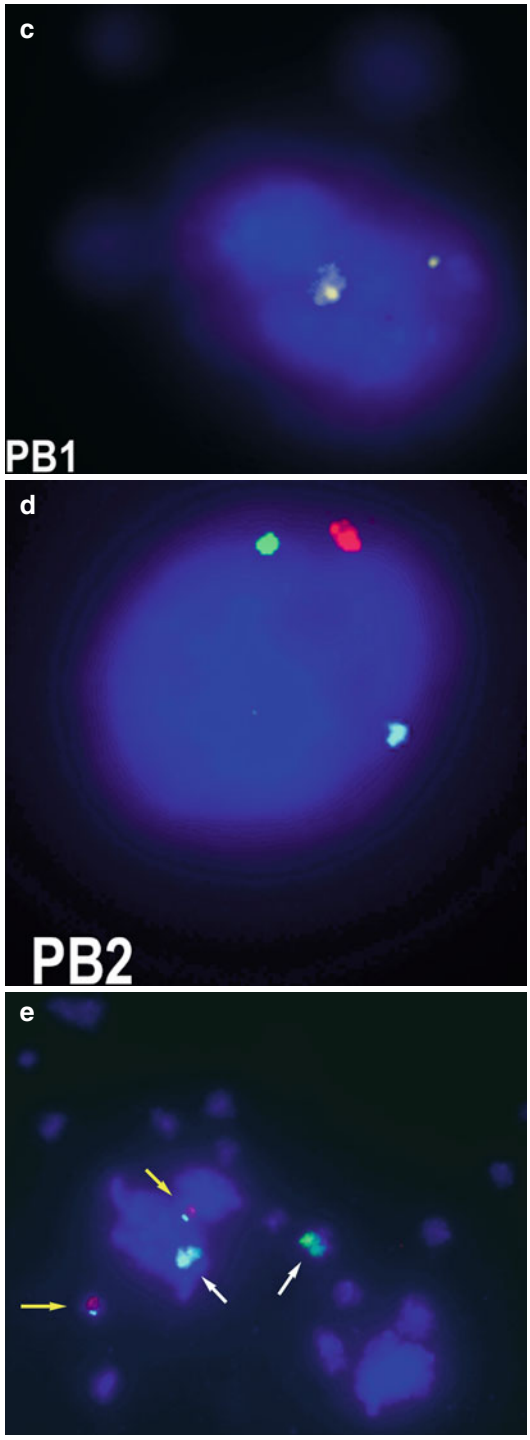



**Fig. 5.26** (continued)

PGD cycles for translocations, polar body approach was applied in 90 cycles from couples with maternally derived translocations. Of 934 oocytes from these cycles tested, FISH results were available in 739 (79%), allowing preselection and transfer of embryos deriving from normal or balanced oocytes in 46 (71.4%) cycles, resulting in 12 (26%) clinical pregnancies, 9 of which yielded the delivery of 12 unaffected children and 3 (25%) were spontaneously aborted (Table 5.14) [79]. The confirmatory testing was possible in two of three spontaneously aborted embryos, showing the presence of de novo translocations different from the expected meiotic outcomes [78, 79].

### 5.3.2 Blastomere Nuclear Conversion by Fusion with Mouse Oocytes

Alternative methods were developed to convert single blastomere nuclei into metaphase chromosomes, following the fusion of single blastomeres with murine or bovine zygotes [80, 81].

To visualize blastomere chromosomes, human single blastomeres were fused with enucleated or intact mouse zygotes at pronuclear stage, known to be at the S – phase of the cell cycle, and the resulting heterokaryons were fixed at the metaphase of the first cleavage division [76, 80]. The commercially available frozen mouse zygotes (Charles River Laboratories, Wilmington, MA) were used as recipient cytoplasts to induce the conversion of blastomere nuclei to metaphase. One to two hours before electrofusion with the human blastomeres, mouse zygotes were thawed, freed of zonae pellucidae using acidic Tyrode's solution, and pipetted to separate PB2. An intact single blastomere was brought into contact with the mouse zygote by agglutination with phytohemagglutinin (Irvine Scientific, Santa Ana, CA), followed by induction of cell fusion using a single direct current pulse. Four hours after fusion, heterokaryons were monitored for signs of the disappearance of pronuclei, and fixed at mitosis following hypotonic treatment.

The entrance of heterokaryons into mitosis, resulting from the fusion of human blastomeres with the intact mouse zygotes, was identified under a dissecting microscope. To avoid monitoring and maintain the heterokaryons in mitosis, they were cultured in the presence of microtubuli inhibitors. Heterokaryons with persisting pronuclei left in culture by the ninth hour after fusion were fixed following an hour's treatment

**Table 5.14** Preimplantation diagnosis for translocations: analysis by conversion of blastomere nuclei into metaphase chromosomes, interphase analysis, and polar body analysis

| Method of analysis | Blastomere conversion | Interphase analysis | PB1 and PB2 | Total |
|---|---|---|---|---|
| Cycles | 227* | 158 | 90 | 475 |
| Embryos/oocytes studied/ with results | 437/383 (88%) | 1,310/1,207 (92.1%) | 934/739 (79.1%) | 2,681/2,329 (86.9%) |
| Embryos transferred (mean) | 297 (1.7) | 213 (1.6) | 71 (1.5) | 581 (1.6) |
| Pregnancies/transfers | 74/173 (43%) | 45/123 (37%) | 12/46 (26%) | 131/354 (37%) |
| Deliveries | 55 (68 children) | 28 (33 children) | 9 (12 children) | 92 (113 children) |
| Spontaneous abortions | 10 (14%) | 9 (20%) | 3 (25%) | 22 (17%) |

*133 by nuclear conversion and 94 by chemical conversion method

with ocadaic acid (OA). This was followed by 10–15 min incubation in a hypotonic solution (0.1% sodium-citrate and 0.6% bovine serum albumin) and then the resulting mitotic heterokaryons were fixed in a cold 3:1 solution of methanol and acetic acid.

Air-dried chromosome plates were assessed by phase contrast analyzed by FISH. These appeared to be useful for PGD of both maternally and paternally derived translocation, but are extremely labor intensive and require the fusion with animal oocytes, followed by fixing the resulting heterokaryons at the metaphase of the first cleavage division. In our work, we fused single blastomeres with enucleated or intact mouse zygotes, which appeared to be 80% efficient, but required the availability of frozen fertilized mouse oocytes and also created ethical issues related to the formation of interspecies heterokaryons.

PGD by fusion approach was performed in 133 of 475 PGD cycles for translocations performed by blastomere testing (Table 5.14). As a result of this procedure, metaphases and S-PCC were obtained in 914 (63%) and 184 (12.7%), respectively, from a total of 1,451 blastomeres in which an attempt was made to visualize the chromosomes. Overall, 75.7% of the tested blastomeres could be investigated using commercially available whole chromosome paints. For the remaining 24.3% (353) blastomeres, 273 (18.8%) interphase nuclei had failed conversion and results were still obtained by interphase FISH analysis but without distinguishing normal from balanced carrier embryos. Fixation of 80 (5.5%) blastomeres revealed the absence of a nucleus and therefore no results were obtained.

This made possible the preselection of normal embryos or those with a balanced chromosomal complement for transfer in 103 (77.4%) cycles. Forty (39%) pregnancies were obtained from these transfers, resulting in birth of 37 healthy children. Only 5 of 40 pregnancies were spontaneously aborted, representing a considerable reduction of spontaneous abortion rate prior to PGD (Table 5.14) (see also Chap. 6).

### 5.3.3 Chemical Conversion Method

To avoid human blastomere fusion with mouse oocytes as well as to simplify the process of conversion from interphase nuclei to metaphase, the chemical conversion method was introduced, which is robust and highly reproducible for practical purposes [82], which was described in Chap. 2, and the example of its application for PGD was presented in Fig. 2.20 (Chap. 2).

As in the other methods, prior to FISH, the slides were pretreated with a 1% formaldehyde solution (Vysis Inc., Downers Grove, IL), followed by digestion of residual cytoplasmic proteins with 0.5 mg/ml pepsin. The strategy of FISH analysis depended on chromosomes involved in the structural rearrangement, the size of the translocated segment(s) and what probes were available commercially. The FISH system was created to be able to identify all segregation outcomes and therefore resulted in the use of a combination of probes, (i.e., locus-specific, centromeric, subtelomeric probes and whole

chromosome paints), as well as required rehybridization [76, 78, 79]. Blastomeres in which metaphase chromosomes and partial chromosome condensation (S-PCC) were obtained could often be investigated with the use of one centromeric enumeration probe and whole chromosome paints for the chromosomes of interest which were commercially available.

The chemical conversion method was applied in 94 of 227 conversion cycles, involving processing 946 blastomeres (from 877 embryos, including some follow-up testing of unbalanced embryos). Metaphases were obtained from 672 blastomeres – a conversion rate of 71%. The remaining which failed to convert, were tested in interphase if a FISH system was feasible to provide a diagnosis however without distinguishing the normal chromosome complements from those embryos which carried the balanced rearrangement. No anucleate blastomeres were observed since this method focuses on the morphologic selection of the appropriate blastomere and nucleus, as mentioned previously.

This is the first experience of the clinical application of the chemical conversion method to PGD of chromosomal rearrangements, which shows that the technique is robust and practical for PGD of translocations. The only difference from the traditional routine procedure of embryo biopsy for PGD (i.e., selection of a cell with a visible nucleus in order to avoid anucleate blastomeres and increase the likelihood of a diagnosis) is the more advanced selection of a suitable blastomere which is closer to entering mitosis. Therefore, the selection of the largest blastomere with —one to two large nucleoli within the cell nucleus which can be identified in embryos at the eight cell stage on day 3 of development would be a first choice for obtaining metaphase chromosomes. Instead of immediate fixation, blastomeres are incubated for several hours until the nuclear envelope breakdown, which is promoted by exposure to caffeine.

It was previously demonstrated that analyzable chromosomes may be obtained in selected blastomeres of four to six cell stage embryos even without any treatment. This was accomplished through close monitoring of embryos on day 2 of embryo development to identify a blastomere in mitosis following nuclear envelope breakdown [83]. However, this early biopsy on day 2 may have an adverse effect on embryo viability since as much as a quarter of the embryo is removed. Although the author reported no detrimental effect on a small group of embryos biopsied at the four to six cell stage, there has been much debate regarding the removal of two cells (a quarter of the embryo) on day 3 from an eight cell stage embryo with regard to detrimental effects [84]. Additionally, waiting for nuclear envelope breakdown prior to biopsy requires the microscopic analysis of the embryo multiple times, removing it from its optimal environment in the incubator. This is particularly labor intensive since nuclear membrane breakdown may take as long as 16 h of monitoring making it less desirable for clinical application.

With the current method, the pretreatment of selected blastomeres on the day 3 with caffeine and colcemid allows the possibility to reduce the potential detrimental effect on embryo viability with the removal of only one blastomere. The additional day in culture allows for further cleavage, and with the removal of only one cell (i.e., one-eighth of the embryonic cell mass of an eight cell embryo), the amount of embryonic cell mass is lessened compared to biopsy of one cell from a four cell embryo, one-fourth the embryonic cell mass, on day 2 of development. In addition, metaphases can be obtained in a shorter timeframe without constant monitoring of the embryos. This is probably due to the caffeine affect on MPF and MAPK activities which lead to earlier nuclear envelope breakdown, premature metaphase, PCC and earlier onset of DNA synthesis that increases the rate of metaphase formation.

The type of translocations performed by chemical conversion method is presented in Table 5.15. As previously reported, the detection rates of embryos suitable for transfer depend on the type of the translocation tested, with a higher rate of unbalanced embryos found in reciprocal translocations cases when compared to Robertsonian translocations. The proportion of

**Table 5.15** List of translocations for which PGD was performed using chemical conversion method

| # patients | # cycles | Karyotype | Probes |
|---|---|---|---|
| 1 | 1 | 46,XX,t(5;16)(p15.3;q24) | WCP 5, WCP 16, CEP 16, tel 5p, tel 16q |
| 1 | 1 | 46,XY,t(12;20)(q23;p13) | WCP 12, CEP 12, CEP 20, tel 12q, tel 20p |
| 1 | 1 | 46,XY,t(6;8)(q21;q13) | WCP 6, WCP 8, CEP 6, CEP 8, tel 6q, tel 8q |
| 1 | 1 | 46,XX t(13;15)(q14;q13) | WCP 13, WCP 15, CEP15, tel13q, tel15q |
| 1 | 1 | 46,XX,t(4;5)(p15.2;q31.3) | WCP 4, WCP 5, CEP 4, tel 4p, tel 5q |
| 1 | 1 | 46,XY,t(2;21)(q31.1;q11.2) | WCP 2, WCP 21, CEP 2, tel 2q, tel 21q |
| 1 | 2 | 46,XY,t(1;2)(q42.3;q37.3) | WCP 1, WCP 2, tel 1q, tel 2q |
| 1 | 2 | 46,XX,t(17;18)(p31.1;pter) | WCP 17, WCP 18, CEP 17, tel 17p, tel 18p |
| 1 | 1 | 46,XY,t(8;11)(p23;p13) | WCP 8, WCP 11,CEP 11, tel 8p, tel 11p |
| 1 | 1 | 46,X,t(X;2)(p11.2;q23) | WCP 2, WCP X, CEP 2, CEP X, tel 2q |
| 1 | 1 | 46,XX,t(6;9)(p25;q31) | CEP 6, tel 6p, tel 9q |
| 1 | 2 | 46,XY,t(6;18)(qter;pter) | WCP 6, WCP 18, CEP 6, tel 6q, tel 18p |
| 1 | 1 | 46,XY,t(5;14)(p10;q10) | WCP 5, WCP14, tel 5p, tel14q, LSI Cri-du-Chat region (5p15.2) |
| 1 | 3 | 46,XX,t(5;20)(q33;q13.1) | WCP 5, WCP 20, CEP 20, tel 5q, tel 20q |
| 1 | 1 | 46,XY,t(1;4)(p32;q21) | WCP 1, WCP 4, CEP 4, tel1p, tel 4q |
| 1 | 1 | 46,XY, t(6;16)(p24;p13.2) | WCP 6, WCP 16, CEP 6, CEP 16, tel 6p, tel 19p |
| 1 | 1 | 46,XX,t(1;14)(p34.3;q22.1) | WCP 1, WCP 14, CEP 1, tel1p, tel14q |
| 1 | 2 | 46,XY,t(4;10)(q21.3;q24.1) | WCP 4, WCP10, CEP 4, tel 4q, tel10q |
| 1 | 1 | 46,X,t(X;17)(p22.1;q21) | WCP17, WCP X, CEP X, tel 17q |
| 1 | 1 | 46,XY,t(18;20)(p11.2;q13.3) | WCP18, WCP 20, CEP 18, CEP 20, tel 18p, tel 20q |
| 1 | 5 | 46,XY,t(4;5)(q33;q22) | WCP 4, WCP 5, CEP 4, tel 4q, tel 5q |
| 1 | 1 | 46,XY,t(13;21)(q14;q11.2) | WCP 13, WCP 21, tel 13q, tel 21q |
| 1 | 4 | 46,XY,t(13;21)(q32;q22) | WCP 13, WCP 21, tel 13q, tel 21q |
| 1 | 1 | 46,XX,t(2;18)(q33;q21.3) | WCP 2, WCP 18, CEP 18, tel 2q, tel 18q |
| 1 | 2 | 46,XX,t(4;16)(q33;p13.1) | WCP 4, WCP16, CEP 4, tel 4q, tel16p |
| 1 | 3 | 46,XY,t(2;4)(q21.1;q31.1) | WCP 2, WCP 4, CEP 4, tel 2q, tel 4q |
| 1 | 1 | 46,XX,t(1;7)(p42.3;q32) | WCP 1, WCP 7, CEP 7, tel 1p, tel 7q |
| 1 | 1 | 46,XY,t(5;6)(q22;q22.2) | WCP 5, WCP 6, CEP 6, tel 5q, tel 6q |
| 1 | 1 | 46,XY,t(3;21)(p21;q22.3) | WCP 3, WCP 21, CEP 3, tel 3p, tel 21q |
| 1 | 1 | 46,XY,t(2;17)(p21;q23) | CEP 17, tel 2p,tel 17q |
| 1 | 1 | 46,XY,t(4;8)(q25;p23.1) | WCP 4, WCP 8, CEP 4, tel 4q, tel 8p |

**Table 5.15** (continued)

| # patients | # cycles | Karyotype | Probes |
|---|---|---|---|
| 1 | 1 | 46,XX,t(16;17)(p13.3;q11.2) | WCP 16, WCP 17, CEP 17, tel 16p, tel 17q |
| 1 | 1 | 46,XY,t(12;15)(p13.3;q24) | WCP 12, WCP 15, CEP 12, CEP 15, tel 15q |
| 1 | 1 | 46,XX,t(8;13)(q22.3;q12) | WCP 8, WCP 13, CEP 8, tel 8q, tel 13q |
| 1 | 1 | 46,XX,t(6;13)(q21;q22) | WCP 6, WCP 13, CEP 6, tel 6q, tel 13q |
| 1 | 3 | 46,XY,t(15;17)(q24;q21.3) | WCP 15, WCP 17, CEP 15, CEP 17, tel 15q, tel 17q |
| 1 | 1 | 46,XY,t(6;8)(q23.3;q21.2) | WCP 6, WCP 8, CEP 6, tel 6q, tel 8q |
| 1 | 1 | 46,XX,t(3;12)(q21;q24.33) | WCP 3, WCP 12, CEP 3, CEP 12, tel 3q, tel 12q |
| 1 | 2 | 46,XY,t(3;6)(q27.1;p21.1) | WCP 3, WCP 6, CEP 6, tel 3q, tel 6p |
| 1 | 1 | 46,XX,t(13;15)(q12.3;q13.3) | WCP 13, WCP 15, CEP 15, tel 13q, tel 15q |
| 1 | 4 | 46,XY,t(6;13)(q23.1;q14.3) | WCP 6, WCP 13, CEP 6, tel 6q, tel 13q |
| 1 | 1 | 46,XX,t(7;14)(q33;q32.3) | WCP 7, WCP 14, CEP 7, tel 7q, tel 14q |
| 1 | 1 | 46,XX,t(13;21)(q32;q22) | WCP 13, WCP 21, tel 13q, tel 21q |
| 1 | 3 | 46,XY,t(4;15)(q21.3;q11.2) | WCP 4, WCP 15, CEP 4, tel 4q, tel15q |
| 1 | 1 | 46,XX,t(11;16)(q23.3;q22) | WCP 11, WCP 16, CEP 11, tel 11q, tel 16q |
| 1 | 1 | 46,XX,t(9;16)(q34.3:p13.1) | WCP 9, WCP 16, CEP 9, tel 9q, tel 16p |
| 1 | 3 | 46,XX,t(4;8)(q27;q24) | WCP 4, WCP 8, CEP 4, tel 4q, tel 8q |
| 1 | 1 | 46,XX,t(4;8)(p16;p23.1) | WCP 4, WCP 8, CEP 4, tel 4p, tel 8p |
| 1 | 1 | 46,XY,t(2;8)(p15;p11.2)inv(10)(p11.2;q21.2) | WCP 2, WCP 8, CEP 2, Cep 8, tel 2p, tel 8p |
| 1 | 1 | 46,XX,t(9;10)(q32;p13) | WCP 9, WCP 10, CEP 10, tel 9q, tel 10p |
| 1 | 1 | 46,XY,t(4;6)(p16;q25.3) | WCP 4, WCP 6, CEP 6, tel 4p, tel 6q |
| 1 | 1 | 46,XY,t(2;17)(p23;q25) | WCP 2, WCP 17, CEP 17, tel 2p, tel 17q |
| 1 | 1 | 46,XY,t(4;11)(q33.3;p13) | WCP 4, WCP11, CEP4, tel 4q, tel 11p |
| 1 | 1 | 45,XX,rob(13;14)(q10;q10) | WCP 13, WCP 14, tel 13q, tel 14q |
| 1 | 1 | 45,XX,rob(13;21)(q10;q10) | WCP 13, WCP 21, tel 13q, tel 21q |
| 1 | 1 | 45,XY,rob(14;21)(q10;q10) | WCP 14, WCP 21,tel 14q, tel 21q |
| 1 | 1 | 46,XY,inv(8)(p23;q22) | CEP 8, tel 8p, tel 8q |
| 1 | 2 | 46,X,inv(X)(p22.3;q13) | CEP X, tel XpYp, tel XqYq |
| 1 | 3 | 46,XY,inv(5)(p15.3;q33.1) | LSI Cri-du-Chat region (5p15.2), tel 5p; tel 5q |
| 1 | 2 | 46,XY, inv(1)(p32.3;q42.13) | WCP 1, tel 1p,tel 1q |
| 1 | 1 | 46,XX,ins(11;5)(q22.2;q31.1q34) | WCP 11, CEP 11, LSI D5S721 (5p15.2), LSI EGR1 (5q31) |

(continued)

**Table 5.15** (continued)

| # patients | # cycles | Karyotype | Probes |
|---|---|---|---|
| 1 | 3 | mosaicism 46,XX ring(15)/45,XX,-15/46,XX dup ring(15) – 39,7 & 4 cells respectively | CEP 15, tel 15q, LSI Prader Willi/Angelman region D15S11 |
| 62 | 94 | | |

*WCP* whole chromosome paint, *LSI* locus specific identifier, *Tel* subtelomeric, *CEP* centromeric enumeration probe

embryos suitable for transfer was not affected by the parental origin in reciprocal translocations, while the rate of unbalanced embryos was significantly lower in Robertsonian translocations of paternal origin. However, neither of the above differences affected significantly the pregnancy rates in our data, which were comparable in reciprocal and Robertsonian translocations, irrespective of parental origin in both types of rearrangements. This may be due, in part, to current improvements in embryo culture systems with a tendency toward making single embryo transfer a real option for patients. Thus the average 1.6 embryos per transfer in our cases were sufficient for yielding clinical pregnancies.

In addition to the provided possibility of testing for small inversions and insertions (Table 5.15), the conversion technique also improves the accuracy of PGD for translocations, as evidenced by our follow-up testing of embryos predicted to be abnormal, and confirmatory prenatal diagnosis of the ongoing pregnancies and newborn testing. As mentioned, the conversion technique also distinguishes balanced from normal embryos, and therefore provides a means to avoid the reproductive risk for carriers of balanced translocations to their next generation.

In each case of translocations, the PGD strategy depends mainly on the chromosomes involved in the rearrangement and the size of the segments, as well as on the origin of translocations. The visualization of single blastomere chromosomes should usually be the method of choice, as it has advantages of differentiation of normal from unbalanced chromosomes, and avoids diagnosis based on split and/or faint locus-specific or sub-telomeric-specific signals. It also allows combining different probes to improve accuracy and follow-up chromatid exchange identified by PB analysis.

In reciprocal translocations, the conversion methods allowed distinguishing 126 balanced from 112 normal blastomeres of maternal origin, and 102 balanced from 105 normal blastomeres of paternal origin. In Robertsonian translocations, 33 balanced were distinguished from 27 normal blastomeres of maternal origin, and 40 balanced from 27 normal blastomeres of paternal origin.

Overall, the conversion approach and corresponding metaphase analysis allowed preselecting and transferring normal or balanced embryos in 173 (76.2%) of 227 PGD cycles, resulting in 74 (43%) clinical pregnancies and 55 (38%) deliveries, with only 10 (14%) spontaneous abortions, which is considerably lower than that observed in the reproductive outcomes of the carriers of chromosomal rearrangements, without PGD application (Table 5.14). This is actually comparable to the results of 158 PGD cycles performed by the blastomere interphase FISH analysis, in which the preselection and transfer of embryos free from unbalanced translocations was possible in 123 (77.8%) cycles, resulting in 45 (37%) pregnancies, 28 (22.7%) deliveries, and 9 (20%) spontaneous abortions (Table 5.14).

The pregnancy outcomes of PGD cycles performed by blastomere testing was not affected by the parental origin of translocations, although there was a higher number of suitable embryos for transfer in paternally derived Robertsonian translocations compared to that of maternally derived Robertsonian translocations (Tables 5.16 and 5.17).

**Table 5.16** Results of PGD for reciprocal translocations

| | Maternally derived | Paternally derived |
|---|---|---|
| Total studied | 1,839 from 199 cycles | 1,569 from 162 cycles |
| # with results | 1,613 (87.7%) | 1,440 (91.8%) |
| Unbalanced | 1,251 (77.6%) | 1,080 (75%) |
| Normal/balanced[a] | 124 (7.7%) | 153 (10.6%) |
| Balanced | 126 (7.8%) | 102 (7.1%) |
| Normal | 112 (6.9%) | 105 (7.3%) |
| Suitable for transfer | 362 (22.4%) | 360 (25.0%) |

[a]Cannot distinguish normal chromosome complement from a balanced rearrangement with interphase analysis

**Table 5.17** Results of PGD for Robertsonian translocations

| | Maternally derived | Paternally derived |
|---|---|---|
| Total studied | 281 from 36 cycles | 406 from 54 cycles |
| # with results | 250 (89.0%) | 384 (94.6%) |
| Unbalanced | 161 (64.4%) | 205 (53.4%) |
| Normal/balanced[a] | 29 (11.6%) | 112 (29.2%) |
| Balanced | 33 (13.2%) | 40 (10.4%) |
| Normal | 27 (10.8%) | 27 (7.0%) |
| Suitable for Transfer | 89 (35.6%) | 179 (46.6%) |

[a]Cannot distinguish normal chromosome complement from a balanced rearrangement with interphase analysis

The overall list of translocations and the probes applied is presented in Table 5.18, presenting experience of the conversion and non-conversion (i.e., interphase and polar body analysis) approaches 500 PGD cycles. 475 of these cycles resulted in preselection and transfer of 581 normal or balanced embryos (1.6 embryos per cycles on the average) in 354 (72%) transfers, which yielded 131 (37%) pregnancies of known outcomes, 92 (31.9%) deliveries of 113 unaffected children, with only 22 (17%) spontaneous abortions (Table 5.14). Of seven of these spontaneous abortions available for testing, one was determined to be an unrelated trisomy 13, two with de novo unbalanced translocations, and one in which a twin pregnancy resulted in a normal fetus and the other was unbalanced after interphase analysis [78, 79].

In contrast, the same patients had a reproductive history of 654 pregnancies prior to PGD, of which 496 (75.8%) resulted in spontaneous abortions, 49 in therapeutic abortions, 9 in stillbirths, and 25 in birth of children with unbalanced translocations. The results strongly support the previous observation of a considerable reduction of fetal loss after the application of PGD, suggesting the positive impact of PGD on the clinical outcome of pregnancies in couples carrying chromosomal rearrangements (see below).

An extremely poor reproductive outcome, with more than three quarters of their pregnancies resulting in spontaneous abortions, may clearly be explained by the poor meiotic outcomes, which vary depending on the type of translocations and their origin. To investigate the meiotic outcome of translocations in relation to the type and origin, a segregation patterns from 130 patients carrying balanced translocations were analyzed, which is presented in Figs. 5.27 and 5.28. The meiotic outcomes were inferred either from PB1 and PB2 (Fig. 5.27), or blastomere analysis (Fig. 5.28), with meoitic outcome detection rates by each of these methods

**Table 5.18** Overall list of translocations for which PGD was performed, FISH probes used and cycle outcomes

| Patients | Cycles | Karyotype | FISH probes | Embryo transfers/pregnancies/deliveries (# of children born) (# of miscarriages*) |
|---|---|---|---|---|
| 23 | 29 | 45,XX,rob(13;14)(q10;q10) | WCP 13, WCP 14, LSI 13 (13q14), tel 13q,Tel 14q | 26/10/6 (7) (4*) |
| 1 | 1 | 45,XX,rob(13;15)(q10;q10) | Tel 13q, tel15q | 0/0 |
| 1 | 1 | 45,XX,rob(13;21)(q10;q10) | Tel 13q, tel 21q | 1/1/1 (1) |
| 1 | 1 | 45,XX,rob(13;22)(q10;q10) | WCP 13, WCP 22, PB panel (chromosomes 13,16,18,21,22) | 1/0 |
| 3 | 5 | 45,XX,rob(14;21)(q10;q10) | WCP 14, WCP 21, LSI 21(21q22.13-q22.2) Tel 14q, tel 21q | 5/3/3 (3) |
| 1 | 5 | 45,XX,rob(14;22)(q10;q10) | WCP 14, WCP 22, Tel 14 q, LSI 22 (22q11.2) | 3/1/1 (1) |
| 35 | 40 | 45,XY,rob(13;14)(q10;q10) | WCP 13, WCP 14, LSI 13 (13q14), Tel 14q | 36/15/12 (12) (3*) |
| 3 | 4 | 45,XY,rob(13;15)(q10;q10) | WCP13, WCP 15, LSI 13 (13q14), Tel 15q | 4/2/2 (2) |
| 1 | 2 | 45,XY,rob(14;15)(q10;q10) | Tel14q, tel 15q | 0/0 |
| 6 | 13 | 45,XY,rob(14;21)(q10;q10) | WCP 14, WCP 21, LSI 21 | 11/4/3 (3)(1*) |
| 1 | 2 | 45,XX,der(4),t(4;13)(q35;q12),-13 | WCP 4, WCP 13, CEP 4, Tel 4q, Tel 13q | 1/0 |
| 1 | 1 | 46,XX,t(1;4)(q23;q31.1) | WCP 1, WCP 4, (CEP 18 as control) | 1/0 |
| 1 | 1 | 46,XX,t(1;7)(p10;p10) | CEP7, tel1p,tel7p | 0/0 |
| 1 | 1 | 46,XX,t(1;7)(q23;p11) | WCP 1, WCP 7, CEP 1 | 1/1/1 (1) |
| 1 | 1 | 46,XX,t(1;7)(p42.3;q32) | WCP1,WCP7,CEP7,tel 1p,tel 7q | 2/0 |
| 1 | 3 | 46,XX,t(1;8)(q42;p11.2) | WCP 1, WCP 8, CEP 1, CEP 8, Tel 1q, Tel 8p | 3/2/1 (1) (1*) |
| 1 | 1 | 46,XX,t(1;8)(q25;p11.2) | WCP 1, WCP 8, CEP 1, CEP 8, Tel 1q, Tel 8p | 0/0 |
| 1 | 1 | 46,XX,t(1;9)(p36.3;p13) | CEP9,tel 1p, tel 9p | 0/0 |
| 1 | 2 | 46,XX,t(1;10)(q32.3;q24.3) | CEP1,CEP10,tel1q,tel10q | 2/1/1 (1) |
| 1 | 2 | 46,XX,t(1;10)(q42.1;q26.1) | Wcp1,wcp10,cep10,tel 1q,tel10q,PB panel | 0/0 |
| 1 | 1 | 46,XX,t(1;11)(q32.1;p15.5) | CEP11, tel1q,tel11p | 1/1/1 (1) |
| 1 | 1 | 46,XX,t(1;13)(q12;q32) | WCP1,WCP13, CEP1, tel 1q, tel 13q | 1/0 |
| 1 | 1 | 46,XX,t(1;14)(p34.3;q22.1) | WCP1,WCP14,CEP1,tel1p,tel14q | 1/0 |
| 1 | 2 | 46,XX,t(1;15)(q32;q26) | WCP 1, WCP 15, CEP 15, CEP 1, Tel 15 q, Tel 1q | 2/1/1 (1) |
| 1 | 3 | 46,XX,t(1;21)(q32;q22.1) | CEP1, tel 1q, tel 21q | 2/0 |
| 1 | 1 | 46,XX,t(1;22)(q25;q11.1) | CEP1, tel 1q, tel22q | 1/0 |
| 1 | 1 | 46,XX,inv(1) | WCP1, tel1p,tel1q | 1/1/1 (2) |
| 1 | 1 | 46,XX,t(2;3)(p13;q29) | WCP 2, CEP 3, Tel 2p, Tel 3q | 1/0 |
| 1 | 1 | 46,XX,(2;4)(p11.1;q31.3) | CEP4, tel2p,tel4q | 2/1/1 (2) |

| | | | | |
|---|---|---|---|---|
| 1 | 1 | 46,XX,t(2;5)(q13;q13) | ECP2,WCP5, CEP2, tel 2q, tel 5q | 0/0 |
| 1 | 1 | 46,XX,t(2;5)(q33;p14) | WCP 2, WCP 5, Tel 2q, Tel 5p | 1/0 |
| 1 | 1 | 46,XX,t(2;6)(q33;p23) | CEP6, tel 2q, tel 6p | 0/0 |
| 1 | 1 | 46,XX,t(2;8)(q11.2;q24.22) | CEP2, CEP8 tel 2q | 1 /1 /1 (1) |
| 1 | 2 | 46,XX,t(2;10)(q31;q11.2) | WCP 2, WCP 10, CEP 2, Tel 2q | 1/0 |
| 1 | 1 | 46,XX,t(2;13)(q22;q33) | WCP 2, WCP 13, CEP 2, Tel 13 q, LSI 13 (13q14) | 1/1/1 (1) |
| 1 | 1 | 46,XX,t(2;13)(q37;q21) | CEP2, tel 2q, tel 13q | 1/1/1 (1) |
| 1 | 2 | 46,XX,t(2;14)(q33;q24.1) | CEP2, tel 2q, tel 14q | 0/0 |
| 1 | 1 | 46,XX,t(2;16)(q37;p13.1) | WCP 2, WCP 16, CEP 16, Tel 2q, Tel 16p | 0/0 |
| 1 | 2 | 46,XX,t(2;18)(q31.1;q21.3) | CEP18, tel 2q, tel18q | 2/0 |
| 1 | 1 | 46,XX,t(2;20)(q37;q11.2) | WCP 2, WCP 20, CEP 2, Tel 20q | 0/0 |
| 1 | 1 | 46,XX,t(3;4)(p13;p35) | CEP4, tel 3p, tel 4q | 0/0 |
| 1 | 1 | 46,XX,t(3;5)(p25;q35.2) | WCP 3, WCP 5, Tel 3p, Tel 5q | 1/0 |
| 1 | 1 | 46,XX,t(3;6)(p13;p15) | CEP6, tel 3p, tel6q | 0/0 |
| 1 | 1 | 46,XX,t(3;6)(p26.2;q21) | WCP 3, WCP 6, CEP 6, Tel 3p, Tel 6q | 1/1/1 (1) |
| 1 | 1 | 46,XX,t(3;7)(p21;q22) | WCP 3, WCP 7, Tel 7q, Tel 3p | 1/1/1 (1) |
| 1 | 1 | 46,XX,t(3;8)(q26.3;q24.3) | CEP8, tel 3q, tel 8q | 1 /1 /1 (2) |
| 1 | 1 | 46,XX,t(3;8)(q27;q22) | WCP 3,WCP 8, Tel 8q, CEP 8, CEP 3 | 1/0 |
| 1 | 1 | 46,XX,t(3;10)(p25;q15.1) | WCP 3, WCP 10, Tel 3p, Tel 10q, CEP 3, CEP 10 | 0/0 |
| 1 | 1 | 46,XX,t(3;12)(p13;q23) | CEP3, CEP12, tel 3p, tel 12q | 1/0 |
| 1 | 4 | 46,XX,t(3;12)(q21;q24.33) | WCP 3, WCP 12, CEP 3, CEP 12, Tel 3q | 4/0 |
| 1 | 1 | 46,XX,t(3;12)(q25;q15) | CEP3, CEP12, tel 3q | 1/0 |
| 1 | 1 | 46,XX,t(3;15)(q13.2;q22.3) | CEP15, tel 3q, tel 15q | 1/0 |
| 1 | 1 | 46,XX,t(3;19)(p25;p13.3) | WCP 3, WCP 19 | 1/0 |
| 1 | 1 | 46,XX,t(3;22)(q13.2;q11.2) | WCP 3, WCP 22, CEP 3, Tel 3q, Tel 22q | 1/1/1 (1) |
| 1 | 1 | 46,XX,t(4;5)(p15.2;q31.3) | WCP4,WCP5,CEP4, tel 4p,tel 5q | 1/0 |
| 1 | 3 | 46,XX,t(4;6)(p14;q21) | WCP4, WCP6, CEP4,CEP6, tel 4p, tel6q | 3/0 |
| 1 | 4 | 46,XX,t(4;8)(p16;p23.1) | WCP4, WCP8,CEP4,tel 4p,tel 8p | 4/0 |
| 1 | 1 | 46,XX,t(4;8)(p16;q24) | CEP 4, Tel 4p, Tel 8q | 0/0 |
| 1 | 3 | 46,XX,t(4;8)(q27;q24) | WCP4,WCP8, CEP4, tel 4q, tel8q | 1/0 |
| 1 | 1 | 46,XX,t(4;10)(q21.3;q11.2) | WCP 4, WCP10,CEP10, tel 4q, tel 10q | 0/0 |
| 1 | 1 | 46,XX,t(4;13)(p11;p12) | CEP4, tel 4p, tel 4q, LSI 13 | 1/0 |
| 1 | 2 | 46,XX,t(4;13)(p14;q31) | WCP 4, WCP 13, Tel 13q | 0/0 |

(continued)

**Table 5.18** (continued)

| Patients | Cycles | Karyotype | FISH probes | Embryo transfers/pregnancies/deliveries (# of children born) (# of miscarriages*) |
|---|---|---|---|---|
| 1 | 2 | 46,XX,t(4;13)(q23;q21) | WCP 4, WCP 13, LSI 13 (13q14), CEP 4, Tel 4q | 1/0 |
| 1 | 2 | 46,XX,t(4;16)(q33;p13.1) | WCP4,WCP16, CEP4, tel 4q, tel 16p | 2/0 |
| 1 | 2 | 46,XX,t(4;22)(q32;q13.1) | CEP4, tel 4q, tel 22q | 2/0 |
| 1 | 2 | 46,XX,t(4;14)(q21.3;q24.3) | WCP 4, WCP 14, Tel 14 q, CEP 4 | 2/0 |
| 1 | 1 | 46,XX,t(5;7)(p14;p21) | WCP 5, WCP 7, Tel 7p | 1/0 |
| 1 | 2 | 46,XX,t(5;10)(q11;q22.1) | WCP 5, WCP 10, CEP 10 | 2/0 |
| 1 | 4 | 46,XX,t(5;10)(q35;p11.2) | CEP10, tel 5q, tel 10p | 4/2/2 (2) |
| 1 | 1 | 46,XX,t(5;11)(p15.1;p15.1) | CEP11, tel 5p, tel 11p | 0/0 |
| 1 | 2 | 46,XX,t(5;13)(q23.1;q14.1) | WCP5,WCP13, tel 5q, tel13q | 0/0 |
| 1 | 2 | 46,XX,t(5;13)(p14;q22) | Cri-du-Chat, tel 13q | 1/0 |
| 1 | 1 | 46,XX,t(5;14)(p16.3;q22) | Tel5p,tel5q,tel14q | 2/0 |
| 1 | 1 | 46,XX,t(5;14)(q35;q32.2) | WCP 5, WCP 14, Tel 5q | 0/0 |
| 1 | 1 | 46,XX,t(5;16)(p15.3;q24) | WCP5,WCP16, CEP16, tel 5p, tel 16q | 0/0 |
| 1 | 1 | 46,XX,t(5;18)(q12;p11.2) | WCP5,WCP18, CEP18, tel 15q, tel18p | 1/1/1 (1) |
| 1 | 1 | 46,XX,t(5;18)(q22;q12.2) | WCP5,WCP18, CEP18, tel 15q, tel 18q | 0/0 |
| 1 | 3 | 46,XX,t(5;20)(q33;q13.1) | Tel 5q, CEP20,tel201 | 2/1/1(1) |
| 1 | 2 | 46,XX,t(5;21)(q11.2;q22) | WCP 5, WCP 21, LSI 21 (21q22.13-q22.2), Tel 21q, Tel 5p, Tel 5q | 1/0 |
| 2 | 3 | 46,XX,t(5;21)(q31;q22.1) | WCP 5, WCP 21, LSI 5 (5q33-q34), LSI 21 (21q22.13-q22.2) | 1/0 |
| 1 | 2 | 46,XX,t(6;7)(p11.2;q11.2) | CEP6,tel6p,tel7q | 0/0 |
| 1 | 1 | 46,XX,t(6;8)(q25;p21.1) | CEP6,CEP8,tel6q, tel8p | 1/1/1 (2) |
| 1 | 1 | 46,XX,t(6;9)(p25;q31) | CEP6, tel6p,tel9q | 0/0 |
| 1 | 1 | 46,XX,t(6;12)(q16.3;p12.2) | CEP6,CEP12,tel12q | 1/1/0 (0)(1*) |
| 2 | 3 | 46,XX,t(6;13)(q21;q22) | WCP 6, WCP 13, LSI 13, Tel 6q, Tel 13q | 1/1/1 (1) |
| 1 | 1 | 46,XX,t(6;14)(q15;q13.1) | WCP 6, WCP 14, CEP 6, Tel 6q, Tel 14q | 0/0 |
| 1 | 1 | 46,XX,t(6;15)(p21.3;q26.1) | WCP 6, WCP 15, CEP 15, Tel 15q | 1/1/1 (1) |
| 1 | 2 | 46,XX,t(6;15)(q23;q26) | CEP6, tel 6q, tel 15q | 2/0 |
| 1 | 1 | 46,XX,t(6;19)(q;p) | CEP6, tel6q, tel19p | 1/0 |
| 1 | 1 | 46,XX,t(7;11)(p11.2;q24.2) | WCP 7, WCP 11, CEP 11, Tel 11q | 1/1/1 (1) |
| 1 | 1 | 46,XX,t(7;13)(q36;q12) | WCP 7, WCP 13, LSI 13 (13q14), CEP 7, Tel 7q | 0/0 |

| | | | | |
|---|---|---|---|---|
| 1 | 1 | 46,XX,t(7;14)(q33;q32.3) | WCP7,WCP14,CEP7, tel7q, tel14q | 1/0 |
| 1 | 1 | 46,XX,t(7;18)(q32;q23) | WCP 7, WCP 18, CEP 18 | 1/0 |
| 1 | 1 | 46,XX,t(7;19)(q22.3;p13.3) | WCP7, CEP7, tel 7q, tel 19q | 1/1/1 (1) |
| 1 | 1 | 46,XX,t(7;19)(q32;p13.2) | WCP 7, WCP 19, CEP 7, Tel 7q, Tel 19p | 1/1/0 (0)(1*) |
| 1 | 3 | 46,XX,t(7;22)(p22.3;p11.2) | CEP 7, Tel 22q, Tel 7p | 1/1/1 (1) |
| 1 | 1 | 46,XX,t(8;9)(q11.2;q32) | WCP8,WCP9,CEP8,CEP9, tel 8q, tel9q | 1/1/1 (1) |
| 2 | 2 | 46,XX,t(8;10)(q22.1;q22.1) | WCP 8, WCP 10, CEP 10, Tel 8q, Tel 10q | 1/0 |
| 1 | 3 | 46,XX,t(8;10)(q24.3;q24.1) | WCP 8, WCP 10, CEP 10, Tel 10q, Tel 8p, Tel 8q | 1/0 |
| 1 | 1 | 46,XX,t(8;10)(p10;p10) | WCP 8, WCP 10, CEP 10, Tel 10p, Tel 8p | 1/0 |
| 1 | 1 | 46,XX,t(8;12)(q21.2;p13.3) | WCP 8, WCP 12, Tel 12 p, CEP 12 | 0/0 |
| 1 | 1 | 46,XX,t(8;13)(q22.3;q12) | WCP8,WCP13,CEP8, tel8q, tel13q | 1/1/1 (3) |
| 1 | 1 | 46,XX,t(8;18)(q22.1;q12.3) | WCP18,CEP18,tel 81, tel 18q | 0/0 |
| 1 | 1 | 46,XX,t(8;18)(q24.13;q21.2) | CEP18, tel8q, tel18q | 0/0 |
| 1 | 2 | 46,XX,t(8;22)(q24.1;q11.2) | WCP 8, WCP 22, CEP 8 | 1/0 |
| 1 | 1 | 46,XX,t(9;10)(q32;p13) | WCP9,WCP10, CEP10,tel 9q, tel 10p | 1/1/1 (1) |
| 1 | 1 | 46,XX,t(9;11)(q13;q13.5) | WCP 9, WCP 11, CEP 11, Tel 11q | 1/1/0(0)(1*) |
| 1 | 1 | 46,XX,t(9;11)(p24;q23.1) | WCP 9, WCP 11, CEP 11, Tel 9p, Tel 11q | 1/1/1 (1) |
| 1 | 1 | 46,XX,t(9;13)(q22.3;q12.3) | WCP 9, WCP 13, CEP 9, LSI 13 (13q14), Tel 9q | 0/0 |
| 1 | 1 | 46,XX,t(9;13)(p21;q21) | WCP 9, WCP 13, CEP 9, Tel 9p, Tel 13q | 0/0 |
| 1 | 2 | 46,XX,t(9;13)(q22;q14) | WCP 9, WCP 13, CEP 9, LSI 13 (13q14), Tel 9q | 0/0 |
| 1 | 1 | 46,XX,t(9;15)(q22.3;q14) | CEP9, tel9q,tel15q | 1/1/1 (1) |
| 1 | 4 | 46,XX,t(9;16)(q34.3)(p13.1) | WCP9,WCP16,CEP9, tel 9q, tel 16p | 4/2/1/(1) (1*) |
| 1 | 1 | 46,XX,t(9;20)(q32;q13.1) | CEP9, tel9q,tel20q | 1/0 |
| 1 | 1 | 46,XX,t(10;11)(p15;q23) | WCP 10, WCP 11, CEP 10, Tel 10 p, Tel 11q | 1/1/1 (3) |
| 1 | 1 | 46,XX,t(10;15)(q23;q21) | WCP 10, WCP 15, CEP 15 | 0/0 |
| 1 | 1 | 46,XX,t(11;13)(q13.1;q33.2) | CEP11, tel 11q, tel13q | 1/0 |
| 1 | 1 | 46,XX,t(11;14)(p15;q24) | WCP 11, WCP 14, CEP 11, Tel 11p | 0/0 |
| 1 | 1 | 46,XX,t(11;14)(p15;q32.1) | WCP 11, WCP 14, CEP 11, Tel 11p | 1/1/1 (1) |
| 1 | 1 | 46,XX,t(11;14)(q22;q32) | CEP11, tel11q, tel14q | 1/0 |
| 1 | 1 | 46,XX,t(11;14)(q22;q23) | CEP11, tel11q, tel14q | 1/0 |
| 1 | 1 | 46,XX,t(11;15)(p15.5;q22.3) | CEp11, tel 11p, tel 15q | 1/1/1 (2) |
| 1 | 1 | 46,XX,t(11;16)(q23;q22) | WCP11,WCP16,CEP11, tel11q,tel16q | 1/1/1 (1) |

(continued)

**Table 5.18** (continued)

| Patients | Cycles | Karyotype | FISH probes | Embryo transfers/pregnancies/deliveries (# of children born) (# of miscarriages*) |
|---|---|---|---|---|
| 1 | 1 | 46,XX,t(11;17)(q13;q21) | CEP11, tel 11q, tel17q | 0/0 |
| 1 | 1 | 46,XX,t(11;18)(p14.2;p11.2) | CEP18, tel11p,tel18q | 0/0 |
| 1 | 1 | 46,XX,t(11;20)(p13;q11.2) | CEP11, CEP20, tel 11p, tel 20q | 1/0 |
| 2 | 5 | 46,XX,t(11;22)(q23;q11.2) | WCP 11, WCP 22, CEP 11, Tel 11q, LSI 22 (22q11.2) | 4/1/1 (1) |
| 1 | 1 | 46,XX/46,XX,t(11;22)(q23.3;q11.2) | CEP11, tel 11q, tel 22q | 1/1/1 (1) |
| 1 | 6 | 46,XX,t(11;22)(q23;q11) | CEP11,tel11q,tel22q | 5/2/1/(1) (1*) |
| 1 | 1 | 46,XX,t(12;15)(p13.3;q13.3) | WCP 12, WCP 15, Tel 12p, Tel 15q, CEP 15 | 1/0 |
| 1 | 3 | 46,XX,t(12;18)(p11.2;p11.2) | CEP18, tel 12p,tel 18p | 2/0 |
| 1 | 4 | 46,XX,t(12;18)(p13.31;q21.32) | WCP 12, WCP 18, CEP 18, Tel 12p, Tel 18q | 2/1/0(0)(1*) |
| 1 | 2 | 46,XX,t(13;15)(q12.3;q13.3) | WCP13,WCP15,CEP15,tel13q,tel15q | 0/0 |
| 1 | 2 | 46,XX,t(13;15)(q14;q13) | WCP13,WCP15,CEP15,tel13q,tel15q | 1/1/1 (2) |
| 1 | 2 | 46,XX,t(13;21)(q32;q22) | WCP13,WCP21, tel13q, tel 21q | 1/0 |
| 1 | 2 | 46,XX,t(14;18)(q24.3;p11.31) | WCP 14, WCP 18, CEP 18, Tel 14q, Tel 18p | 1/0 |
| 1 | 1 | 46,XX,t(14;20)(q24.1;q11.2) | CEP20, tel14q, tel20q | 0/0 |
| 1 | 1 | 46,XX,t(14;20)(q31;q13.2) | CEP20, tel14q, tel20q | 1/1/1/(1) |
| 1 | 2 | 46,XX,t(14;21)(q24.1;q22.1) | WCP 14, WCP 21, Tel 14q | 1/1/1 (1) |
| 1 | 2 | 46,XX,t(16;17)(p13.3;q11.2) | WCP 16, WCP 17, CEP 17, Tel 16p, Tel 17q | 2/2/2 (3) |
| 1 | 1 | 46,XX,t(16;17)(p13.3;p13.1) | CEP16,CEP17, tel 16p, tel17p | 0/0 |
| 1 | 2 | 46,XX,t(16;18)(p13.1;p11.2) | CEP16, tel 16p, tel 18p | 2/0 |
| 1 | 2 | 46,XX,t(16;19)(q13;p13.3) | WCP16,WCP19,CEP16, tel 16q, tel 19p | 0/0 |
| 1 | 2 | 46,XX,t(17;18)(p13;pter) | WCP17,WCP18,CEP17,tel17p,tel18p | 2/1/1 (2) |
| 2 | 2 | 46,XX,t(19;22)(q13.3;q11.2) | Tel 19p, tel19q,tel22q | 1/0 |
| 1 | 4 | 46,XX,inv(X)(p22.3;q13) | CEP X, Tel Xp/Yp, Tel Xq/Yq | 3/2/2 (2) |
| 1 | 1 | 46,X,t(X;2)(p11.2;q23) | WCP2,WCPX,CEP2,CEPX, tel 2q | 1/0 |
| 1 | 1 | 46,X,t(X;17)(p22.1;q21) | WCP17,WCPX,CEPx,tel17q | 1/0 |
| 1 | 1 | 47,XX,+fis(19)(p10),+fis(19)(q10) | Tel 19p, Tel 19q | 1/0 |
| 1 | 1 | 46,XX/46,XX,+21,der(21;21)(q10;q10) | PB panel, tel21 | 0/0 |
| 1 | 3 | 46,XY,t(1;2)(q42.3;q37.3) | WCP1,WCP2,tel1q,tel2q, PB panel | 1/0 |

| | | | | |
|---|---|---|---|---|
| 1 | 1 | 46,XY,t(1;3)(p34.3;q26.2) | WCP1,WCP3,CEP3,tel1p,tel3q,,CEPx < CEPY, tel21 | 1/1/1 (1) |
| 1 | 1 | 46,XY,t(1;3)(p36.1;q27) | WCP 1, WCP 3, CEP 1, Tel 1p, Tel 3q | 1/1/1 (1) |
| 1 | 1 | 46,XY,t(1;3)(q42.3;q29) | WCP 1, WCP 3, CEP 3, Tel 1q, Tel 3q | 1/0 |
| 1 | 1 | 46,XY,t(1;4)(q41;p22) | CEP 4,tel 1q,tel 4p | 0/0 |
| 1 | 1 | 46,XY,t(1;4)(p32;q21) | WCP1,WCP4,CEP4,tel1p,tel4q | 1 /1 /1/ (2) |
| 1 | 1 | 46,XY,t(1;7)(q11;p13) | WCP 1, WCP 7, CEP 7, Tel 1q, Tel 7p | 0/0 |
| 1 | 8 | 46,XY,t(1;8)(p13;q23) | WCP 1, WCP 8, CEP 8, CEP 1,Tel 1q | 5/0 |
| 1 | 1 | 46,XY,t(1;8)(q42.1;q24.1) | WCP1,WCP8, tel 1q, tel8q | 1 /1 /1 (2) |
| 1 | 1 | 46,XY,t(1;10)(p13;p11.2) | CEP10, tel 1p,tel1 0p,tel 1q | 1/0 |
| 1 | 2 | 46,XY,t(1;15)(p13;q21.2) | CEP15,tel1p,tel15q | 0/0 |
| 1 | 2 | 46,XY,t(1;16)(q32.1;q12.1) | CEP16, tel 1q, tel16q | 2/1/1 (1) |
| 1 | 2 | 46,XY,t(1;21)(p13;q11.2) | WCP 1, WCP 21, CEP 1 | 2/0 |
| 1 | 1 | 46,XX,t(1;22)(q25;q11.1) | CEP1, tel 1q,tel 22q | 1/0 |
| 1 | 1 | 46,XY,t(2;3)(q21;p13) | WCP 2, WCP 3, CEP 2,Tel 2q, Tel 3p | 0/0 |
| 1 | 3 | 46,XY,t(2;3)(p25;q13) | CEP2, tel 2p, tel 3q | 3/1/1 (1) |
| 1 | 3 | 46,XY,t(2;4)(q21.1;q31.1) | WCP2, WCP4, CEP4, tel 2q, tel 4q | 2/1/1 (1) |
| 1 | 2 | 46,XY,t(2;5)(q11.2;q33.3) | CEP2, tel 2q, tel5q | 2/0 |
| 1 | 1 | 46,XY,t(2;6)(p13;p25) | CEP2, CEP6,tel 2p, tel 6p | 1/0 |
| 1 | 1 | 46,XY,t(2;7)(p13;q36) | CEP7, tel 2p, tel7q | 1/1/1 (1) |
| 1 | 1 | 46,XY,t(2;7)(q32.1;q33) | CEP7, tel 2q, tel7q | 1/1/1 (1) |
| 1 | 1 | 46,XY,t(2;8)(p15;p11.2) inv(10)(p11.2;q21.2) | WCP2,WCP8, tel8p,CEP2,CEP8, PB panel | 1/0 |
| 1 | 1 | 46,XY,t(2;9)(q36;p22) | CEP9, tel 2q, tel 9p | 0/0 |
| 1 | 1 | 46,XY,t(2;11)(p13.3;p15.1) | WCP 2, WCP 11, CEP 2, CEP 11, Tel 2p | 1/0 |
| 1 | 1 | 46,XY,t(2;11)(q33;q23.3) | CEP11, tel2q, tel11q | 1/0 |
| 1 | 1 | 46,XY,t(2;14)(q32.2.;q32.3) | CEP2, tel 2p, tel 2q, tel14q | 1/0 |
| 1 | 1 | 46,XY,t(2;17)(p21;q23) | CEP17, tel 2p, tel 17q | 0/0 |
| 1 | 1 | 46,XY,t(2;17)(p23;p25) | WCP2,WCP17,CEP17, tel 2p, tel 17q | 2/1/1 (1) |
| 1 | 3 | 46,XY,t(2;18)(p13;q11) | WCP 2, WCP 18, CEP18 | 2/1/1 (1) |
| 1 | 1 | 46,XY,t(2;19)(p25.1;p13.11) | WCP 2, WCP 19, Tel 2p, Tel 19p, CEP 4 | 1/1/0 (0) (1*) |
| 1 | 1 | 46,XY,t(2;21)(q31.1;q11.2) | WCP2, WCP21, CEP2, tel 2q, tel21q | 1/1/0 (0) (1*ect) |

(continued)

**Table 5.18** (continued)

| Patients | Cycles | Karyotype | FISH probes | Embryo transfers/pregnancies/deliveries (# of children born) (# of miscarriages*) |
|---|---|---|---|---|
| 1 | 6 | 46,XY,t(3;5)(p26;q35.1) | WCP 3, WCP 5,CEP3, tel3p, tel 5q, | 5/1/1 (1) |
| 1 | 4 | 46,XY,t(3;6)(q27.1;p21.1) | WCP 3, WCP 6, CEP 6 Tel 3q, Tel 6p | 4/2/2 (2) |
| 1 | 1 | 46,XY,t(3;7)(p24;p21) | WCP 3, WCP 7, Tel 3p | 1/0 |
| 1 | 2 | 46,XY,t(3;10)(q21;p14) | WCP 3, WCP 10, CEP 10 | 0/0 |
| 1 | 1 | 46,XY,t(3;10)(q21;q24.1) | WCP 3, WCP 10, CEP 10, Tel 3q, Tel 10q | 1/0 |
| 1 | 3 | 46,XY, t(3;10)(q27;q25.2) | CEP10, tel 3q, tel 10q | 2/2/2 (2) |
| 1 | 1 | 46,XY,t(3;14)(q13.3;q24.1) | CEP3, tel 3q, tel14q | 1/0 |
| 1 | 1 | 46,XY,t(3;15)(q23;q13) | CEP15, tel 3q, tel 15q | 1/0 |
| 1 | 2 | 46,XY,t(3;21)(p21;q22.3) | WCP3,WCP21,tel 3p,tel21q | 1/0 |
| 1 | 1 | 46,XY, t(3;21)(q12;q11.2) | Tel 3p, tel 3q, tel 21q | 1/1/0/(0) (1*) |
| 1 | 5 | 46,XY,t(4;5)(q33;q22) | WCP4,WCP5,CEP4,tel4q,tel5q | 5/1/0 (0) (1*) |
| 1 | 1 | 46,XY,t(4;6)(p16;q25.3) | WCP4,WCP6,CEP6,tel4p.tel6q,PB panel | 2/1/1 (2) |
| 1 | 1 | 46,XY,t(4;6)(q25;q21) | WCP 4, WCP 6, CEP 6, Tel 6q | 0/0 |
| 1 | 2 | 46,XY,t(4;8)(q25;p23.1) | WCP4,WCP8, CEP4, tel 4q, tel 8p | 1/0 |
| 1 | 2 | 46,XY,t(4;10)(q21.3;q24.1) | WCP4,WCP10, CEP4, tel 4q, tel10q | 1/0 |
| 1 | 3 | 46,XY,t(4;11)(q33.3;p13) | WCP 4, WCP 11, CEP 4, Tel 4q, Tel 11p | 2/1/1 (1) |
| 1 | 1 | 46,XY,t(4;13)(q35;q22.1) | WCP4, WCP13, tel 4q, tel 13q | 0/0 |
| 1 | 1 | 46,XY,t(4;14)(p16;q23) | CEP4, tel 4p, tel 14q | 0/0 |
| 1 | 2 | 46,XY,t(4;15)(q21.3;q11.2) | WCP4,WCP15,CEP4, tel 4q, tel 15q | 2/0 |
| 1 | 1 | 46,XY,t(4;17)(q31.1;p13) | WCP 4, WCP 17, CEP 4, Tel 4q, Tel 17p | 1/0 |
| 1 | 1 | 46,XY,t(5;6)(q22;q22.2) | WCP 5, WCP 6, tel 5q, tel 6q | 1/0 |
| 1 | 1 | 46,XY,t(5;7)(q21.2;q36) | WCP 5, WCP 7, Tel 7q, CEP 7, Tel 5p | 1/1/1 (1) |
| 1 | 2 | 46,XY,t(5;8)(p15.3;p21.1) | CEP8, tel 5p, tel 8p | 2/1/1 (1) |
| 1 | 2 | 46,XY,t(5;10)(q35.2)(q26.13) | WCP5,WCP10,CEP10, tel5q, tel10q | 0/0 |
| 1 | 1 | 46,XY,(5;12)(p14;p13.33) | CEP 12, Tel 5p, Tel 12p | 0/0 |
| 1 | 1 | 46,XY,t(5;14)(p10;q10) | WCP5,WCP14,tel 5p, trl14q, Cri-du-Chat | 1/1/1 (2) |
| 1 | 2 | 46,XY,t(5;15)(q31;q22) | CEP15, tel5q,tel15q | 1/0 |
| 1 | 1 | 46,XY,t(6;7)(q23;q36) | WCP 6, WCP 7, CEP 7 | 1/1/1 (1) |
| 1 | 1 | 46,XY,t(6;8)(q21;q13) | WCP6,WCP8,CEP6,CEP8, tel6q, tel8q | 1/0 |
| 1 | 2 | 46,XY,t(6;8)(q21;q32.1) | WCP 6, WCP 8 | 1/0 |

| | | | | |
|---|---|---|---|---|
| 1 | 1 | 46,XY,t(6;8)(q23.3;q21.1) | WCP6,WCP8,CEP6,tel6q,tel8q | 2/0 |
| 1 | 1 | 46,XY,t(6;9)(p12;q34.1) | CEP6,CEP9, tel 6p | 0/0 |
| 1 | 1 | 46,XY,t(6;10) | CEP6,CEP10, tel 6q, tel10q | 1/1/0 (0) (1*) |
| 1 | 4 | 46,XY,t(6;13)(q23.1;q14.3) | CEP6, WCP13,WCP6,tel 13q, tel6q | 3/2/2 (3) |
| 1 | 2 | 46,XY,t(6;13)(q23;q32) | CEP6, tel 6q, tel13q | 2/1/1 (1) |
| 1 | 1 | 46,XY,t(6;16)(q21;q13) | CEP6, tel 6q, tel16q | 1/1/1(1) |
| 1 | 1 | 46,XY,(6;16)(p24;p13.2) | CEP6,CEP16, WCP6, WCP16,tel6p,tel16p | 1/1/0 (0) (1*ect) |
| 1 | 4 | 46,XY,t(6;17)(p25;p13) | CEP6, tel6p, tel17p | 4/1/1 (1) |
| 1 | 2 | 46,XY,t(6;18)(qter;qtr) | WCP6, WCP18, CEP6, tel6q, tel18q | 2/1/1 (1) |
| 1 | 2 | 46,XY,t(6;21)(p12.2;q22.2) | CEP6, tel 6q, tel 21q | 2/1/1 (1) |
| 1 | 1 | 46,XY,t(6;21)(q22.2;q22.2) | CEP6, tel 6q, tel 21q | 1/1/1 (1) |
| 1 | 1 | 46,XY,t(7;10)(q36;p11.2) | WCP 7, WCP 10, CEP 10, Tel 7q, Tel 10p | 1/0 |
| 1 | 1 | 46,XY,t(7;12)(p15.1;p13) | CEP7, tel 7p, tel 12q | 0/0 |
| 1 | 1 | 46,XY,t(7;21)(p11.2;q22.2) | WCP7,WCP21,CEP7,tel 7p, tel21q | 1/0 |
| 1 | 1 | 46,XY,t(8;10)(q22.1;q22.1) | WCP 8, WCP 10, CEP 10, Tel 8q | 1/1/1 (2) |
| 1 | 1 | 46,XY,t(8;11)(p23;p13) | WCP8,WCP11,CEP11,tel8p,tel11p | 1/1/1 (1) |
| 1 | 2 | 46,XY,t(8;19)(p23.1;q13.3) | WCP 8, WCP 19, CEP 8, Tel 8p, Tel 19q | 2/1/1 (1) |
| 1 | 1 | 46,XY,t(9;11)(p22;q23) | WCP 9, WCP 11, CEP 11, Tel 9p, Tel 11q | 1/0 |
| 1 | 2 | 46,XY,(10;11)(p11.1;q25) | CEP10, tel 10p,tel11q | 2/1/1(1) |
| 1 | 1 | 46,XY,t(10;13)(p12;p11.2) | WCP 10, WCP 13, CEP 10 | 1/0 |
| 1 | 1 | 46,XY,t(10;16)(p10;q10) | CEP10, tel 10p, tel 16q | 1/0 |
| 1 | 1 | 46,XY,t(10;22)(q24.1;p12) | WCP 10, WCP 22, LSI 22 (22q11.2), Tel 10q, CEP 10 | 1/1/1 (1) |
| 1 | 2 | 46,XY,t(10;22)(q25;q13.3) | WCP 10, WCP 22, LSI 22 (22q11.2), Tel 10q, CEP 10 | 2/0 |
| 1 | 1 | 46,XY,t(11;20)(p15;q13.12) | WCP 11, WCP 20, Tel 11p | 1/1/1 (1) |
| 1 | 1 | 46,XY,t(11;22)(q13.1;q13.3) | CEP11,tel11q,tel22q | 0/0 |
| 1 | 1 | 46,XY,t(11;22)(q23.3;q11.2) | CEP 11, Tel 11q, LSI 22 (22q11.2) | 0/0 |
| 1 | 1 | 46,XY,t(11;22)(q23;q11.2) | CEP11, tel 11q, tel22q | 1/1/0 (0)(1*) |
| 1 | 2 | 46,XY,t(11;22)(q25;q12) | WCP 11, WCP 22, CEP 11, Tel 11q | 1/0 |
| 1 | 2 | 46,XY,t(12;15)(p13.3;q24) | WCP12,WCP15,CEP12,CEP15, tel 15q | 1/1/1 (2) |
| 1 | 1 | 46,XY,t(12;19)(p13.3;q13.4) | CEP12, tel 12p, tel 19q | 1/0 |

(continued)

**Table 5.18** (continued)

| Patients | Cycles | Karyotype | FISH probes | Embryo transfers/pregnancies/deliveries (# of children born) (# of miscarriages*) |
|---|---|---|---|---|
| 1 | 1 | 46,XY,t(12;20)(q23;p13) | WCP12,WCP20,CEP12, tel12q, tel20p | 1/1/1 (1) |
| 2 | 3 | 46,XY,t(13;20)(q22;q11.2) | WCP 13, WCP 20 | 0/0 |
| 1 | 4 | 46,XY,t(13;21)(q32;q22) | WCP13,WCP21,LSI13, tel13q, tel21q | 1/1/1 (1) |
| 1 | 1 | 46,XY,t(13;21)(q14;q11.2) | WCP13,WCP21,tel13q,tel21q | 1/1/0 (0)(1*) |
| 1 | 1 | 46,XY,t(14;15)(q32.2;q26.1) | CEP15, tel14q,tel15q | 1/1/0 (0) (1*) |
| 1 | 2 | 46,XY,t(14;20)(q24.1;q11.2) | WCP14,WCP20,tel14q,tel20q | 0/0 |
| 1 | 1 | 46,XY,t(15;16)(q13;q13) | WCP 15, WCP 16, CEP 15 | 1/0 |
| 1 | 3 | 46,XY,t(15;17)(q24;q21.3) | WCP15,WCP17, tel 15q,tel 17q,CEP15,CEP17 | 3/1/1 (2) |
| 1 | 1 | 46,XY,t(16;20)(q12.3;q13.1) | WCP 16, WCP 20, CEP 16, CEP 20, Tel 16q, Tel 20q | 1/0 |
| 1 | 3 | 46,XY,t(18;20)(p11.2;q13.3) | WCP 18, WCP 20, CEP 18, Tel 18p, Tel 20q | 3/2/1 (1) (1*) |
| 1 | 2 | 46,XY,inv(5)(p13;q13) | Xcyte 5 (Metasystems), Tel 5p, Tel 5q | 2/0 |
| 1 | 3 | 46,XY,inv(5)(p15.3;q33.1) | LSI 5p, tel 5p,tel 5q,Cri-du-Chat | 3/1/ 1(1) |
| 1 | 1 | 46,XY,inv(8)(p22;q22) | CEP8, tel 8p, tel8q | 1/0 |
| 1 | 1 | 46,XY, inv(9) | Conversion failed/no result | 0/0 |
| 1 | 1 | 48,XY,idic(15)(q11.1) | CEP 15, Tel 15q, 15q22,15p11.2 | 1/0 |
| 1 | 1 | Del17p13.1 | CEP17, tel 17p, p53 | 1/1/0 (0) (1*) |
| 1 | 3 | 46,XX,r(15) | CEP15,tel15q, PWP | 2/0 |
| 1 | 1 | 46,XY,t(9;6)(q22;q15q23.1) | G-Banding, WCP 6, WCP 9, CEP 6 | 0/0 |
| *330* | *500* | | | *365/141/115(135)(26*)* |

*WCP* whole chromosome paint, *LSI* locus specific identifier, *Tel* subtelomeric, *CEP* centromeric enumeration probe, *G-Banding* Giemsa Stain, *# of miscarriages

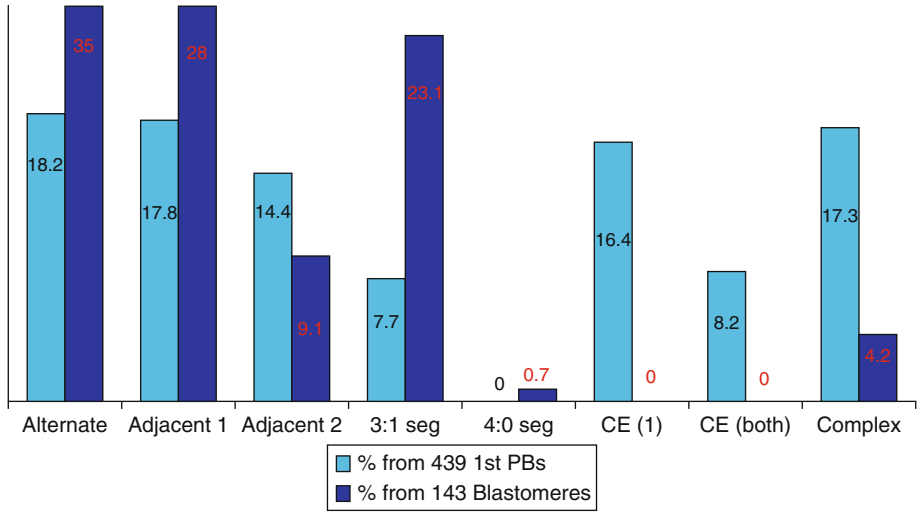



**Fig. 5.27** Distribution of segregation patterns. Bar graph demonstrating the distribution of observed segregation modes for female reciprocal translocations by PB1 analysis versus inferred segregation patterns by blastomere analysis

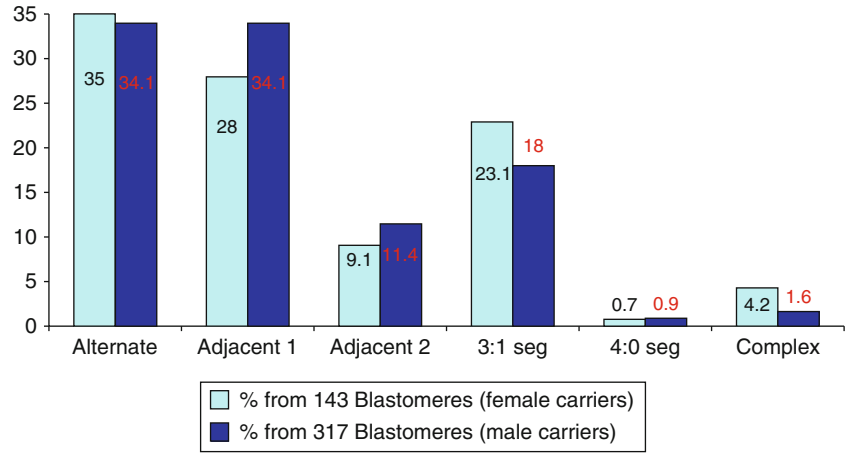



**Fig. 5.28** Distribution of segregation patterns. Bar graph demonstrating the distribution of inferred segregation modes for female reciprocal translocations compared to male reciprocal translocations

being comparable, except for chromatid exchanges, detected only by sequential PB1 and PB2 analysis (16.4%), and complex errors which were higher in PB analysis (17.3% vs.4.2%); 3:1 segregation was found more frequently in blastomere analysis (7.7% vs. 23%).

Segregation patterns for paternally and maternally derived translocation showed similar tendencies, predominantly represented by alternate (35 and 34%, respectively) and adjacent I (28 and 34%, respectively), and lower adjacent II (9.1 and 11.4%, respectively). These meiotic outcomes may explain the proportion of balanced and unbalanced embryos detected, which were predicted in 77.6% embryos obtained from maternally derived reciprocal translocations, leaving only 22.4% suitable for transfer, including 7.8% balanced, 6.9% normal and 7.7% - balanced/normal. On the other hand, unbalanced embryos were predicted in 75% embryos obtained from paternally derived reciprocal translocations, leaving 25% embryos suitable for transfer, including 7.1% balanced, 7.3% normal and 10.6% - balanced/normal. Testing of embryos for maternally derived Robertsonian translocations resulted in prediction of 64.4% unbalanced embryos, with the remaining

35.6% embryos suitable for transfer, which included 13.2% balanced, 10.8% normal and 11.6% - balanced/normal. Similarly, the testing of embryos for paternally derived Robertsonian translocations allowed identification of 53.4% unbalanced embryos, with the remaining 46.6% suitable for transfer, including 10.4% balanced, 7% normal and 29.2% - balanced/normal. Overall clinical pregnancies were obtained in 37% of transfer cycles, with 26% overall delivering healthy children. The data on the meiotic outcome may explain the observed 85% spontaneous abortion rate in patients prior to undertaking PGD procedure, which was reduced to 17% after PGD, demonstrating the tremendous positive impact of PGD on the clinical outcome of pregnancies in couples carrying translocations.

However, in some rearrangements, a prediction of the unbalanced embryos rate may present a challenge, such as in couples with mosaicism for translocation, or highly complex translocation in both parents. We performed a PGD for male patients with mosaicism for balance reciprocal translocation 46,XY,t(10;11)(q23;q23/46,XY), with the application of specific FISH probes. Of six embryos tested by interphase blastomere analysis, three were unbalanced, and three normal/balanced, two of which were transferred resulting in an unaffected pregnancy.

In the other unique case, PGD was performed for a consanguineous couple, with both the patient and her husband carrying balanced Robertsonian translocation (der(13;14)(q10;q10)), in homozygous status for this rearrangement (44,XY,der(13;14;q10;q10) x 2) (Fig. 5.29). Two PGD cycles were performed for this couple, with unaffected embryos identified for transfer in each cycle. It is understood that no normal embryos could be formed in these cycles, so only balanced embryos could have been selected for transfer, with two such embryos available for transfer in the first (Fig.5.29) and two in the second PGD cycles; however, neither resulted in clinical pregnancy, presenting difficulty for interpretation of possible effect on such a complex rearrangement.

Although the application of the conversion technique to visualize chromosomes in single blastomeres improves the accuracy of the diagnosis by analysis of metaphase chromosomes using a combination of commercially available probes, a high frequency of mosaicism in the cleavage stage embryos, arising from anaphase lag or nuclear fragmentation, still presents problems for diagnosis. Follow-up analysis of unbalanced embryos, including those in which chromatid malsegregation or recombination was identified by the analysis of PB1, and subsequent testing of PB2 inferred a balanced or normal embryo, revealed a mosaicism rate of 41%. Different cell lines were present, including normal or balanced, which if investigated only by embryo biopsy, may have led to misdiagnosis. Therefore, PGD strategy for maternally derived translocations may use PB1 and PB2 testing, applying also the blastomere nucleus conversion technique only if further testing.

We have also observed that the embryos with unbalanced chromosome complements have the potential to reach the blastocyst stage of embryo development in extended culture. Of 250 unbalanced embryos identified with FISH cultured for a further period, 78(31%) reached the blastocyst stage, confirming our previous results, that some of the detected chromosomal rearrangement may not be lethal in preimplantation development, being eliminated either during implantation, or postimplantation development [85], explaining an extremely high spontaneous abortion rate in couples carrying translocations. As will be shown in Chap. 6, our data is also in the agreement with the previous reports suggesting as much as six-fold reduction of spontaneous abortions in PGD cycles for translocations [86–88].

In addition to avoiding the use of the expensive and time-consuming customized breakpoint-spanning probes [89], the conversion technique used in the present study was highly accurate based on the follow-up testing of embryos predicted to be abnormal, and confirmatory prenatal diagnosis of the ongoing pregnancies or testing of the newborn babies. The method also allows balanced and normal embryos to be distinguished, which cannot be achieved by currently available interphase FISH analysis.

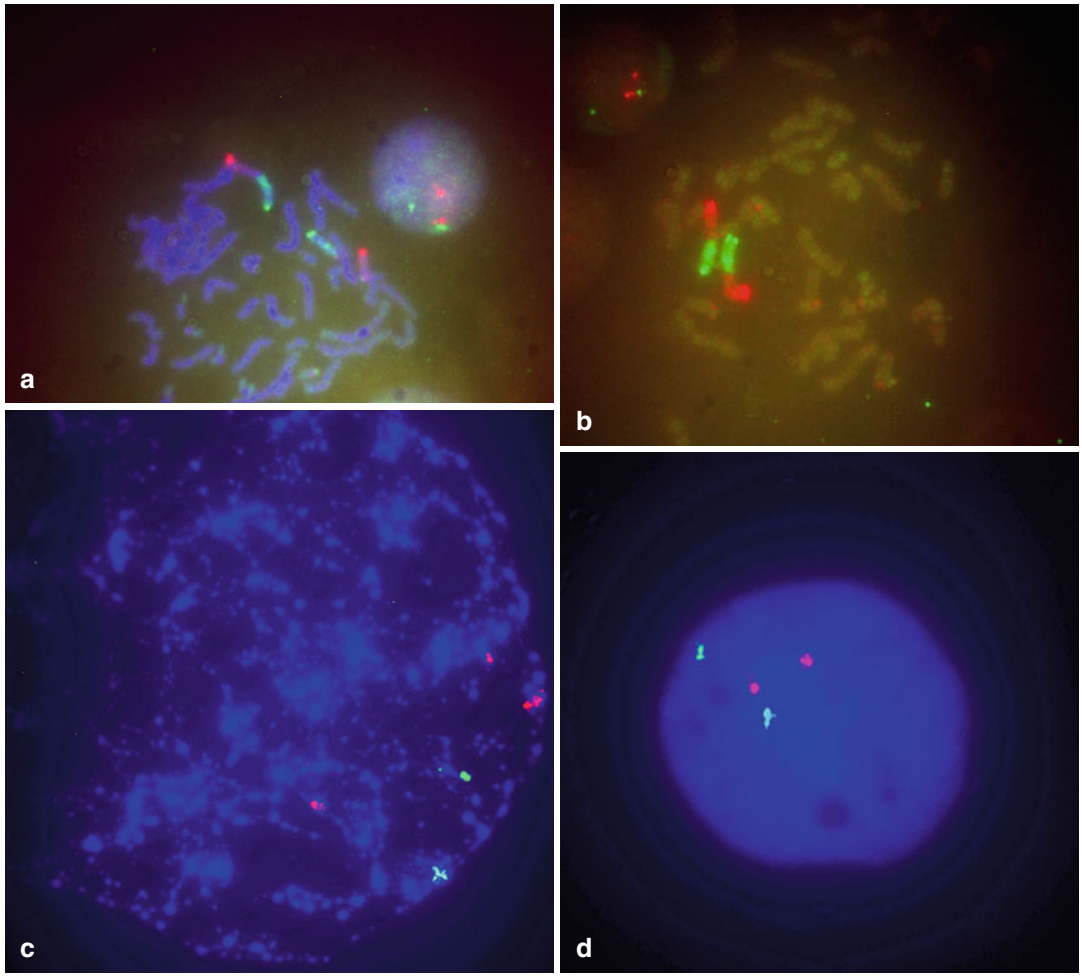



**Fig. 5.29** PGD in a family with both maternally and a paternally derived robertsonian translocation 45,XX,der(13;14)(q10;q10) with involvement of both pairs of chromosomes 13 and 14 (44,XY,der(13;14)(q10;q10),der(13;14)(q10;q10)). Testing was done by interphase nuclei analysis, using probes for Tel 13q in *green* and Tel 14q – in *red*. Balanced embryos are represented by 2 signals of each color, while any odd numbers of any or both signals identify unbalanced embryo. Since both paternal chromosomes are derivatives, no normal embryo can be produced. (**a**) Fluorescent in situ hybridization (FISH) analysis of metaphase chromosomes of a peripheral blood lymphocyte of the patient (female partner). Chromosome 13 is identified by whole chromosome paint (WCP) 13 (*green*) and telomeric probe 13q (*green*), while chromosome 14 – by WCP 14 (*red*) and telomeric14q (*red*). The derivative is identified by fluorescence of both WCP and both telomeric probes in *green* and *red*. (**b**) FISH analysis of metaphase chromosomes of the male partner (father). Two derivatives present. (**c**) FISH image of a nucleus obtained after embryo biopsy presenting 1 signal of 13q and three signals of 14q (unbalanced). (**d**) A normal number of chromosomes, evident in a nucleus by presence of 2 signals of 13q and 14q (balanced)

Because PGD is still practically the only hope for couples with translocations to have an unaffected child of their own without fear of repeated spontaneous abortions or having affected children, increasing numbers of PGD cycles for this indication have recently been performed. The current experience now includes a few thousand clinical cycles, resulting in hundreds of clinical pregnancies and births of unaffected children [78, 79, 86–93]. This experience provides strong evidence that the practice of PGD for couples carrying balance chromosomal rearrangements, which

is clearly the only option for having unaffected offspring, also reduces the six-fold increased risk of spontaneous abortions for these couples. Our data on comparing the pregnancy outcomes before and after PGD in translocations carriers, demonstrated considerable reduction of spontaneous abortions rate from 87.8% to 17.8%, and improvement of take-home baby rates from 11.5% to 81.4% after PGD (see Chap. 6). The extended experience reported here, which represents the world's second largest experience of PGD for translocations, further confirms the clear beneficial effect of PGD on couples carrying balanced translocations.

A few approaches have been developed for improving the accuracy and outcome of PGD of translocations, such as haplotyping by the use of a multiplex fluorescent PCR of highly polymorphic markers [94] and most recently microarray technology, which already replacing all other methods.

This method may be applied together with testing for aneuploidy and single gene disorders, and does not require much preparatory work. Despite being extremely expensive, this technique is already used in practical application to PGD for translocations [95], and, according to our experience, is highly accurate, as also demonstrated in most recent reports of the application of microarray analysis for a combined PGD for aneuploidy and translocations [96, 97]. As the major limitation of this approach is still extremely high cost, of great importance is the progress, currently achieved in this area by Bluegnome (Cambridge, UK), which involves both lowering the cost of the procedure, which is most essential for the future application, and improving the accuracy of PGD for chromosomal rearrangements by array-CGH.

The presented data suggests that patients with translocations, who are at an extremely high risk for spontaneous abortions, should be informed about the availability of PGD for this indication. Awareness of the availability of PGD will permit such couples to establish pregnancies, which are unaffected from the onset, and offer the opportunity to have children of their own, instead of multiple unsuccessful attempts of prenatal diagnosis and subsequent termination of pregnancy. The application of the above approaches and particularly microarray technology will make it possible to perform PGD for a wide variety of structural rearrangements independent of the availability of specific fluorescent probes.

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# Clinical Outcome of Preimplantation Genetic Diagnosis

6

As mentioned in the introduction, after over two decades of application to clinical practice, PGD is no longer a research tool, but become an established procedure, considered by patients as a realistic option to reproduce responsibly without risk of having an affected pregnancy. With such an option, the at-risk couples can achieve the desired family size with no much difference from the couples without the known inherited risk. As will be described below, the available data of approximately hundred thousands of PGD cycles performed by the present time, suggest that the procedure is safe, accurate and reliable, and should be offered to those at need for the procedure, who will otherwise not reproduce because of fear of affected pregnancy or prenatal diagnosis followed by termination of pregnancy [1, 2].

## 6.1 Safety of PGD

Although the majority of PGD cycles are still performed in the USA and Western Europe, increasing numbers are reported from the Eastern Mediterranean and Asian countries. Indications for PGD are also expanding, with more PGD cases being performed for the conditions that have never been practiced in traditional prenatal diagnosis, such as preimpantation gender determination for social reasons [3–5], common late onset diseases with genetic predisposition, and preimplantation HLA typing. However, the majority of PGD cycles are still performed for chromosomal disorders, with the ratio of PGD cycles for chromosomal and single gene disorders, approximately 3:1.

The clinical outcome data are presently available from 7,126 PGD cycles performed in our center, and from 15,885 PGD cycles collected from 39 different centers by ESHRE PGD Consortium [5]. These resulted in 1,775 (25%) and 2,881 (18%) clinical pregnancies per initiated cycles, respectively, and birth of 4,227 healthy children, overall (1,504 and 2,723, respectively), with the multiple pregnancies observed in over one-third of the cases. The overall congenital malformation rate was under 5%, which is not different from population prevalence, of which only half were attributable to the major abnormalities.

No differences were found also in the recent report of the results of a longstanding systematic follow-up study from the world's second largest PGD center [6], which presented the physical findings at birth and up to 2 months of age for 995 children born after PGD in comparison to 1,507 children born after ICSI. Comparison was made for prematurity, mean birth weight, very low birth weight (<1,500 g), perinatal death, major malformations and neonatal hospitalizations in singletons and multiples born following PGD versus ICSI. Compared with ICSI, fewer multiples born following PGD presented a low birth weight.

The detailed testing of consecutive series of 581 children, born after blastomere-based PGD, showed even lower major malformations (2.13%) compared to that in 2,889 ICSI children (3.38%), despite significantly higher overall perinatal death

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rate in the post-PGD children. A thorough, systematic, study of PGD offspring based on a 2-month exam also supported the above data. There were a total of 563 PGD liveborns, 18 stillborns, and nine neonatal deaths. Among these were 300 liveborns after PGD for single gene disorders, seven stillborns and four neonatal deaths; the other cases were after PGD for aneuploidy testing. No differences were found in structural malformations between PGD and ICSI offspring–2.13% vs. 3.38%, respectively. There further were no differences between offspring resulting from PGD for single gene disorders and PGD for aneuploidy. There proved to be no differences in singleton in respect to stillborns, liveborns, or neonatal deaths. In multiple gestations, PGD offspring showed increased perinatal deaths. This is in agreement with our data, showing the major congenital anomaly rate similar –1.7% of 1,230 babies born after PGD [7] (Table 6.1). Average maternal age in this retrospective study was 34.8 years with multiple pregnancies in 24% of cases, with 22.4% twins and 1.6% triplets. Spontaneous delivery in only 49.1% of pregnancy, with cesarean section performed in the rest. Intrauterine growth retardation (IUGR) was found in 3%. Only 33.1% of couples followed recommendation for prenatal diagnosis and 66.9% declined the confirmation testing. Imprinting disorders was in 1/1,230, which was Beckwith Weidermann Syndrome.

In no study have anomalies been disproportionately clustered in any given organ system in either cohort further offering assurance. So PGD does not introduce any extra risk to the overall medical condition of newborn children, and the differences are due to multiple pregnancies [5–8].

**Table 6.1** Major congenital abnormalities observed in 1,027 babies born after PGD

| | |
|---|---|
| Congenital Heart Defects | 9 |
| Limb reduction defect | 1 |
| Syndactyly toes | 1 |
| Thumb abnormality | 1 |
| Hip dysplasia | 1 |
| Malrotation of gut | 1 |
| TE-fistula | 1 |
| Torticolis | 1 |
| Anencephaly | 1 |
| Hypospadia | 3 |
| Pectus excavatum | 1 |
| Total | 21 (1.7%) |

[a]In addition 55 minor abnormalities were seen, including 40 hemangiomas and 12 birth marks [7]

## 6.2 Diagnostic Accuracy of PGD

Over 20,000 PGD cycles have currently been performed for single gene disorders, with the outcome data available for hundreds of clinical pregnancies and babies, suggesting an extremely high accuracy of PGD (see also Chap. 3). Both polar body (PB) based approach and embryo biopsy methods have been used extensively and were shown to be safe and extremely accurate in the leading PGD centers. The most recent report on the PGD accuracy (as described in detail in Chap. 3) involving the testing of a total of 9,036 oocytes by sequential first (PB1) and second (PB2) PB removal, demonstrated 97% amplification efficiency with embryo transfer in 84.2% of the initiated cycles [9]. As a result of this approach, only two misdiagnoses were described in PB testing of over 9,000 oocytes, including one in PGD for fragile-X, and the other in PGD for myotonic dystrophy (see Chap. 3). Both of these misdiagnoses were due to undetected ADO, as only one, or two linked markers, respectively, were available for testing, clearly insufficient for accurate diagnosis. Assuming that these misdiagnoses were observed in 790 PB-based PGD transfer cycles, the accuracy rate of this approach was as high as 99.7% per transfer.

Similar accuracy rate per transfer was reported in this center's overall experience, which is presently the world's largest PGD series for Mendelian disorders, with the majority of PGD cycles still performed by embryo biopsy [9]. This series of 2,158 PGD cycles, performed for 239 genetic conditions, resulted in 677 (40.2%) unaffected pregnancies and birth of 690 healthy children, with a total of only four misdiagnoses (including the above two cases, mentioned). One of these misdiagnoses was due to undetected ADO in PGD for cystic fibrosis (CF) in a mutant double heterozygous embryo, erroneously diagnoses as unaffected carrier [10], and the others were

due to transfer of embryos with predicted low accuracy in PGD for fragile-X, muscular dystrophy and beta-thalassemia, when the couples opted to transfer the embryo tested normal based on insufficient number of markers, leaving the probability for ADO (Chap. 3).

A high accuracy rate was also reported in the above world's second largest PGD experience for monogenic disorders, mentioned, which documented only 0.6% misdiagnosis rate in PGD of 1,443 PGD cycles, one in PGD for myotonic dystrophy and three in PGD for Charcot-Marie-Tooth disease (CMT1A), due to errors in linkage analysis in preparation to PGD [6]. However, the accuracy is much lower in a collection of PGD data from many centers, because of differences in experiences and expertise [11, 12].

## 6.3 Reproductive Outcome of PGD

As shown in (see Chap. 3), an extremely high pregnancy rate (approximately 41.3%) was observed in PGD for single gene disorders, despite transferring of only two embryos per cycle on an average. This may be explained by the fact that these are fertile couples of younger reproductive age under 35 years, compared to the poor prognosis IVF patients referred for aneuploidy testing. It is also of interest that the pregnancy rate in PGD cycles for HLA typing, combined with aneuploidy testing was significantly higher, compared to that in PGD for HLA typing without aneuploidy testing (Table 4.9 in Chap. 4).

In PGD for chromosomal disorders, there is the further obvious interest in PGD for translocations, because of a strong PGD impact on reducing the spontaneous abortion rate in the carriers of balanced translocations [13–17]. The majority of these cycles have been performed in the two largest US centers [13–17], with increasing number of PGD for translocations in other centers worldwide as well. The available experience demonstrates a clear advantage of PGD for translocations over traditional prenatal diagnosis, as shown in Chap. 5, attributable to a poor meiotic outcome, particularly in reciprocal translocations. As mentioned, the accuracy of PGD for translocation has been improved by the introduction of increasing number of subtelomeric probes and the technique of blastomere nucleus conversion to metaphase, currently performed by chemical methods [16], which allows a reliable testing for any complex chromosomal rearrangement (Chap. 5). In addition, PCR-based approaches for translocation detection have been introduced, including haplotyping [18] and microarray technology, which may be performed together with PGD for monogenic disorders [19] and HLA typing (our unpublished data), further improving the accuracy of the procedure. The experience of approximately 5,000 PGD cycles for chromosomal rearrangements, accumulated at the present time, further confirms the previous observations on sixfold reduction of spontaneous abortion rate in translocation carriers (see below), making PGD a preferred option for chromosomal translocations over traditional prenatal diagnosis. Although, the proportion of PGD cycles with detected balanced or normal embryos for transfer was not sufficiently high, especially in reciprocal translocations, the transfer of these normal or balanced embryos resulted in pregnancy rates comparable to those PGD cycles performed for Mendelian disorders (Chap. 5).

As described in Chap. 5, more than half of the preimplantation embryos are chromosomally abnormal from the onset, so have to be avoided from transfer in IVF patients of advanced reproductive age. These biological data provide the background for clinical application of aneuploidy testing, making it obvious that the recent controversy about PGD application in IVF is not about its benefit, as the transfer of chromosomally abnormal embryos should clearly be avoided, but solely concerns the accuracy and reliability of the testing. The high aneuploidy prevalence in oocytes and embryos makes it obvious that without the detection and avoidance of chromosomally abnormal embryos, there is a 50% chance of transferring the abnormal embryos, destined to be lost during implantation or postimplantation development. So, in addition to the clear benefit of avoiding aneuploid embryos from transfer, which contributes to the improvement of the pregnancy outcome of poor prognosis IVF patients, this

should also improve the overall standard of medical practice, upgrading the current selection of embryos by morphological criteria to include the testing for aneuploidy.

The expected obvious benefit of avoiding aneuploid embryos from transfer may explain the widespread application of aneuploidy testing, representing up to 80% of the PGD cycles performed worldwide in an effort to preselect the embryos with highest developmental potential. It is not surprising that most of the large studies have demonstrated the clinical benefit of aneuploidy testing, in terms of the improved IVF outcome through higher implantation rates and reduction of spontaneous abortions in poor prognosis IVF patients, including those of advanced reproductive age, repeated IVF failures and recurrent spontaneous abortions [20–27]. However, none of these studies were randomized, nor had sufficient case numbers to detect a significant increase in live birth rates. Randomized clinical trials in the US (and many other countries) have been difficult to perform because of the high associated cost and the self-pay nature of IVF, as well as the lack of sufficient funding for human embryo research.

## 6.4 Controversy in Assessing Clinical Outcome of Preimplantation Aneuploidy Testing

PGD is still a highly sophisticated procedure, involving the oocyte and/or embryos biopsy, which may have detrimental effect on embryo development if not performed up to the standard [1, 2, 25]. This implies to the FISH technique applied on single cells as well, also requiring sufficient training and experience due to present limitations of the FISH procedure. So the failure of observing the positive effect of aneuploidy testing on reproductive outcome in a few smaller studies may be due to possible methodological shortcomings [28–31]. This may be due to potential detrimental effect of removing two blastomeres instead of one, according to the present PGD guidelines [1, 2], in the first two randomized studies, which definitely could have reduced the implantation potential of the biopsied embryos to the extent that could not be bridged even by preselection of aneuploidy-free embryos [28, 29]. The point is well supported by indirect evidence from the study that assessed the loss of two cells from —five- to eight-cell embryos after freezing and thawing. It was shown in this study that the loss of two cells impacted implantation potential of the embryo by more than 50%, whereas the loss of one cell decreased it by only 10% [32]. The loss of implantation potential proved proportional to the number of cells lost; a single cell loss decreased implantation rate by 10% and two by 20%. The embryo biopsy impact can be validated by such cryopreservation data, since these data reflect embryo damage caused by routine loss of one or more cells – a condition similar to removal of cells for PGD. Overall, the potential improvement in ART outcome caused by selecting against abnormal embryos through PGD should far outweigh the potential damage caused by the biopsy procedure (10%) if only one cell is removed.

Without taking into consideration these technical details, the data was erroneously misinterpreted as the lack on PGD impact of the pregnancy outcome, although even the absence of the differences between PGD and non-PGD groups in the above studies, may have suggested the beneficial effect of preselection of aneuploidy-free embryos, in terms of compensating a detrimental effect of two-cell biopsy at the day 3.

The other report of randomized study that failed to detect the positive effect despite single blastomere biopsy, was also with serious technical problems, providing significant clues as to why PGD failed [30]. In contrast to the experience of laboratories where large numbers of PGD cycles have been performed with low rates of failed analyses (<5% no results) have been encountered, the frequency of biopsied embryos without results in this study was as high as 20%, with the implantation rate only 6% in cycles in which biopsy was performed but diagnosis failed, while these embryos with no results were still replaced. Taking into consideration 14.7% implantation rate in the control non-PGD group, this suggests 59% reduction in implantation potential due

to the biopsy procedure. On the other hand, implantation rate of the biopsied embryos with normal results was 16.8%. Thus, had these been more in line with an expected (10%) proportional reduction of implantation potential, but not such an extensive damage to the embryos as observed, the result should have been positive.

So the study seems to demonstrate that, in inexperienced hands, PGD for aneuploidies can be detrimental. Although it is difficult to identify the problem in the biopsy procedure resulting in such a detrimental effect with an extraordinary reduction of implantation rate, the most reasonable explanation may be the problem with the inappropriate equipment used, that could have compromised the embryo survival [30]. Another factor definitely contributing to the poor results could be the failure to use DNA probes for chromosomes 15 and 22 that account for at least 10% of abnormalities in cleavage-stage human embryos, which could have further reduced the selection potential of the technique. Unfortunately, no confirmatory analysis of the abnormal nontransferred embryos was performed, to evaluate the possible error rate of PGD procedure.

Finally, the average number of available embryos for biopsy and testing was only 4.8, clearly affecting the appropriate preselection of embryos for transfer. It was previously demonstrated that there must be at least six embryos for biopsy and potential replacement in order to detect an increase in live birth rate after PGD [25, 26]. Because in this study many patients must have had only —two to three embryos biopsied; and even if biopsy and diagnosis were done optimally, there would have been little beneficial effect. Probably admitting this, the authors defined their procedure as "screening," which presumes the possibility of errors and the need for the additional confirmatory diagnosis, which, however, was not applied.

Despite the above methodological shortcomings, which have been heavily criticized in the literature [33–36], the ASRM Practice Committee misinterpreted this in favor of transferring embryos without aneuploidy testing [37]. This suggests the alternative of incidental transferring of chromosomally abnormal embryos, as every second oocyte or embryo obtained from poor prognosis IVF patients is chromosomally abnormal, and if not avoided from transfer are destined to be lost before or after implantation. In fact, only one in ten of the chromosomally abnormal embryos may survive to the recognized clinical pregnancy, 5% survive to the second trimester, and 0.5% reach the birth, suggesting that the majority are eliminated before or during implantation, being a major cause of a miserable implantation rate in poor prognosis IVF patients, and explaining a high fetal loss rate in these patients without PGD. This has been demonstrated by testing of products of conception from poor prognosis non-PGD IVF patients, which confirmed the high prevalence of chromosomal aneuploidy in the absence of PGD. Of 273 cases tested, 64.8% were with chromosomal abnormalities, up to 79% of which could have been detected and avoided from transfer using PGD [38].

Although randomized controlled studies may, of course, still be useful to quantify the clinical impact of preselection of aneuploidy-free zygotes for genetic counseling purposes, it is also obvious that in order to achieve the expected benefit, the testing should first of all not damage the embryo viability and be performed accurately according to the available standards, mentioned. In other words, there seems to be actually no controversy in the uselessness of aneuploid embryos transfer, the major issue being the safety and reliability of aneuploidy testing, which will no doubt, be further improved in the near future.

## 6.5 Reproductive Outcome Before and After PGD in Same Couples

In the absence of sufficient data of the well-designed randomized controlled studies, the beneficial impact of PGD have been also demonstrated by the comparison of reproductive outcome in the same patients with and without PGD, with the assumption that the previous reproductive experience of the patients may serve as an appropriate control for PGD impact. In the two large series, overall comprising over 500 couples, implantation, spontaneous abortions and

take-home baby rates were analyzed before and after PGD, demonstrating significant improvement after PGD [39, 40]. This included an almost fivefold improvement in implantation rate, and threefold reduction of spontaneous abortion rate, which contributed to more than twofold increase of take-home baby rate after PGD, suggesting the obvious clinical usefulness of aneuploidy testing for IVF patients with poor reproductive performance (Fig. 6.1). These have been further confirmed by a number of reports presented during the 8th PGDIS 2008 Barcelona Conference, with the total of reproductive outcomes analyzed in close to 1,000 patients [41]. The impact of PGD is even higher in translocation patients, with considerable reduction of spontaneous abortion rate after PGD (Fig. 6.2), resulting in a corresponding increase of the take-home baby rate [39, 40].

In the light of these data, the current IVF practice of selection of embryos for transfer based on morphologic criteria may hardly be an acceptable procedure for poor prognosis IVF patients. In addition to an extremely high risk of establishing an affected pregnancy from the onset, this will significantly compromise the very poor chances of these patients to become pregnant, especially with the current tendency of limiting the number of transferred embryos to only two, leaving only a single embryo on the average with a potential chance of reaching the term. Although culturing embryos to the day 5 (blastocyst) before transfer may allow, to some extend, to preselect developmentally more competent embryos compared to the day 3, at least some aneuploid embryos will still be capable of developing to blastocyst [42, 43]. As we demonstrated in Chap. 5, an unexpected high proportion of embryos even with autosomal monosomies reach the blastocyst stage, as well as unbalance translocations. So these abnormal embryos will not be eliminated in the current shift to the blastocyst transfer and may implant and lead to fetal loss, compromising the outcome of pregnancies resulted from the

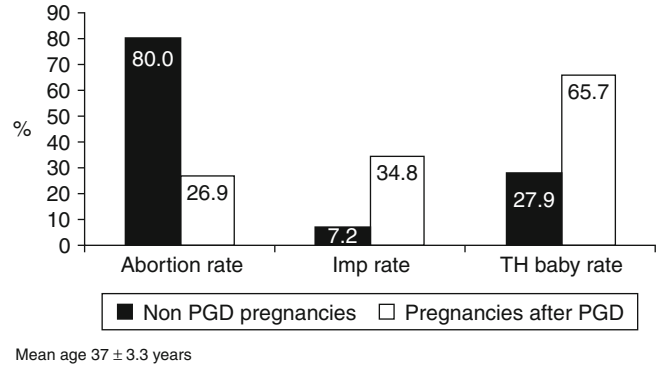


**Fig. 6.1** Bar graph representing the obstetric history of 432 patient/pregnancies before and after preimplantation genetic diagnosis for aneuploidy. Retrospective analysis shows a significantly lower abortion rate, a significantly higher implantation rate and a greater than twofold increase in the take home baby rate. Of these PGD patients 82.1% were greater than 35 years of age

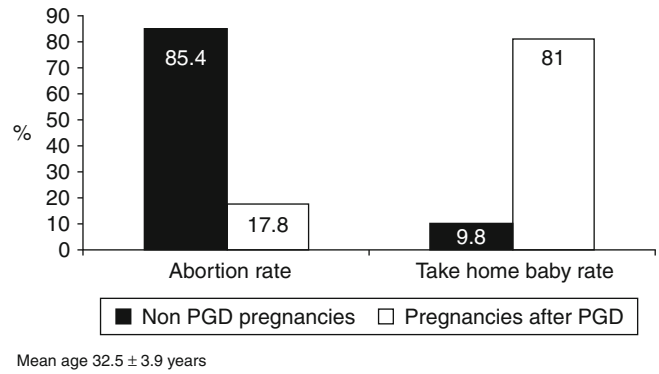


**Fig. 6.2** Bar graph representing the retrospective analysis of the previous obstetric history in relation to the 44 patient/pregnancies that occurred following preimplantation genetic diagnosis for chromosome translocations. A significantly lower abortion rate (15.9%) was realized along with a significantly higher take home baby rate of 82.9%

implanted normal embryos in multiple pregnancies. In fact, multiple pregnancies represent a severe complication of IVF, as can be seen from the mortality rate associated with triplet pregnancies, including stillbirth, neonatal and perinatal death of 32/1,000, 79/1,000 and 109/1,000, respectively [44]. The risk of acquired disorders is also increased following the birth of twins and triplets compared to singleton birth. For example, the estimated rate of cerebral palsy is 8.8/1,000 in twins, 16.9/1,000 in triplets, compared to 2.7/1,000 in spontaneous conceptions. This is even higher in survivors to 1 year, which is 7.3/1,000 in twins, 29/1,000 in triplets, in contrast to 1.6/1,000 in singletons [45, 46]. As these children are also characterized by low birth weight, the higher prevalence of neurological, psychomotor and mental abnormalities is also observed. So the avoidance of multiple pregnancies is one of the urgent problems of ART, which may, in future, be avoided by preselection and transfer of a single blastocyst with the greatest developmental potential to result in healthy pregnancy. Such testing is presently possible for the nuclear abnormalities and may soon become realistic for cytoplasmic disorders as well. Thus, multiple pregnancies may, in future, be avoided by preselection and transfer of a single aneuploidy-free blastocyst with the greatest developmental potential to result in healthy pregnancy, as there seems to be no reason of deliberately transferring chromosomally abnormal embryos, destined to be lost during implantation or post-implantation development.

It may, therefore, be predicted that, with the future improvement of safety and accuracy, PGD may definitely contribute to improving the overall standards of the assisted reproduction practices, by substituting the presently practiced selection of embryos for transfer using morphologic parameters by the preselection of chromosomally normal embryos with the higher potential to result in pregnancy.

However, patients should be aware of the limitations of the methods for aneuploidy testing, as at least half of chromosomally abnormal embryos are with mosaicism, which is the major challenge in the improving the accuracy of PGD for aneuploidies, performed by embryos biopsy. As overall prevalence of aneuploidies in oocytes and embryos seems to be comparable, suggesting that mosaic embryos may originate from the aneuploid oocytes through the process known as "trisomy rescue," the further improvement of PGD accuracy may, in future, require testing of both oocytes and the resulting embryos. This may be achieved by a sequential biopsy of both PB1 and PB2 and single blastomere from the resulting embryo, so that both meiotic and mitotic errors could be excluded. In addition, the information of both the oocyte and the embryo chromosome sets will make it possible detecting the potential uniparental disomy cases, which may be present in one-third of the normal disomic embryos, originating from trisomic oocytes. Collecting of this unique information may also be useful in finding a possible explanation for at least some of the cases of imprinting disorders, such as BWS, as reported in one of our cases, in association with assisted reproductive technology, discussed above in more detail. The fact that more than half of the IVF patients are 35 years and older, and that more than half of their oocytes are with aneuploidies, avoiding the transfer of the embryos resulting from these oocytes through PGD for aneuploidies should be useful in avoiding the transfer of embryos with possible imprinting disorders.

Thus, detection and avoidance of transfer of the embryos with genetic abnormalities using PGD is an important alternative to the embryo transfer based on morphological criteria in the current IVF practice. Although originally introduced for preexisting genetic conditions as an alternative option to prenatal diagnosis, PGD has become of special value for assisted reproduction practices, because genetic factors contribute considerably to the infertility problems. With the majority of the IVF patients being of advanced reproductive age, PGD provides an obvious tool for preselection and avoidance from transfer of the embryos with the age-related aneuploidies, the major contributors to spontaneous abortions and implantation failure.

## 6.6 Possible Impact of Aneuploidy Origin

As described in Chap. 5, preliminary data suggest that there may be differences in the embryo viability depending on the meiotic origin of aneuploidy. On one hand, there is no difference in prevalence of aneuploidy originating from the first and second meiotic division, but only the trisomies of the first meiotic origin are found in recognized pregnancies and at birth. On the other hand, some of the chromosome-specific errors (such as chromosome 16 and 22 errors) originate predominantly in meiosis II, although exclusively meiosis I error of these chromosomes observed in spontaneous abortions. Although the mechanism of such differences in embryo viability depending on the meiotic origin is not understood, it may be due to loss of heterozygosity or higher homozygosity of the embryos originating from meiosis II errors of the genes located in respective chromosomes, which may lead to imprinting of paternal or maternal genes located in these chromosomes. Although there is no proof of the established imprinting genes in the chromsomes in question, there are a few case reports of a possible imprinting on chromosome 16, affecting fetal development or being associated with cancer (see Chap. 5).

There may be also other factors affecting the reproductive outcome of aneuploidy testing, which include a particular stage of preimplantation development when biopsy procedure is performed. For example, as described in Chap. 5, there is discrepancy between PB1 data and that obtained at the cleavage stage. Namely, the PB1 data show predominantly missing signals, predictive of predominant trisomy at the cleavage stage. In contrast, the data obtained in PGD for aneuploidy at the cleavage stage show the predominance of monosomies, which contradict to the findings in recognized pregnancies as well. In fact, significant proportion of the cleavage stage monosomies have not been confirmed after reanalysis (Chap. 5), and many of them are not detected at the blastocyst stage. This may suggest that some monosomies are eliminated before implantation and have no biological significance, reflecting the poor viability of monosomic embryos and their degenerative changes.

The high rate of mosaicism will also heavily contribute to false-positive and false-negative results. The fact that the overall mosaicism prevalence does not show a relationship with maternal age may suggest that a significant proportion of mosaicism is either artifactual and of no clinical relevance, or simply transitional without affecting the embryo viability. It may also be the consequence of degenerative processes in the embryos prior to embryo arrest. On the other hand, a certain fraction of mosaicism is still dependant on maternal age, probably deriving from the aneuploid zygotes, such as trisomics, some of which may result in disomic embryos, due to selective disadvantage of abnormal cells, with also a chance of forming uniparental disomies in one-third of them. Such cases were incidentally detected in PGD for single gene disorders, as well as in the process of haplotyping for preimplantation HLA typing.

The data may explain the above-mentioned controversy on the clinical impact of PGD for aneuploidies, as majority of centers perform aneuploidy testing at the cleavage stage, which may not be the ideal choice for aneuploidy detection, unless it can be coupled with additional analysis, such as PB analysis or blastocyst biopsy. To further clarify the utility of each of the approaches, studies based on sequential PB and embryo biopsy may be useful, to investigate the relative impact of each of these tests in improving the accuracy of detection of aneuploidy-free embryos for transfer.

## 6.7 Possible Impact of 24 Chromosome Aneuploidy Testing

One of important limitation of the available material is that only limited number of chromosomes was tested, so the transfer of embryos with undetected chromosome anomalies could have contributed to poor reproductive outcome. This has currently been overcome by the application of microarray technology for 24 chromosome aneuploidy testing. So on one hand, the choice of an appropriate stage for biopsy procedure, and

on the other, the application of 24 chromosome aneuploidy testing, may allow improving the accuracy of the procedure and quantifying the actual impact of preselecting of aneuploidy-free embryos for transfer on the IVF efficiency.

Recent reports on the application of 24 chromosome testing have already demonstrated beneficial impact. There were two major approaches, one based on PB biopsy, and the other performed on blastocyst biopsy samples, as it provides sufficient material not only to identify the loss or gain of specific chromosomes, but also detect the possible mosaicism, exceeding 10%.

The first approach was recently undertaken to determine whether PB1 and PB2 biopsy approach followed by 24 chromosome testing enables a reliable, timely and accurate identification of maternal contribution to the chromosomal status of the corresponding zygote [47, 48]. For this purpose, array-based CGH analysis was used, which allowed completing both PB1 and PB2 analysis within 12 h, on day 2 of preimplantation development, to perform fresh transfer in participating centers. Accurate identification of the maternal contribution to the chromosomal status of the zygote was achieved in more than 90% of cases, the remaining showing the diagnostic problem mainly due to amplification failure or high noise of the signals.

Follow-up testing of corresponding abnormal zygotes showed concordance rate of aneuploidies to the PB1 and PB2 results in 130 of 138 (94%) zygotes, one of which appeared to be with different aneuplody compared to prediction, while the remaining seven were euploid, despite the expected aneuploid results. The latter was explained by possible compensation of aneuploidy of PB1 by corresponding aneuploidy in PB2 or sperm. As expected the aneuploidy rate was higher than that detected by FISH, which could have missed 23.7% of the corresponding aneuploidies.

Although at least one zygote was predicted to be affected in 41 of 42 cycles, euploid zygotes were available for transfer in 23 cycles. Overall, 39 euploid embryos were transferred (1.6 per cycle on an average), resulting in eight clinical pregnancies (33% per transfer), and birth of seven unaffected children (the implantation rate was 26% per embryo transferred). Although, the data is not sufficient for conclusions, the results of this study suggest that the array CGH analysis of PB1 and PB1 for 24 chromosome testing is accurate and reliable and allows performing PGD in time for fresh transfer, resulting in an acceptable clinical outcome.

Improved clinical outcome was reported also in CGH and array-CGH testing for 24 chromosome coupled with blastocyst biopsy on day 5 and vitrification for embryo cryopreservation [49–55]. While CGH technique is too much time consuming and labor intensive, which make its future use problematic, array-CGH and Single Nucleotide Polymorphism arrays (SNPa) are in increased use, showing significant impact of preselection of aneuploidy-free embryos on the outcome of PGD using 24 chromosome aneuploidy testing. However, clearly, the data are preliminary and not sufficient for conclusions. As these techniques allow analytical automation, avoiding the human-factor contribution to the results, they seem to be a method of choice for the future. While it may be predicted that preimplantation genetic counseling and aneuploidy testing will soon become a standard practice for IVF patients of advanced maternal age, it cannot be excluded that preselection of aneuploidy-free embryos may appear even of higher value for younger IVF patients, because of much higher number of oocytes available for testing. So the IVF patients will need to be informed about the availability of PGD, so they could be able to use the option of preimplantation genetic counseling. This will definitively contribute to improving the standards of the assisted reproduction practices, substituting the presently practiced "blind" selection of embryos for transfer, using morphological parameters, by the preselection of chromosomally normal embryos with the highest possible potential to result in pregnancy. As a tool for a reliable preselection of aneuploidyfree embryos, PGD will potentially contribute not only to prevention of the birth of children with chromosomal disorders, but will also be a useful tool for the improvement of the efficiency and standards of assisted reproduction.

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# 7 Preimplantation Diagnosis and Establishment of Disease and Individual Specific Human Embryonic Stem Cell Lines

As described in Chap. 4, PGD provides an exciting possibility for obtaining HLA matched stem cells for treatment of siblings with bone marrow disorders. This involves preselection of HLA matched embryos in couples with affected siblings requiring the compatible stem cell transplantation, obviating the need for therapeutic cloning, which is highly controversial at the present time. However, in emergency situations, it may be too late to wait until the HLA matched child is born, so other possible approaches need to be developed. For example, some of the tested embryos fail to reach the developmental stage to be considered for transfer, so they might be used for establishment of human embryonic stem cell (hESC) lines. Alternatively, partially matched embryos may be also considered for this purpose, provided the patients provide the consent. Although, in principal a single cell removed from cleaving embryo together with the embryo biopsy may allow establishing of hESC from this single cell for using as a stem cell source for transplantation before the birth of HLA matched child, this does not seem practical, as removing additional material from the embryos at this stage may totally compromise the viability of the embryo.

The technique for the establishment of hESC lines was introduced more than a dozen years ago from the inner cell mass of the preimplantation blastocyst [1]. To provide hESC lines for research purposes, the NIH repository of ESC lines was set up, which initially contained 78 lines, of which only 11 have met NIH scientific criteria, including the presence of L-alkaline phosphatase (TRA-2-39), Oct-4, high molecular weight glycoproteins (antibodies TRA-1-60, TRA-1-81), stage specific embryonic antigens (SSEA-3, SSEA-4), euploid karyotype and teratoma formation in SCID mice [2]. The list of hESC lines in the NIH registry is currently being extended, following Executive Order 13505, entitled "Removing Barriers to Responsible Research Involving Human Stem Cells." The Order was issued by President Obama on March 9, 2009, lifting the ban regarding the use of Federal funds for human embryonic stem cell research (NIH Stem Cell Information Page). However, the U.S. Court of Appeals overturned this permission of federally funded research in October 2010, which was in effect until July 27, 2011, when this court ruling was dismissed by Federal Judge. This currently allows continuance of federal funding for stem cell research, using the NIH hESC lines registry, which contained 136 lines at the end of 2011, of which nine are with different genetic abnormalities (see below).

## 7.1 Sources for Establishing Human Embryonic Stem Cell Lines

Although according to the standard procedure, the main sources of ESC are the human blastocysts [1–3], the morula stage embryos were also attempted, as well as embryonic germ cells [4–6]. The first attempt to isolate ESC from morula stage embryos was reported almost 50 years ago in rabbit embryos [7], and then was also achieved from the morula stage embryos in mink, mouse and

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cow, which were reported to be similar to ESC isolated from the blastocyst stage embryos [4–6].

The first ESC lines from the morula stage human embryos were obtained in our hESC program, which met all the above NIH criteria [8]. The method involves the removal of zona pellucida from morula stage embryos, placement of the embryo under middle density feeder layer for several days, spreading the resulting primary cells outgrew into the feeder layer, which are then disaggregated with EDTA or EGTA, and transfer (loose cells) back to the feeder layer to proliferate, isolating and propagating the fast proliferating colonies further.

Approximately, one quarter of the morula stage embryos resulted in the initial outgrowth, yielding ESC lines in 20% of cases, which is not significantly different from the efficiency of the derivation of hESC lines originating from blastocyst. The typical human morula derived stem cells are shown in Fig. 7.1. In contrast to the above methods, the establishment of the human ESC from blastocyst involves immunosurgery, requiring the isolation and placement of the inner cell mass (ICM) on a feeder layer. In fact, this method is more practical and presently used as a standard procedure for the establishment of hESC lines. As mentioned, no morphological differences between human ES-cells originating from ICM and from morula were observed, including the pattern of the above marker expression mentioned (Table 7.1), with the only exception that the morula derived ESC are more heterogeneous (Fig. 7.1). The established human ESC lines were maintained in vitro up to 15 passages before freezing in sufficient amounts, with the control thaw out, with no differences in the efficiency of obtaining ESC lines depending on the source.

To induce the differentiation of human ES cells into different cell types, the cells were cultured to form embryonic bodies (Fig. 7.2), which were isolated with subsequent disaggregation and plating of clusters of cells. The cultured clusters of cells showed a wide range of cell types belonging to ectoderm, endoderm and mesoderm, and spontaneously differentiated in vitro into a variety of cell types, including neuron-like cells with dendrites and contracting primitive cardiocyte-like cells. The directed differentiation of ES cells was achieved also for ciliated epithelia of the lung, secretary epithelia in the gut like structures, insulin-producing beta cells, and the production of hematopoietic precursor cells, following a co-culture of embryonic stem cells with mouse bone marrow cell line [9–12]. It was shown that the neural progenitors differentiate in-vitro into astrocytes, oligodendrocytes and mature neurons, which were able to respond to host signals and were capable of constructing the neuronal and glial lineages, following their transplantation into the developing brain [13]. Controlling differentiation into pure populations of

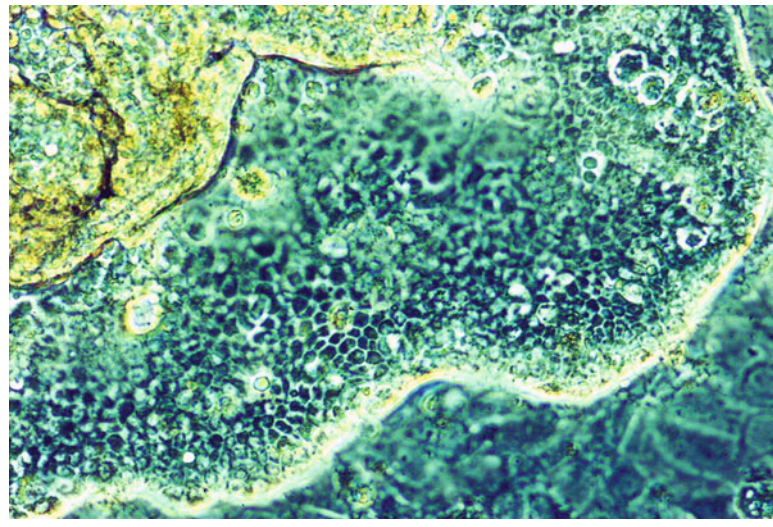

**Fig. 7.1** Morphology of ES-cells derived from morula

**Table 7.1** Markers for human ES-cells derived from different stages

| Cell lines | TRA-2-39 (AP) | Oct-4 | SSEA-3 | SSEA-4 | TRA-1-60 | TRA-1-80 |
|---|---|---|---|---|---|---|
| ICM-ES | + | + | + | + | + | + |
| Bd-ES | + | + | + | + | + | + |
| Md-ES | + | + | + | + | + | + |

*ICM-ES* inner cell mass derived SC, *Bd-ES* blastocyst derived SC, *MD-ES* morula derived ES

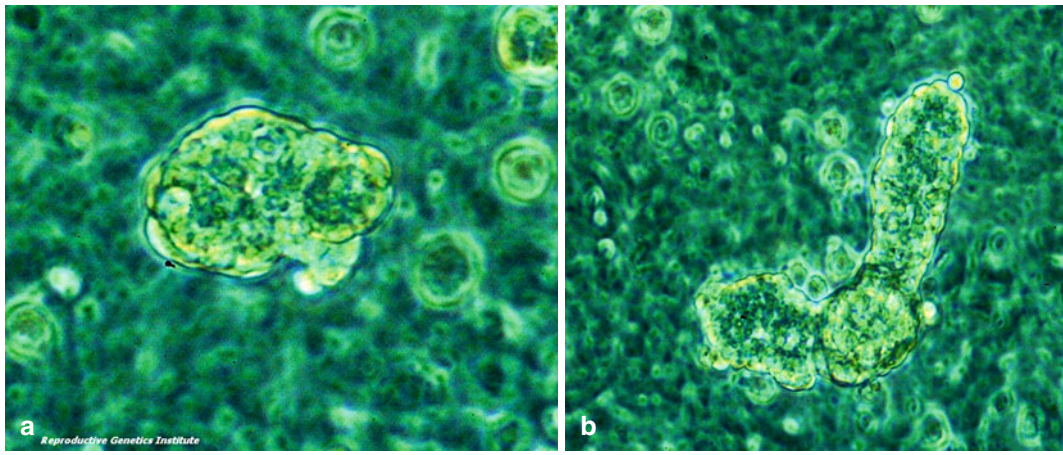


**Fig. 7.2** Formation of embryonic bodies in the process of differentiation in vitro. Two different morula derived ES-cell lines are shown on the *left* and *right*

specific neural cells eventually formed the basis of therapy for some neurodegenerative disorders and spinal injuries, recently approved for application in humans in a few clinical trials. A considerable progress has been achieved also in differentiation of hESC lines into hematopoietic cells, which may in future be used for transplantation treatment of congenital and acquired bone marrow failures [14]. These developments provide an obvious potential for the forthcoming therapeutic use of hESC lines in clinical practice.

## 7.2 Human Embryonic Stem Cell Lines with Genetic Disorders

The above developments made it possible to initiate the establishment of repository of hESC lines with different genetic abnormalities from PGD derived embryos as a novel source for derivation of hESC lines [15–19]. During the PGD practice, the mutation free embryos are transferred back to the patients, while those with genetic abnormalities are donated for research and used for establishment of ESC lines, as described above. This also provides a unique opportunity of investigating the potential of establishing ESC lines depending on the genotype.

### 7.2.1 Human Embryonic Stem Cell Lines with Chromosomal Disorders

Based on our ongoing PGD work described in previous sections, we attempted the derivation of hESC lines from the embryos with a variety of single gene and chromosomal disorders, which showed a poor outcome of hESC lines from the embryos with autosomal aneuploidies [20]. Also due to expected selective disadvantage of abnormal cells in culture, some of the hESC lines deriving from chromosomally abnormal embryos appeared to have normal karyotype, which was also reported by other authors [21, 22]. On the other hand, it was also reported that there may be a risk of the de novo chromosomal abnormalities in the process of the propagation and maintenance of hESC lines [23]. One of such incidental

chromosomal abnormalities was detected in the hESC line, distributed from NIH registry to more than hundred research laboratories around the world. This human ES cell line was obtained from the Wisconsin-based stem cell registry WiCell, and have had a normal karyotype 46, XX which was stable through the establishment and maintenance for several months, with the ability to differentiate with the formation of neural and beating cardiac muscle cells. Karyotyping changes, involving the gain of the chromosome 17q, were observed in three independent hESC lines on five independent occasions, together with the occasional gain of chromosome 12, which was suggested to be attributable to a selective advantage of these cells to the propagation of undifferentiated cells. This phenomenon created a concern over the use of hESC lines for stem cell-based therapies, because cytogenetic changes may be only the part of genetic abnormalities acquired in the process of the establishment, maintenance and differentiation of hESC lines.

The most recent International Stem Cell Initiative [24] analyzed 127 human embryonic stem cell lines, from 39 laboratories worldwide, including the lines from our collection, for genetic changes occurring during culture. Most cell lines were analyzed at an early and late passage, and were shown to remain cytogenetically normal, but a progressive tendency to acquire karyotypic changes on prolonged culture were also observed, commonly affecting chromosomes 1, 12, 17 and 20. SNP analysis revealed that genomic structural variants also appeared sporadically but no common variants related to culture were observed on chromosomes 1, 12 and 17. However, overlapping structural variants were acquired on chromosome 20p11.21 during culture of multiple cell lines, suggesting the anti-apoptotic gene, BCL2L1, as the most likely driver of culture adaptation in this chromosomal region

This also makes initial genetic testing of the embryos used as a source of ESC lines one of the basic requirements, making PGD derived embryos an excellent source for obtaining hESC lines, because the embryos obtained from PGD are well tested, with the genotype of the potential ESC line known from the onset. As will be described below, the efficiently of obtaining ESC lines from the embryos with single gene disorders is not affected (see the list of hESC lines with different genetic abnormalities), while it is not the case for chromosomal abnormalities, which may significantly affect the outcome, and may depend on the origin on aneuploidy [20].

A few hESC lines with chromosomal disorders were reported previously, including those with trisomy 13, triploidy and 2 with mosaicism [2, 25]. Our collection of hESC lines contains 14 hESC lines obtained from the embryos with chromosomal disorders, including four lines with translocations, one with trisomy 21, one with trisomy 13, one with trisomy 14, one with triploidy, two with trisomy 12 and four with aneuploidy of sex chromosomes, including 45, X plus Marker chromosome, 47, XXX, and two with 47, XXY, one of which was derived from the same embryo that was the source of the hESC line with Emery–Dreifuss (carrier) type muscular dystrophy (see further details in Table 7.2).

However, this is only a small proportion of those embryos from which the derivation of hESC lines were attempted, as the majority of the attempts failed. In fact, as shown in Chap. 5, not all embryos could actually even reach to blastocyst stage, to attempt the establishment of ESC lines, depending also on the origin of aneuploidy, which may also have impact on the success of obtaining hESC lines.

To investigate the possible impact of aneuploidy type on the establishment of hESC lines, the efficiency of obtaining hESC lines were investigated depending on the origin of aneuploidies [20]. The first group included those obtained from the embryos with exclusive PB diagnosis, where the blastocysts originated from zygotes tested positive for monosomy, trisomy or multiple errors in all cells. Second group was also diagnosed by PB1 and PB2 analysis, originating from blastocyst with balanced karyotype, following the sequential meiosis I and meiosis II errors. Such embryos either have a stable diploid karyotype or showed a predisposition for further post-zygotic and thus mitotic non-disjunction events, as described in Chap. 5.

**Table 7.2** List of hESC lines with cytogenetic abnormalities (14 lines)

| | ID # | Stem cell karyotype | PGD results |
|---|---|---|---|
| 1 | hESC-104 | 69,XXY | Triploid |
| 2 | hESC-145 | 47,XXY | N/A |
| 3 | hESC-168 | 46,XX, der(4) t(4;13) | translocation t(4;13) |
| 4 | hESC-184 | 47,XX,+13 | Trisomy 13 |
| 5 | hESC-208 | 47,XX,+14 | N/A |
| 6 | hESC-245 | 47,XXY | Emery-Dreifuss muscular dystrophy, X-linked; EDMD |
| 7 | hESC-252 | 47,XXX | N/A |
| 8 | hESC-257 | 47,XY + 12 | N/A |
| 9 | hESC-278 | 47,XY + der(21)t(2,21) | translocation t(2;21) |
| 10 | hESC-300 | 46,X + mar | N/A |
| 11 | hESC-310 | 46,XX (t14;17) | translocation t(14;17) |
| 12 | hESC-321 | 47,XX + 21 | Trisomy 21 |
| 13 | hESC-339 | 46,XX iso (17q) | translocation t(17;18) |
| 14 | hESC-359 | 47,XX + 12 | N/A |

The remaining group was either with normal PB1 and PB2, or without PB analysis, with abnormalities deriving from mitotic errors, detected by blastomere analysis, usually as mosaicism.

The data showed that the plating efficiency for hESC originating from blastocyst carrying a proven meiotic error was 12%, compared to that for hESC originating from blastocysts with balanced meiotic (21.6%) or mitotic errors (9.1–12.2%). So the plating efficiency differ significantly depending on the origin of aneuploidy, with very low plating efficiency from the embryos with prezygotic aneuploidy, such as those originating from zygotes with MI or MII errors. It was previously shown, that a mononuclear zygote could produce a hESC line with normal diploid karyotype (46, XX) [22], which may be due to a regular fertilization event forming asynchronous pronuclei, which resulted in a normal blastocyst and subsequently euploid hESC line. Karyotypically normal hESC lines may be produced from the embryos with chromosomal abnormalities detected by blastomere biopsy [21], probably due to selective advantage of normal cell lines in the embryos with undetected mosaicism.

The highest efficiently of obtaining hESC lines (21.6%) was observed from the embryos with a balanced chromosome abnormality as a result of sequential errors in the first and second meiotic division, which is comparable to the success rate of 20–25% in obtaining hESC lines from chromosomally normal embryos in our and other experiences.

### 7.2.2 Genetic Disease Specific Human Embryonic Stem Cell Lines

Our present collection of hESC lines represents the world's largest repository, containing 87 hESC lines with genetic and chromosomal disorders, which was derived from our ongoing PGD practice [17–19]. These lines are clearly of great importance for understanding the mechanisms of the phenotypic realization of genetic defects and for the development of new approaches for their possible treatment. A few hESC lines with genetic disorders were also reported, and also available in NIH registry, including cystic fibrosis, Charcot-Marie-Tooth disease, Duchenne Muscular Distrophy, congenital nephrotic syndrome, spinal muscular atrophy, and Marfan syndrome [2, 16].

Our overall success rate of the establishment of hESC lines with the use of the above techniques mentioned was 20–25%, which is not different from the success rate from the embryos with normal genotype. The established hESC lines were tested for alkaline phosphatase, stage specific antigens SSEA-3 and SSEA-4, high molecular

weight glycoproteins or tumour rejection antigens, TRA-1-60 and TRA-1-80, and Oct-4, and maintained in vitro for up to over a dozen passages before freezing in sufficient amounts.

In addition to the above 14 hESC lines with chromosomal abnormalities, our collection of genetic specific hESC lines contains 35 with autosomal dominant (Table 7.3), 24 autosomal recessive (Table 7.4) and 14 with X-linked disorders (Table 7.5), which are frozen in sufficient quantities and available at different passages. The examples of the derivation of hESC lines with single gene disorders, including thalassemia (HBB), neurofibromatosis type I (NF1), Marfan syndrome (FBN1), myotonic dystrophy (DMPK), Becker muscular dystrophy (BMD) and fragile-X syndrome (FMR1), are described below.

## Marfan Syndrome (FBN1)

Marfan syndrome is autosomal dominant disease caused by mutation of the gene located in chromosome 15 (15q21.1). The disease leads to skeletal abnormalities, including scoliosis, chest wall deformity, tall stature and abnormal joint mobility. Ectopia lentis occurs in up to about 80% of patients and almost always is bilateral. The leading cause of premature death is progressing dilation of the aortic root and ascending aorta causing aortic incompetence and dissection.

**Table 7.3** List of hESC lines with autosomal dominant disorders (35 lines)

| |
|---|
| Breast cancer, familial (BRCA2); affected (N/IVS7 GT del) ($n=1$) |
| Breast cancer, familial (BRCA2); affected (N/IVS7 GT del) and multiple endocrine neoplasia, type I; men1 affected (N/3036 4 bp del) ($n=1$) |
| Huntington disease; HD, affected, expansion ($n=7$) |
| Marfan syndrome; MFS, affected, G7712A/N ($n=1$) |
| Dystrophia myotonica 1, affected, expansion ($n=2$) |
| Neurofibromatosis, type I; NF, affected, ($n=7$) |
| Torsion dystonia 1, autosomal dominant; DYT1, affected, exon 7 GAG deletion ($n=3$) |
| Treacher Collins-Franceschetti syndrome; TCOF, affected (Nt. 4374 ins. A/N) ($n=3$) |
| Tuberous sclerosis TYPE 1, affected ($n=2$) |
| Popliteal pterygium syndrome; PPS, affected ($n=1$) |
| Facioscapulohumeral muscular dystrophy 1A; FSHMD1A, affected ($n=7$) |

**Table 7.4** List of hESC lines with autosomal recessive disorders (24 lines)

| |
|---|
| Hemoglobin – alpha locus; HBA, affected (– –/– –) ($n=1$) |
| Hemoglobin – beta locus; HBB, affected (cd39 / IVS1–110) ($n=1$) |
| Hemoglobin – beta locus; HBB, affected (cd8 + G /619del) ($n=1$) |
| Hemoglobin – beta locus; HBB, affected (HbS/HbS – sickle cell anemia) ($n=1$) |
| Hemoglobin – beta locus; HBB, affected (IVS1-5/ Cd8 + G) ($n=1$) |
| Hemoglobin – beta locus; HBB, affected (IVSI-6 / IVSI-6) ($n=1$) |
| Hemoglobin – beta locus; HBB, affected (unknown/ IVSII-1) ($n=1$) |
| Hemoglobin – beta locus; HBB, affected (unknown/ IVSII-1) ($n=1$) |
| Hemoglobin – beta locus; HBB, carrier (N/IVS 1–1) ($n=1$) |
| Hemoglobin – beta locus; HBB, carrier (N/IVS1–110) ($n=1$) |
| Cystic fibrosis; affected (ΔF508/1,717–1 G>A) ($n=1$) |
| Cystic fibrosis; affected (ΔF508/1,717–1 G>A) ($n=1$) |
| Cystic fibrosis; affected (ΔF508/ΔF508) ($n=1$) |
| Cystic fibrosis; affected (ΔF508/ΔF508) ($n=1$) |
| Cystic fibrosis; affected (ΔF508/ΔF508) ($n=1$) |
| Cystic fibrosis; affected (ΔF508/ΔF508) ($n=1$) |
| Cystic fibrosis; affected (N1303K/ΔF508) ($n=1$) |
| Cystic fibrosis; affected (W1282X/R117C) ($n=1$) |
| Fanconi anemia, complementation group A; FANCA, carrier of 14 bp deletion, ($n=1$) |
| Spinal muscular atrophy, type I; SMA1, affected, exon 7 deletion ($n=2$) |
| Sandhoff disease, affected ($n=3$) |

**Table 7.5** List of hESC lines with X-linked disorders (14 lines)

| |
|---|
| Albinism, ocular, type I; OA1, (c.251del C), affected male ($n=1$) |
| Albinism, ocular, type I; OA1, (N/c.251del C), carrier ($n=1$) |
| Adrenoleukodystrophy; ALD, (1,801 del AG) affected male ($n=1$) |
| Muscular dystrophy, Becker type; BMD, affected male ($n=1$) |
| Muscular dystrophy, Duchenne type; DMD, affected ($n=2$) |
| Muscular dystrophy, Duchenne type; DMD, carrier ($n=2$) |
| Emery-Dreifuss muscular dystrophy, X-linked; EDMD, affected male ($n=3$) |
| Emery-Dreifuss muscular dystrophy, X-linked; EDMD, carrier ($n=1$) |
| Fragile site mental retardation 1, affected male, expansion ($n=1$) |
| Fragile site mental retardation 1, carrier female ($n=1$) |

As can be seen from the pedigree presented in Fig. 7.3, the couple presented for PGD because female partner was a carries of G7712A mutation in exon 62. PGD was performed using PB1 and PB2 analysis, which predicted eight mutant, and eight mutation free oocytes. Three embryos resulting from these oocytes were transferred, and three of those that reached blastocyst stage were frozen. One of the mutant embryos, resulting from oocyte #5 was donated for research and used for establishment of ESC line, which was confirmed to contain G7712A mutation in exon 62 (hESC–154).

## Thalassemia (HBB)

The list of mutations for which we performed PGD was presented in Chap. 3. Thalassemias are among the most common single gene disorders, requiring the lifelong blood transfusion and iron chelation therapy, with the only radical treatment being the HLA compatible bone marrow transplantation, as described in Chap. 3 and 4. As shown in these sections, thalassemia is one of the major indications for PGD combined with preimplantation HLA typing, so this provided also the possibility of the establishment of thaslassemia specific ESC lines, one of which is shown in Fig. 7.4.

As seen from the pedigree, the parents were the carriers of different mutations, father carrying Cd39 and mother IVS I-110 mutation, both being the most common mutations in the Mediterranean region. They had one previous child affected with severe beta-thalassemia, requiring HLA compatible bone marrow transplantation. So the couples requested PGD and HLA typing, which was performed by blastomere

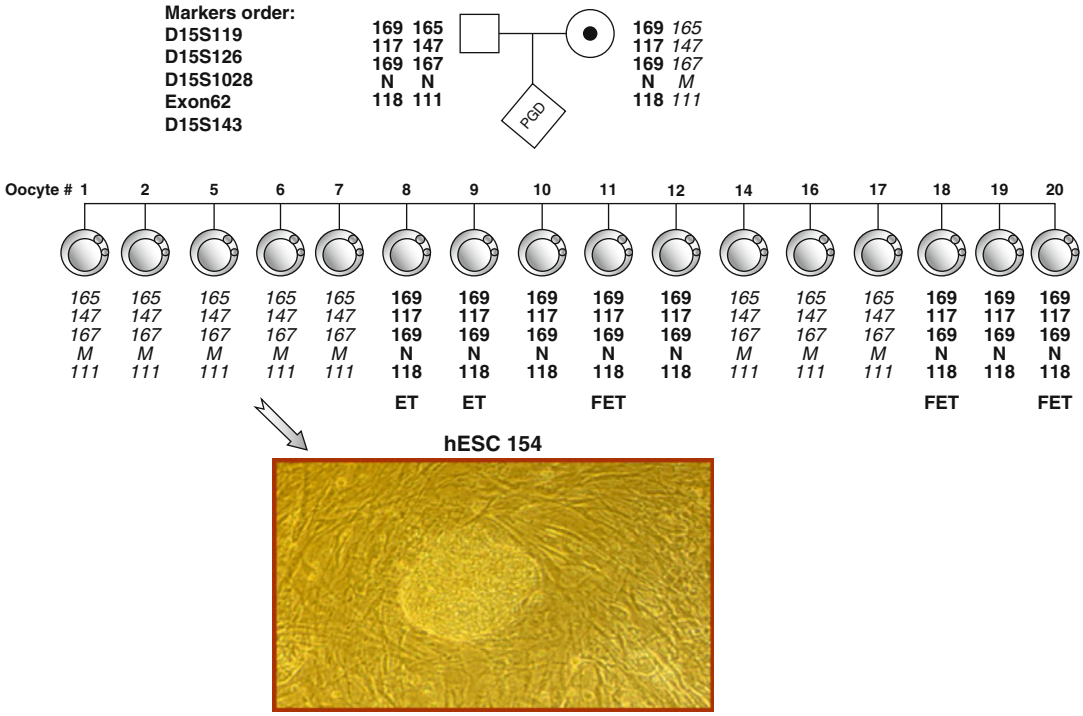


**Fig. 7.3** PGD for Marfan syndrome with the establishment of embryonic stem cell line. *Upper panel* – Family pedigree with paternal and maternal haplotypes (marker order is given on left) showing the unaffected pregnancy as a result of PGD. *Middle panel* – Results of sequential PB1 and PB2 analysis of 16 oocytes, 8 of which were predicted to be mutation-free, and the other 8 affected. Two embryos were transferred back to patient (*ET*), three were frozen and used for frozen embryo transfer, while one of the affected embryos (embryo #5) was donated for research and used for establishment of ESC line, shown on the *lower panel*, with confirmed affected genotype

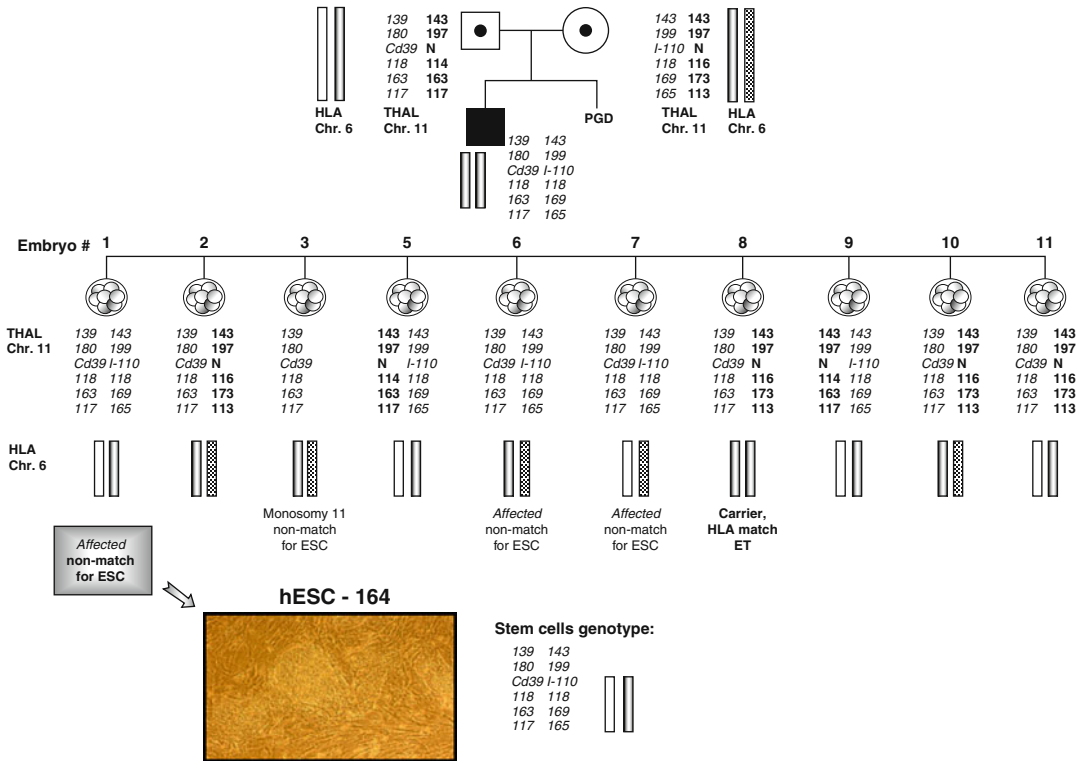


**Fig. 7.4** PGD for thalassemia and HLA typing, with the establishment of embryonic stem cell line. *Upper panel* – Family pedigree with paternal and maternal haplotypes: father is carrier of Cd39 mutation, mother – IVSI-110; HLA hoplotypes as darker and lighter bars, darker corresponding to haplotypes of sibling with thalassemia. *Middle panel* – Results of blastomere biopsy analysis of ten embryos, six of which were predicted to be unaffected, one being also HLA match to the affected siblings, which was transferred (embryo # 8). Of the remaining four embryos, one was monosomic for chromosome 11 of paternal origin with mutation Cd39, and three were affected, one of which (embryo #1 was donated for research and used for establishment of ESC line, shown on the *lower panel*, with confirmed double heterozygous affected genotype Cd39/ IVSI-110

biopsy and multiplex PCR analysis, involving simultaneous testing of both mutation and HLA markers, describe in Chap. 4. Of 10 embryos tested, three were affected (embryos #1, 6 and 7), one contained only one chromsome 11 with Cd 39 mutation (embryo #3), while the remaining six embryos were carriers of one of the mutations. Only one of these embryos was a full HLA match and transferred. Of the three affected embryos, embryo # 1, which was also an HLA non-match, was donated for research and resulted in the establishment of ESC line, hESC – 164. The follow up testing showed that the cells are double heterozygous affected for Cd39 and IVSI-110 mutations.

## Fragile-X Syndrome (FRM1)

As described in Chap. 3, FRM1 is one of the most common genetic disorders with prevalence of one in every 2,000 children, which is characterized by moderate to severe mental retardation, microorchidism, large ears, prominent jaws, and high pitched, jocular speech, resulting from an expansion of a CGG repeat. As seen from the pedigree (Fig. 7.5), the couple presented for PGD with previous three unsuccessful pregnancies, one of which resulted in birth of affected child with fully expanded allele. PGD was performed by PB1 and PB2 testing for maternal mutation, using simultaneous amplification of seven linked polymorphic

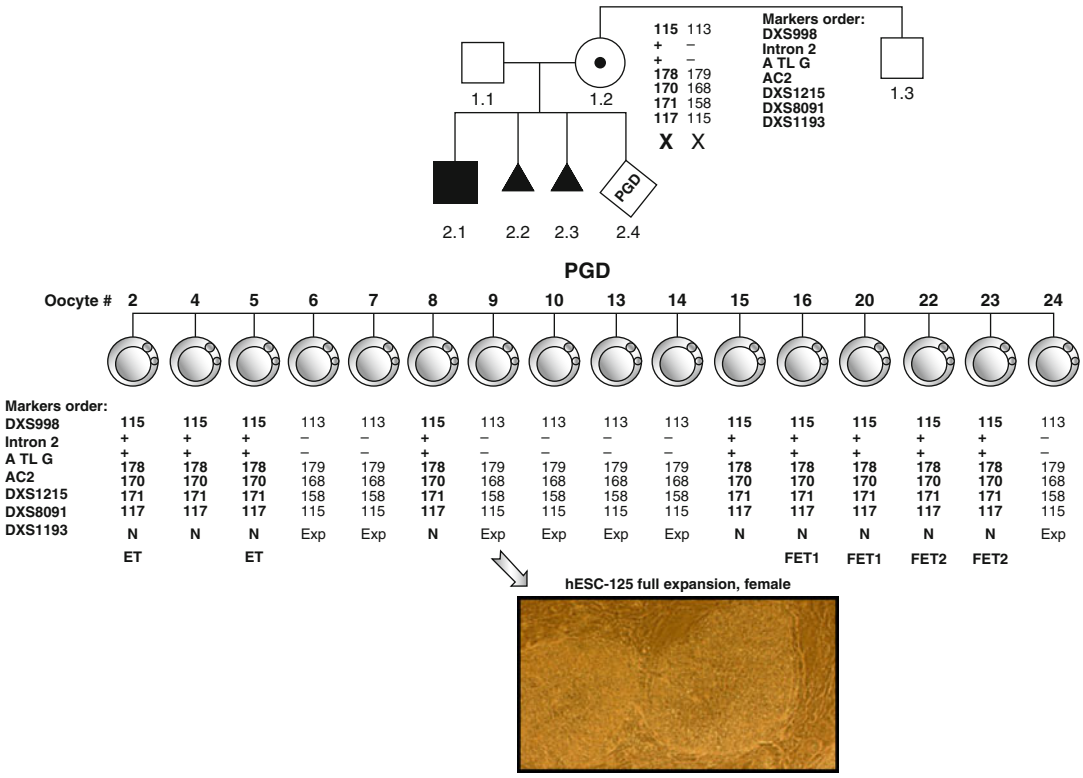


**Fig. 7.5** PGD for Fragile X syndrome (FMR1) with establishment of embryonic stem cell line. *Upper panel* – family pedigree, showing maternal [1, 2] haplotypes, and reproductive history with one affected son (*2.1*), two spontaneous abortions and unaffected pregnancy following PGD. *Middle panel* – results of polar body analysis of 16 oocytes, 9 of which were predicted to be unaffected, and 7 with the expansion. Two of the embryos deriving from mutation free oocytes (oocytes #2 and 5) were transferred and four frozen. Of seven affected embryos originating from the mutant oocytes, one (embryos deriving from oocyte #9) was donated for research and used for establishment of ESC line, which is shown at *Bottom panel*. The ESC line was confirmed to have female genotype with fully expanded allele

markers, which showed that 9 of 16 tested oocytes were free of mutation, which were either transferred or frozen for the future use by the couple. Of seven embryos, resulting from oocytes containing the mutant gene, embryo # 9 was the source of embryonic stem cell line, confirmed to contain the fully expanded allele of FRM1 gene.

## Myotonic Dystrophy (DMPK)

Similar to FRM1, DMPK is an autosomal dominant dynamic mutation in chromosome 19 (19q13.2-13.3), resulting from the expansion of triplet repeat, with prevalence of 1 in 8,000 live births. This is a severe neurodegenerative disorder characterized by myotonia, muscle wasting in the distal extremities, cataract, hypogonadism, defective endocrine functions, and cardiac arrhythmias. As mentioned in Chap. 3, DM was one of the most frequent indications for PGD, which also provided possibility for obtaining affected embryos for the establishment of the ESC lines with DMPK. One of PGD cycles for the maternally derived mutation is presented in Fig. 7.6, showing the results of blastomere biopsy of six embryos, which resulted in the transfer of two mutation free embryos (embryos #5 and 7). Two of the remaining four embryos were with aneuploidies, monosomy 16 in embryo #8 and

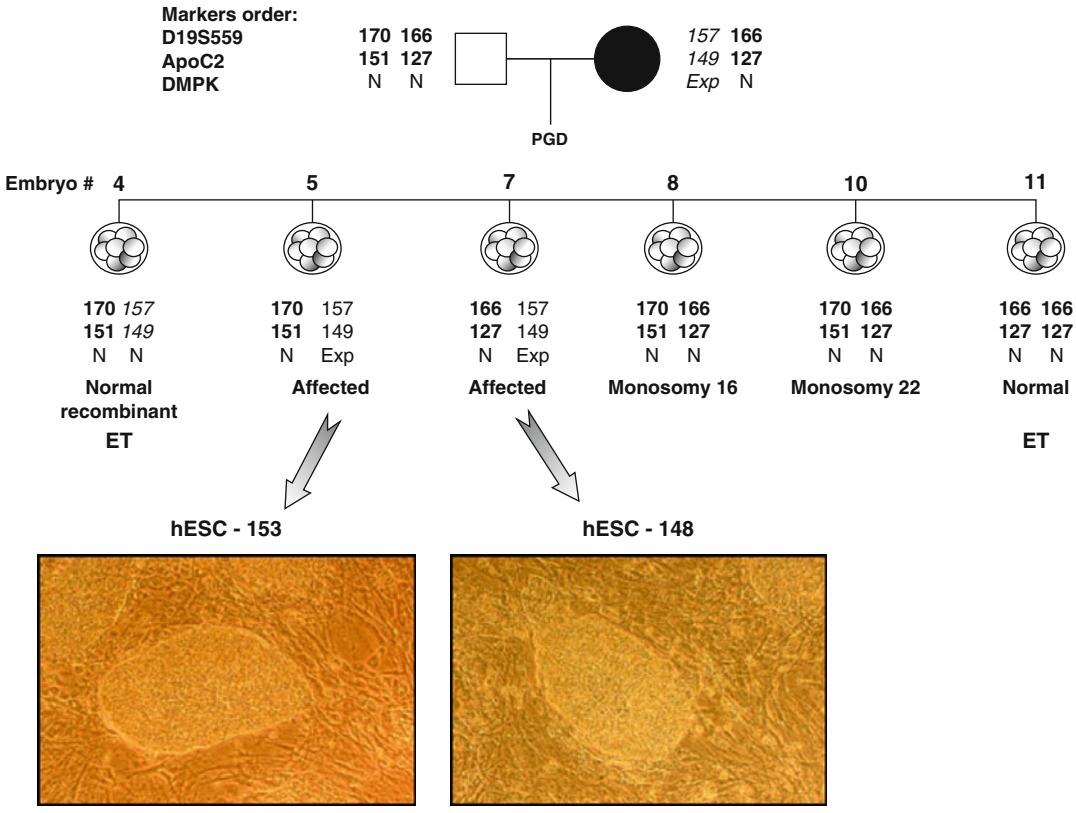


**Fig. 7.6** PGD for myotonic dystrophy and aneuploidy with establishment of two mutant embryonic stem cell lines. *Upper panel* – paternal and maternal haplotypes (marker order is given on left). *Middle panel* – results of blastomere analysis of six embryos, two of which were predicted to be mutation-free and transferred (embryos #4 and 11), two were mutation free but chromosomally abnormal (embryo #8 with monosomy 16 and #10 with monosomy 22), and the remaining two were with expansion (embryos #5 and 7). These latter embryos were used for establishment of ESC lines, shown on the *Lower panel*, with confirmed affected genotypes

monosomy 22 in embryo 10, and two affected embryos were donated for research and resulted in the establishment of two embryonic stem cell lines, hESC-148, and hESC -153.

### Neufibromatosis Type I (NF1)

PGD for this case was described in Chap. 3, resulting in the birth of healthy unaffected twins. Three of the embryos with mutation Trp->Ter (TGG->TGA) in exon 29 of NF1 gene were donated for research and used for the establishment of three ESC lines with NF1, shown in Fig. 7.7.

### Becker Muscular Dystrophy (BMD)

Becker muscular dystrophy is an X-linked disorder located in Xq21.2, resembling Duchenne muscular dystrophy (DMD), which is the most common form of muscular dystrophy. The clinical features of BMD, however, are more benign and realized later than DMD, which typically presents in boys aged 3–7 year. As shown in pedigree of the couple presenting for PGD (Fig. 7.8), there were three affected boys in the family of the mother, who is a carrier of the deletion in intron 45 of the gene. Of eight embryos tested for deletion using seven polymorphic markers, only one

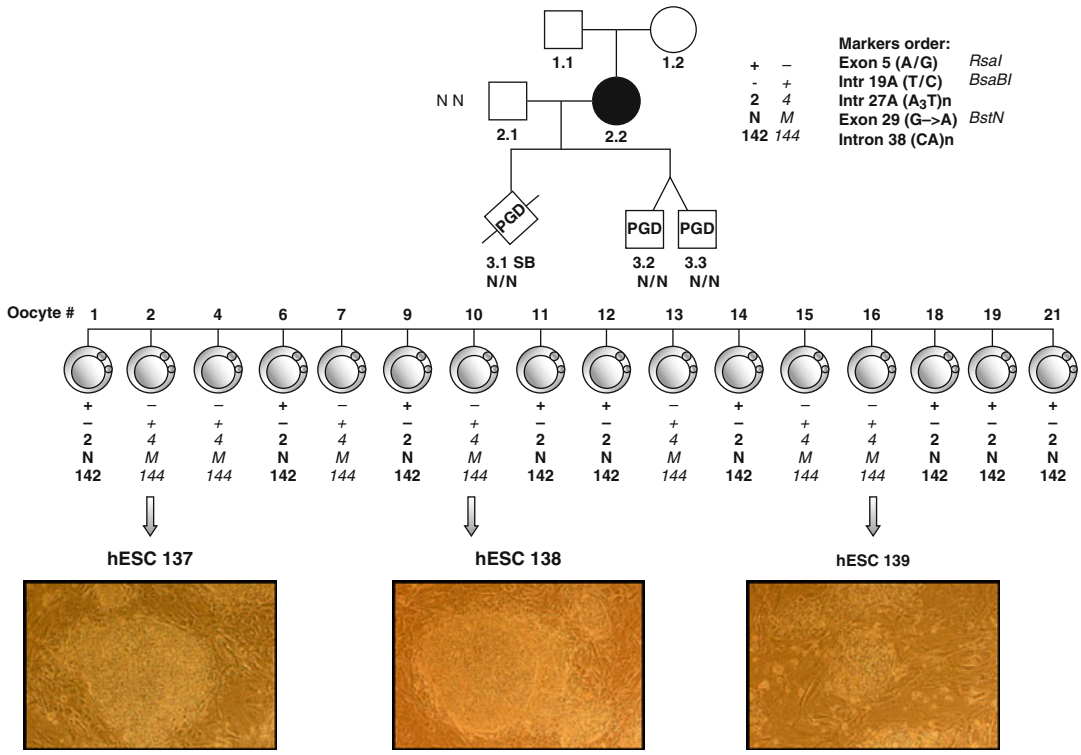


**Fig. 7.7** PGD for neurofibromatosis type 1 (NF1) with the establishment of three ESC lines with NF1. *Upper panel* – family pedigree showing affected mother (*2.2*) with de-novo NF1 mutation. Two pregnancies were obtained from the transfer of mutation free embryos following *PGD*, one of which resulted in a stillbirth (*3.1*) and the other in a healthy twin pregnancy (*3.2* and *3.3*), all three babies being confirmed to be unaffected. *Middle panel* – results of polar body analysis of 16 oocytes, 7 of which were predicted to be mutant, and 3 of the embryos deriving from these mutant embryos were donated for research (embryos #2, 10 and 16). These embryos were used for establishment of ESC lines, shown on the *Lower panel*, which were confirmed to be affected

was mutant, so donated for the establishment of embryonic stem cell line. Of the remaining seven normal embryos, four reached the blastocyst stage, of which two were transferred and two were frozen for subsequent transfer. The established ESC line, hESC–170, was tested by the same set of markers used for PGD, and was confirmed to contain the mutant gene.

## 7.3 Development of Individual Specific hESC Lines

The extensive experience of stem cell transplantation treatment shows that the success rate has depended heavily on the finding of a human leukocyte antigen (HLA) matched donor [26, 27]. Therefore, the potential of the future cellular therapeutic interventions for many congenital and acquired disorders will depend on the production of the HLA identical patient-specific hESC lines. Although such a possibility was reported to be feasible [28–30], many problems still remain unresolved, the key issue being the ability to turning differentiated specialized somatic cells into ESC, to culture such cells in sufficient quantities and reprogram embryonic stem cells into different specialized cells.

Nuclear reprogramming of human fibroblasts has been performed through hybridization between fibroblasts and hESC [28], and fusion of hESC cytoplasts with adult lymphocytes and fibroblasts [30]. Although hESC were demonstrated to reprogramme adult somatic cells, the

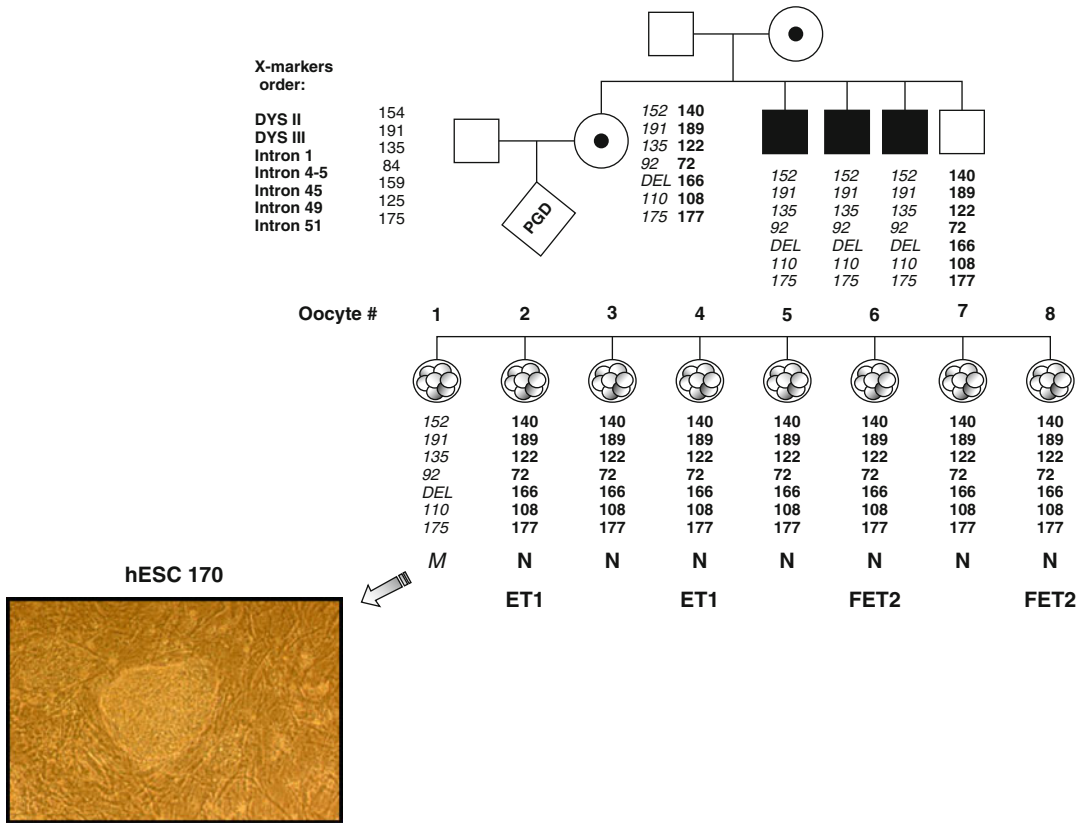


**Fig. 7.8** PGD for Becker muscular dystrophy (BMD) with the establishment of ESC line with BMD. *Upper panel* – family pedigree showing a carrier mother who had three affected brothers with the deletion. *PGD* allowed having an unaffected pregnancy. *Middle panel* – results of blastomere analysis of eight embryos, identifying only one mutant embryo, which was the source for the establishment of ESC line, shown on the *lower panel*, which was confirmed to be affected

resulting hybrid cells contained tetraploid DNA, including the contribution from both hESC and donor somatic cells [28], while the isolated colonies of the resulting hESC cybrids appeared to contain the cells with recipient nuclei, in addition to the cells with somatic donor cell nuclei [30].

The design of stembrid technology is based on the use of the female hESC for reprogramming of male adult lymphocytes or fibroblasts, with its outcome evaluated by the appearance of the cells with male karyotype and Oct-4 and TRA-2-39 markers in the resulting proliferating colonies. The details of the suppression of hESC nuclei, enucleation of hESC, and fusion of hESC cytoplasts with somatic cells were described elsewhere [30]. In brief, isolated colonies of typical hESC morphology obtained after 7–10 days of culture were transferred into separate wells of a 48-well dish and cultured for 2 weeks before being passaged and tested for stemmness by fluorescein isothiocyanate for TRA-2-39 and tetramethylrhodamine B isothiocyanate for Oct-4, followed by analysis by fluorescence in-situ hybridization (FISH).

The somatic cell nuclei fused with hESC cytoplasts were shown to proceed with cell division, resulting in the establishment of cybrid cells with male karyotype. It was demonstrated that the mitotic donor (lymphocytes) nuclei in cytoplasts start synthesizing Oct-4, not typical for the donor nuclei, with its gradual increase despite originating from differentiated cells. Similarly, the colonies derived from the differentiated cell nuclei

fusion with the cytoplasts of hESC also expressed Oct-4/TRA-2-39, while FISH analysis showed that the resulting colonies contained cells with XY karyotype, although mixed with recipient XX nuclei. All isolated colonies were positively tested also for SSEA3, SSEA4, TRA-1-60, TRA-1-80 as well as Oct-4/TRA-1-39. The cybrid cells demonstrated the typical hESC morphology and stemmness, as shown by positive Oct-4 and alkaline phosphatase, providing the evidence of the replacement of hESC nuclei by the nuclei of somatic cells.

The results suggested feasibility of a complete replacement of hESC nuclei by the nuclei of donor somatic cells. However, only a small proportion of cells were derived from donor lymphocytes or fibroblasts, with no pure population of cybrid ESC yet isolated. In addition to these cybrid cells, the hybrids between the donor and non-enucleated hESC were obtained with XY/XX, XXYY/XX and XXY/XX karyotypes.

The obtained cybrid cells, with complete replacement of hESC nuclei by the nuclei of somatic cells, also demonstrated the typical SC morphology and the presence of Oct-4 and L-alkaline phosphatase, confirming the 'stemmness' of the resulting cybrids. However, the efficacy of the method was not sufficiently high, requiring considerable improvement before it could be considered for obtaining individual specific hESC, with the required somatic cell nuclei, as no methods are yet available for isolation and purification of hESC cybrid colonies. The further improvement of this stembrid technology may be useful for the construction of individual specific hESC lines, which may provides the prospect of future stem cell therapy, also avoiding the controversy of the use of human oocytes for production of hESC.

Many of the above problems mentioned have been solved by the induced pluripotent stem cells (iPSC) which makes possible to develop a custom-made hESC for future cell therapy [31, 32]. This involves the induction of overexpression of four main genes responsible for stemmness, including Oct -3/4, Sox2, Klf4 and c-Myc, which result in pluripotency and differentiation characteristics similar to hESC. However, it is still not clear if these iPSC may provide an alternative to hESC, as they are not identical in DNA microarray analysis, and it is not known if they are safe due to significant gene modification and possible tumorigenic potential.

## 7.4 Human Embryonic Stem Cell Lines Resistant to HIV

The first HIV-positive individual has recently been successfully cured via bone marrow transplantation from the unrelated donor, who was chosen not only by human leukocyte antigen (HLA) matching, but also for having a homozygous polymorphism in the chemokine receptor 5 (CCR5) gene CMKBR5 [33]. Although CCR5del32 allele was linked to HIV resistance long time ago, providing an immense promise of being able to treat HIV positive patients [34], finding acceptable donor matches homozygous for the CCR5del32 presented a real challenge. Of course, as in other conditions treatable by allogeneic bone marrow transplantation, finding an HLA match *related* donor is ideal, but probability of finding such a match is extremely low [35]. Further, this is almost unrealistic if there is a need for a related or even unrelated donor with a specific allele, like CCR5-del32. Clearly, transplantation from an adult unrelated donor is limited by the availability of fully HLA matched donors, while an increased HLA disparity provides for lower survival rates and a higher chance of graft-versus-host disease (GVHD).

The use of unrelated umbilical cord blood (UCB), which is a valuable source of hematopoietic stem cells, is presently an established alternative to bone marrow transplantation. Its potential advantages was expected to be the possibility of using UCB with one or two HLA mismatches, reducing conditioning intensity and avoiding the risk of severe GVHD [36]. These effects seemed to be due to the "naïve" nature of umbilical cord lymphocytes [37]. However, the major disadvantage of UCB is a reduced engraftment, which is due to the limited number of CD34 stem cells obtainable from a UCB sample, limiting the application of UCB transplantation to older children.

On the other hand, with the current progress in stem cell research, there is presently an important potential alternative source of stem cells for transplantation, residing in human embryonic stem cell (hESC) lines, which are readily available in a few centers around the world [2, 17, 18]. Since embryonic stem cells may be expected to have even more "naïve" immunological features compared to UCB stem cells, they should allow for similarly high acceptable HLA disparities, thereby making patient-donor matching much more permissible, to avoid aggressive conditioning before and after transplantation, a critical advantage for patients with serious health conditions [38, 39]. Also, one of important advantages of this option is that hESC collections provide an unlimited source, as they may be expanded virtually without limit, so a single hESC line can potentially be used for the transplantation treatment of any number of recipients.

As described above, we have presently the world's largest collection of hESC lines [17, 18], which has been screened for the presence of the CCR5-del32 allele, revealing 12 hESC lines containing this gene, of which one is unique parthenogenetic line with two copies of CCR5-del32 [40]. To perform screening for the CCR5del32 deletion, the cells were removed from cryogenic storage, washed in PBS, and were placed directly into a lysis solution, consisting of 0.5 μL of 10× PCR buffer, 0.5 μL of 1% Tween 20, 0.5 μL of 1% Triton X-100, 3.5 of water, and 0.05 μL of Proteinase K (20 mg/mL in 0.5-mL PCR tube). After spinning down, the samples were covered with one drop of mineral oil and incubated at 45°C for 15 min in a thermal cycler. Proteinase K was then inactivated at 96°C for 20 min. Hot start PCR was performed at 72°C for 10 min, followed by denaturation at 95°C for 3 min. Round 1 PCR master mix consisted of dNTP 400 μM, 1× PCR buffer, Taq polymerase (2 U), 1.5 mM magnesium dichloride, 6% dimethylsulfoxide, and 0.5 μM of outside upstream and downstream primers for the mutation in a final volume of 50 μL. The cycling conditions for the first round of PCR were as follows: 95°C for 30 s, 55°C for 1 min 30 s, 72°C for 45 s, for five cycles; during the following 23 cycles, the annealing temperature was gradually decreased from 55° to 45°; with a final incubation at 72°C for 10 min.

The outer primers CCR5-1 GCGTCTCTCC CAGGAATCATC and CCR5-2 GATTCCCGAG TAGCAGATGACC for performing the first round of amplification, and inner primer CCR5-3 GCGT CTCTCCCAGGAATCATC for second round of hemi-nested (CCR5-1 and CCR5-3) PCR. The cycling conditions for the second round of PCR were as follows: 92°C for 30 s, 55°C for 30 s, 72°C for 30 s, for a total of 30 cycles; and followed by a final 10- min incubation at 72°C. Primers for chromosome specific microsatellite markers were added to identify the copy number and parental origin of the polymorphic markers, used for aneuploidy testing of chromosomes 13, 16, 18, 21, 22 and X, as described in Chap. 2. The PCR product was assessed by gel electrophoresis and Ethidium Bromide staining, with the normal allele corresponding to the 141 bp and the CCR5-del32 allele to 109 bp bands.

Of a total of 137 hESC lines tested, 125 were without deletion, 11 were heterozygous, and one was with two copies of the genes with the deletion. The latter was established from the blastocyst deriving from partenogenetic embryo (46, XX), with two copies of all maternally derived genes, as demonstrated by polymorphic markers for X-chromosome, and chromosomes 13, 16, 18, 21 and 22.

The frequency of the CCR5-del32 allele in the studied material (4.7%) is comparable to data published in earlier studies [41], making it realistic to predict the possibility of identification of potentially useful hESC lines conferring resistance to HIV even in smaller collections. Assuming that more than 327 hESC lines are now available in our collection, the testing of the whole material could have led to finding of more than two dozens of hESC lines containing the CCR5-del32 allele conferring resistant to HIV infection.

It may be expected that with the establishment of larger repositories of hESC lines, there may be a possibility to perform a search for finding HLA match for HIV patients. It was predicted that a bank of 150 donor cell lines may already provide a chance of finding full match of HLA-A, HLA-B,

and HLA-DR for up to 20% recipients [42]. With the present progress in the differentiation of hESC into hemopoietic stem cells [14], this material may appear a readily available source of bone marrow transplantation. Clinical implication of the resistant hESC lines will further be widened with the progress in transplantation treatment with unrelated stem cells having significant HLA disparity following the sophisticated immunosuppression therapy and conditioning of the recipients, which may soon appear routine.

The availability of a hESC lines containing CCR5-32 bp deletion, and particularly a parthenogenetic lines with two copies of this allele, may have particular potential for research into the mechanisms of conferring resistance to HIV, the results of which could lead to new treatments to this most devastating disease. Moreover, the prospective advantages of clinical therapies derived from hESC lines will likely hold true for many other congenital and acquired diseases. Our repository has a large collection of hESC lines, which provides a unique opportunity to screen available hESC lines for polymorphisms associated with susceptibility and/or resistance to different common diseases, such as cancer. So this study provides the first evidence that such screening is productive for finding hESC lines with rare mutations which may prove invaluable to the future stem cell therapy of severe disorders for which there is no available treatment

## 7.5 Progress in Study of Disease Specific hESC Lines

Although initially the major goal of the establishment of human ES cell lines was the development of the cell-replacement therapies, it is presently obvious that human ESC lines will have an important role in the studies of mechanisms of genetic disorders through generating the sources of normal and genetically abnormal cells and tissues. The ability to obtain the hESC lines with specific genetic disorders, that could produce unlimited quantities of the disease tissue where the disease has a genetic basis, makes it realistic to undertake research on the primary disturbances of the cellular processes in the genetically abnormal cells and to identify the molecular mechanisms that might be blocked to prevent the disease progression. Therefore, there is obvious need for establishment of ESC lines originating from embryos with genetic and chromosomal abnormalities, to provide the basis for understanding of the mechanisms of phenotype realization of genetic defects and for the development of new approaches for their possible treatment.

There are presently a few studies of primary mechanisms of genetic disorders, using hESC lines with Fragile –X, Down's syndrome, Huntington's disease (HD), thalassemia and sickle cell disease. The results were reported for the latter three conditions, with unique findings concerning the development and differentiation of the primary progenitors.

One of the conditions investigated in many laboratories is Huntington's disease (HD), which is an autosomal dominant late onset disorder caused by an expanded CAG repeat region in exon 1 of the *HTT* gene, affecting approximately 1 in 10,000 individuals word-wide. Hallmarks of the disease pathology include the formation of polyglutamine HD protein fibrillar aggregates, disruption of the normal transcriptome and CAG repeat expansion in striatal neurons of the brain. There is no cure for HD and potential treatments have proven to be largely ineffective. So HD hESC lines serve as valuable in vitro human models to better understand the mechanisms of pathogenesis that eventually lead to neurodegeneration in HD patients.

Two hESC lines from our collection of hESC lines, obtained from affected PGD embryos, carrying partial ($CAG_{37}$) and fully ($CAG_{51}$) penetrant disease alleles, were used. Following treatment with noggin to induce neural differentiation, the HD positive lines were capable of forming Pax-6 positive neurospheres. However, there was evidence of apoptosis within HD neurospheres that was exacerbated by increased CAG repeat length. On further differentiation using specific growth factors, HD neurospheres formed striatal neurons and astrocytes, although the frequency of these neuronal subtypes was significantly less than HD negative neurospheres. These findings suggest

that the HD mutation has a detrimental affect on neural differentiation. The nature of the high levels of apoptosis were tested using fluorescent probes, as this may be due to loss of mitochondrial function in the HD positive lines. In PCR studies of CAG repeat stability, it was shown that ~ 0.5% of HD neuronal cells have either contractions (1–5 repeats) or expansions (1–6 repeats). Whether oxidative stress can further exacerbate this pathology is also being investigated, which together with the above and future studies may improve understanding of HD pathologies and provide a source of affected neurons for high throughput screening to identify more effective therapies [43].

The other studied conditions were thalassemias and sickle cell disease. By comparing erythropoietic differentiation of hESC lines from sickle cell disease with control lines, it was demonstrated that In vitro-expanded sickle cell erythroblasts were comprised of homogenous populations of $CD71^{+}CD36^{+}$ and $CD71^{+}CD235a^{+}$ progenitors, and underwent developmentally appropriate embryonic and fetal, but limited adult hemoglobin switching, similar to control. However, in comparison to unaffected erythroblasts from control hESCs, the mutant SSD erythroblasts had a relatively shortened lifespan, suggesting an inherent defect in primitive erythropoietic progenitor expansion. hESC – derived erythrocytes could also be infected in vitro with malaria parasites, thus demonstrating the utility of modeling important pathogenic processes in normal and diseased erythropoiesis using the disease–specific –hESC [14].

These results and those forthcoming from other similar ongoing studies using the available hESC lines with genetic and chromosomal disorders introduce important paradigms for using genetic disease specific hESCs for understanding the primary mechanisms of congenital disorders.

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# Social, Ethical, and Legal Aspects

# 8

Preventive approaches to congenital disorders always raise ethical problems, because traditionally the emphasis should be on treatment rather that on the avoidance of birth of children with congenital disorders, which is unfortunately unavailable for most of genetic disorders at the present time. On the other hand, the most ethically acceptable preventive approaches are indeed those that involve the primary preventive measures, which are better tolerated by society, than the secondary preventive measures involving pregnancy termination. As described in Chap. 1, one of the best examples of the most efficient primary preventive measure may be a population-based fortification of the major foodstuffs by folic acid containing multivitamins, which has been demonstrated to result in significant reduction of neural tube defects and congenital malformations overall. Still such programs have been introduced only in a few populations, so the lack of similar preventive measures in most of communities may be the reason that thousands of children with congenital disorders continue to be born, who otherwise might have been born healthy, which, therefore may represent an important legal, social and ethical issue. What is of special importance is that these primary preventive measures are ethically acceptable in any population, because they provide the actual gain in infants free of congenital malformations, rather that the avoidance of birth of affected children.

The same is true for preimplantation genetic diagnosis (PGD), which is also a primary preventive measure, although applied on a family level, allowing the genetically disadvantaged couples to produce unaffected children of their own, who might not otherwise be born at all because of fear of these couples to reproduce and face prenatal diagnosis and termination of pregnancy [1, 2]. So any legal restrictions of these patients' choices may only force them to achieve their goal by traveling to other countries where the regulations regarding PGD are more liberal. The available reviews on the status of PGD in different countries [3–8] show that the international legal practices range from explicit legalization (e.g., the Netherlands, United Kingdom, France, Spain) over more or less "lawless control" as in Belgium and the United States, to legal prohibition through restrictive laws, as in Italy, Germany, Austria or Switzerland. However, even in these countries, there is a tendency to ease such legal restrictions. For example, there is no interdiction of PGD in Austria, neither through the Law on Reproductive Medicine, nor through the Law on Genetic Engineering, unless the polar body or blastomere biopsy would be misinterpreted as "interference in the germ cell lineage" which would be prohibited [7]. In France, PGD is under the control of CNMBRDP, which is a governmental commission controlling also IVF [4, 5]. According to the regulation, an agreement is required to perform the embryo biopsy and genetic/FISH testing, the evolution of which and indications being the subject for a follow-up by the representatives on a regular basis. Only a few centers are allowed to perform PGD for the initial 5 years, the subject for the renewal afterwards; the regulations are

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under the "ethical" law, requiring a forthcoming re-examination. Similarly, PGD in the United Kingdom is regulated by HFEA, which is also a governmental organization, which provides the license for performing PGD and also has to approve any new condition to be performed. For example, PGD for chromosomal aneuploidies, practiced for more than 10 years in many other countries, has been allowed by HFEA much later. HFEA also initially refused to allow preimplantation HLA typing without PGD and then changed its position. Finally, PGD and many aspects of IVF were forbidden in Italy by the Act of Parliament almost for 7 years, according to which only three oocytes were allowed to be aspirated for fertilization in vitro, clearly following the opinions of the hierarchy of the Roman Catholic Church [9]. Of course, this made PGD impossible in Italy until only recently when this law was finally lifted, despite the fact that this country has been among the most active ones involved in the development and application of PGD for genetic and chromosomal disorders [2].

As described in Chap. 2, with the introduction of the methods of preconception diagnosis by polar body (PB) diagnosis, PGD has become ethically acceptable even in countries with restrictive laws, such as in Germany, where no manipulations are allowed after conception. According to the German Embryo Protection Act, the fertilized, viable ovum is already an embryo in the sense of the law [10], so no manipulation is allowed that could potentially damage it, despite the fact that approximately 120,000 abortions are performed annually in this country. Removing and examining blastomere in PGD, which is destroyed in this process, is punishable with a prison sentence, for up to 3 years or a fine. So PGD is an undisputable violation of this Act, as the life of a human being (namely the life of this biopsied cell) is destroyed. In these circumstances, PB testing is the only way to avoid violation of this law, as no embryo is formed by completing diagnosis prior to fusion of male and female pronuclei (see Chap. 2). In fact, this approach may also resolve the ethical issues of PGD in Austria, Switzerland, Malta, and other strictly Catholic countries [7] and may, in future, make PGD an acceptable procedure even in those countries, where no preventative measures have ever been allowed on religious grounds. No doubt that preconception diagnosis will also make PGD even more attractive in Muslim countries, where blastomere biopsy is currently acceptable and a more preferred option over prenatal diagnosis. Preconception diagnosis may no longer be restricted to the maternally derived genetic abnormalities, because the possible progress in sperm duplication may, in future, allow also sperm testing for the paternally derived abnormalities prior to fertilization (see Chap. 2).

On the other hand, the above law restriction on PGD, have stimulated the progress in the development of PGD technique, as could be observed in Italy, after introductions of restriction on IVF and PGD by the Roman Catholic Church. As the fertilization of no more than three oocytes was allowed, and PGD was prohibited, PB analysis was introduced to test the mature eggs prior to fertilization, so to avoid the use for fertilization of the oocytes with meiosis I errors (see Chap. 5).

The available experience show that PGD has become a routine procedure in an increasing number of countries, such the United States and Belgium, where no strict governmental regulations for PGD exist. It is, therefore, not surprising that the largest experiences in PGD for genetic and chromosomal disorders have been accumulated in these countries. The guidelines and standards for appropriate PGD practice have recently been developed by Preimplantation Genetic Diagnosis International Society (PGDIS) and ESHRE and may be followed to achieve the required standards of PGD [11, 12]. There are also regulations developed by the national scientific societies, such as in Japan, where no active PGD program is currently available, but there are regulations for PGD developed by the National Society of Obstetrics and Gynecology and by the Japan Society of Human Genetics, which provides the guidelines for genetic diagnosis [8].

The ethical issues of PGD have been recently evolving together with the development of the methods and with the expansion of the PGD indications. Initially, when PGD was applied only to pre-existing conditions, with the only goal of avoiding the risk of birth of children with genetic

disorders, PGD allowed avoiding traditional prenatal diagnosis and termination of pregnancy, so making prevention of genetic disorders more ethically acceptable. Some of the couples may have had the experience of the repeated pregnancy terminations before having a normal child, while the others could not accept prenatal diagnosis and termination of pregnancy at all. So PGD initially was an important alternative, so that the at-risk couples had the choice of either going through prenatal diagnosis and termination of pregnancy, or controlling their pregnancy outcome by testing the oocytes or embryos before implantation, to secure that the pregnancy is unaffected from the onset. Accordingly, not informing the genetically disadvantaged couples about PGD availability, which may have affected their possible choices, may present an important ethical and legal issue. It is especially important for those conditions, such as translocations, carriers of which have an absolutely miserable pregnancy outcome. As mentioned in Chap. 5, analysis of meiotic outcome in carriers of translocation leaves a little chance for prenatal diagnosis to be useful in identifying a balanced or normal fetus, as the carriers of translocations have more than 80% prospect of losing their pregnancy by spontaneous abortions. So, PGD for such couples is clearly the only hope, providing a realistic option of having unaffected children of their own.

As shown in Chap. 3, PGD will have the increasing practical implications with the current progress in the improvement of the quality of life, the life expectancy and the possibility to reproduce by the genetically affected patients. For example, the life expectancy in cystic fibrosis (CF) patients may presently be no different from the normal individuals, who may be able to procreate and have their own children. Similarly, with the success in stem cell transplantation, children with thalassemia may be radically cured, and these so-called ex-thalassemics require PGD to avoid 50% risk of producing their own thalassemic children. As seen from Chap. 3, PGD has already been applied for homozygous or double heterozygous affected individuals with CF, thalassemias and phenylketonuria (PKU), who were able to have their own unaffected children following PGD. On the other hand, this may still create the feeling that some extreme variations of the genotype are rejected by society, so the couples may face a complex decision of transferring back the embryos with different genotypes. For example, some couples may elect to transfer the embryos carrying the affected genes, such as for deafness or achondroplasia, so using PGD to conceive a disabled child, which sets a poor precedent for the patients facing complex familial decisions [13].

The important breakthrough from the ethical and social point of view was the introduction of PGD for the diseases with genetic predispositions, especially when it has become possible to avoid the transfer of the embryos carrying the genes predisposing to common disorders of adult life. Although there is no difference in the application of PGD for early or late onset disorders with genetic predisposition from the application of PGD to chromosomal disorders and autosomal recessive metabolic disorders with the onset at birth or early childhood, the discomfort of PGD for disorders with genetic predisposition can be explained by the fact that this has been controversial or even unacceptable in the practice of prenatal diagnosis. The same diagnosis is of course possible by chorionic villus sampling (CVS) or amniocentesis with the only difference that if the fetus would appear carrying the gene predisposing to late onset diseases with genetic predisposition, such as Alzheimer disease (AD) or other late onset diseases with genetic predispositions, described in Chap. 3, the couple would have to make an important decision of pregnancy termination. This could hardly be justified on the basis of genetic predisposition alone, taking into consideration that the clinical manifestation of the disease might not be realized at all in some proportion of cases. Alternatively, PGD technology allows genetic testing of human eggs and embryos before pregnancy, therefore, making it totally realistic to establish only potentially normal pregnancies without a disease with early or late onset disorder with genetic predisposition. Thus, the prospective at-risk parents have to be informed about the availability of the PGD technology, to

allow them to make the decision themselves about their reproductive options.

This is not similar for PGD for Hantington's disease (HD), which despite being also the late onset disease, always progressing and leading to death within approximately 15 years after start. Prenatal diagnosis is still controversial, as selective abortion will not be acceptable because the child might still expect many disease-free years. The well-known "nondisclosure PGD" is obviously the best option for these couples, as asymptomatic individuals with risk of carrying HD may be offered PGD to test embryos without ever being informed about the specific test results.

The situation is even more controversial for PGD of late onset common disorders, which may never be presented during the whole lifespan. On the other hand, with no current prospect for treatment of most late onset diseases with genetic predisposition, such as AD, which may arise despite presymptomatic diagnosis and follow-up, prevention of inherited predisposition to late onset disease may be the only possible option for the couples at risk, because the carriers of mutations causing the above group of diseases not only have up to 100% lifetime risk of developing a disease, but also pass this genetic predisposition to their children. The extremely difficult life experience of families affected by any catastrophic early or late onset inherited disorder, seeing suffering from the disease and being anxious that they themselves will be soon affected, make them responsible to ensure that future generation will not be faced by the same difficulties [14, 15].

As for helping couples with their fully responsible decision to use the option of PGD to avoid the inheritance of a causative gene to their progeny, such as a gene for AD, although one of the partners may not be around to see this child grow up, of course societal discussions on the issue will be of great use [16]. First of all, the situation when only one parent supports a child to grow up and takes the responsibility for his or her future is not rare. On the other hand, this is not much different from that in parents who may get cancer or killed in a car accident, which are the main killers in western countries. At least, using PGD is better than having children without testing, because these children will have 50% chance of having AD or other dominantly inherited predisposition to severe late onset disorders with genetic predisposition. The possibility that there may be some approaches to prevent the clinical manifestation of these disorders in carriers of the mutant gene should not be excluded either.

PGD for common late onset disorders provides a novel nontraditional option for patients, who may wish to avoid the transmission of the mutant gene predisposing to their potential children. This may appear for some patients the only reason for undertaking pregnancy, as the pregnancy may be established free from an inherited predisposition from the very onset. Because, as mentioned, such diseases never present at birth or early childhood and even later may not be expressed in 100% of the cases, the application of PGD is still controversial. However, with no current prospect for treatment of many of them, which may arise despite presymptomatic diagnosis and follow-up, PGD may be offered as the only relief for such at-risk couples.

Therefore, prospective parents should be informed about this emerging new technology, so they could make their choice between seizing their reproduction and forgoing pregnancy free from late onset disorders with genetic predisposition. This seems to be ethically more acceptable, than a denial of the information on the availability of PGD. Presented results of PGD for the early or late onset disorder in Chap. 3, demonstrate the extended practical implications of PGD, providing prospective couples at genetic risk with wider reproductive options for having unaffected children of their own.

One of emerging indications for PGD, presenting complex ethical issues is predisposition to different forms of cancer (see Chap. 3). For example, PGD for breast cancer, caused by BRCA1 and BRSA2 genes, is being performed for increasing number of cases, despite the high cost of the procedure. PGD for breast and ovarian cancer has recently been also allowed by HFEA, despite the lack of appropriate guidelines for its use. It is expected that PGD will be used selectively, depending on the gene mutation, factors around a particular condition, age of onset, treatability, the

average penetrance, and the medical history of the individual family.

Even more complicated decision for PGD may concern the inherited cardiac diseases, for which no preclinical diagnosis and preventive management may exist and which may lead to premature or sudden death. The cumulative experience of PGD for inherited cardiac diseases, presented in Chap. 3, showed first results of PGD for familial hypertrophic and dilated cardiomyopathy, which introduces the option for couples carrying cardiac disease predisposing genes to reproduce without much fear of having offsprings with these genes at risk for premature or sudden death. However, it is still not clear how complex the ethical concerns are in relation to PGD for these common disorders, which may not be realized even in the whole lifespan.

One of the important ethical issues of PGD is also preimplantation HLA typing, because PGD for this indication is done for the benefit of a potential recipient rather than for the embryo itself, particularly when there is no need for testing of causative gene [13, 17, 18]. This may lead to feelings of moral outrage in some, while others may justify the action as saving a child's life from a severe disease. It is of interest that the majority of Americans are supportive of using PGD to ensure that an infant will provide an HLA match to donate stem cells or even tissue to an older sibling [19].

However, attitudes may be different depending on whether the genetic testing in the embryo is done or not. If preimplantation HLA typing is performed in combination with PGD, with the primary purpose being testing for causative gene, such as in case of Fanconi anemia (FA) [20], it appeared morally more acceptable, than preimplantation HLA typing as a sole purpose. The example of the latter situation may be leukemia or sporadic Diamond-Blackfan anaemia (DBA) in older children that may be cured by HLA-matched stem cell transplantation [21], which, however, does not present any benefit to the embryos tested. For example, as mentioned, such parents have initially been denied permission for preimplantation HLA typing in the UK. The moral dilemma stands also on the need of parents to have another child. However, preimplantation HLA typing as the sole reason is currently allowed also in the UK.

Some issues associated with preimplantation HLA typing are related to the actual indications for preimplantation HLA testing, which seem to be similar to the indications for stem cell transplantation, because preimplantation HLA typing has the objective of improving the access to an HLA identical stem cell transplant, which is the key in achieving an acceptable engraftment and survival in stem cell therapy. No doubt that the indications will be modified with progress in treatment of bone marrow disorders. For example, with current success in cure rate by chemotherapy, acute lymphoid leukemia (ALL) and acute myeloid leukemia (AML) may no longer be an indication [22], but the option of the stem cell therapy should still be available for patients, taking into consideration a sizeable proportion of patients, for whom chemotherapy was not effective and who may still require compatible stem cell transplantation, especially if the parents plan to have another child anyway. So all the conditions, for which bone marrow or cord blood stem cell transplantation is required, are also indications for preimplantation HLA typing.

The other important issue is the applicability of preimplantation HLA typing for sporadic conditions, such as DBA, for which there are also inherited forms. Accordingly, the inherited forms might require PGD for the mutations involved, to exclude the risk of transplantation of compatible stem cells, which might contain exactly the same mutation as the sibling [23]. For example, there are already known mutations causing DBA, such as one in the gene encoding ribosomal protein S19 on chromosome 9, and another gene mapped to chromosome 8 (see Chap. 4). It cannot be excluded, that additional mutations will be found for some of the other sporadic forms, also requiring PGD [24]. Still without such information, the couples have to make the decision about the need for undertaking transplantation, because of serious iron overload in the patients requiring urgently the compatible stem cell transplantation from the family member. Therefore, all known mutations causing the disease should be excluded by detailed mutation testing in parents and affected children,

and this should also be confirmed by the ongoing follow-up studies of the HLA-matched children born after preimplantation HLA typing.

The other controversial issue involves the written consent form, which is signed by parents for the embryo, similar to the situation when parents sign the consent form for umbilical cord blood stem cell collection and storage. It may be also argued that parents do not actually need a baby, and have it merely as a means to save its older sibling, so it would then become a commodity in some peoples' eyes, although parents usually claim that another child is needed for their family anyway, the decision which is solely parents' right.

Although at the present time, only umbilical cord blood stem cells are being collected from the "designer babies" at birth, presenting no harm for the baby, it is argued that the same approach may be used for organ donation. While with the progress in differentiation of cord blood stem cells into the other types of cells [25], such possibilities cannot be entirely excluded. It should be mentioned that preimplantation HLA typing also allows avoiding many ethical issues of reproductive and therapeutic cloning, as it provides more ethically acceptable option of selecting an HLA-matched progeny, rather than obtaining custom-made embryonic stem cells following somatic nuclear transfer and cloning.

Of special ethical concern is a nonmedical use of PGD for sex selection, which has been considered acceptable for social reasons in the US, provided that it is applied for selection of sex of the second or subsequent children [26, 27]. On the other hand, in some countries, such as India or Jordan, PGD is legally used for sex balancing, which seems to be also well justified [28, 29], as it is a part of reproductive autonomy, privacy in reproductive decision making and the moral superiority of preimplantation selection over sex selection abortion [30, 31]. However, it may also be argued that PGD for gender determination reinforces existing sexism and the expectation of conformity to stereotypical gender norms, and inconsistent with the ideal of parents having unconditional love for their children [32, 33]. Despite this opposition and also the opinion of American Society of Reproductive Medicine [26], American College of Obstetricians and Gynecologists [34] and HFEA [35], that the creation of embryos to select sex or enhance gender variety in the family is an inappropriate way to allocate medical resources, the use of PGD for this purpose is steadily increasing, with approximately 3,000 PGD cycles conducted annually only in the USA [36]. While the majority of studied cases were performed for medical reasons or together with PGD for genetic conditions and aneuploidies [37–39], increasing number of cases is performed for non-medical reasons [40–42]. For example, the special study performed to investigate moral attitudes and beliefs of the couples pursuing PGD solely for the purpose related to sex selection showed that the motivations for requesting gender determination includes a desire to limit family size, concerns about parental age, and financial concerns [43]. Although one of the main desires is to achieve a gender-balanced family, it was also shown that the majority of couples (78%) were seeking sex selection in order to have a boy [41, 42].

Finally, PGD raises many ethical issues, which are not unique to its clinical practice and instead are the same as in assisted conception [13]. One of the major criticisms concerns the selection of the embryos according to certain genetic parameters and destruction of others. In fact, the selection of a few embryos for transfer from approximately a dozen available after hyperstimulation is a routine practice of IVF, the remaining embryos being either frozen or discarded. Such embryo selection is usually done routinely based on morphological criteria, which has the goal of identifying the embryos with highest developmental potential. PGD, on the other hand, allows the improvement of the embryo selection, by applying genetic tests, which has shown that perfectly morphologically normal embryos may be chromosomally abnormal and so destined to be lost during pre- and postimplantation development. As described in Chap. 5, approximately half of oocytes and embryos obtained from women of advanced reproductive age are chromosomally abnormal, suggesting that it might no longer be an acceptable practice to select embryos on morphological grounds. In other words, the advent of PGD is a natural evolution of assisted

reproduction, allowing replacement of an almost "blind" selection of embryos on morphological grounds by chromosomal testing, to ensure the transfer of chromosomally normal embryos, with the objective of improving the chances of IVF patients to become pregnant. It may be hoped that the genetic testing of the oocytes and embryos may be further extended also for cytoplasmic abnormalities, which together with testing of nuclear abnormalities will, in future, allow the identification of a single viable embryo for transfer, which will ensure the highest possible efficiency of IVF, allowing a singleton unaffected pregnancy and birth of a healthy baby.

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# Index



A. Kuliev, *Practical Preimplantation Genetic Diagnosis*,
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