



## Reprogenetics: Preimplantational genetics diagnosis

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### Abstract

Preimplantational Genetics Diagnosis (PGD) is requested by geneticists and reproductive specialists. Usually geneticists ask for PGD because one or both members of the couple have an increased genetic risk for having an affected offspring. On the other hand, reproductive specialists ask for embryo aneuploidy screening (PGS) to assure an euploid embryo transfer, with the purpose to achieve an ongoing pregnancy, although the couple have normal karyotypes. As embryonic aneuploidies are responsible for pre and post implantation abortions, it is logical to considerer that the screening of the embryonic aneuploidies prior to embryo transfer could improve the efficiency of the *in vitro* fertilization procedures. Nevertheless, it is still premature to affirm this until well-designed clinical trials were done, especially in women of advanced age where the rate of embryos with aneuploidies is much greater. Although the indications of PGD are similar to conventional prenatal diagnosis (PND), PGD has less ethical objections than the PND. As with the PGD/PGS results only unaffected embryos are transferred, both methods can avoid the decision to interrupt the pregnancy due to a genetic problem; this makes an important difference when compared to conventional prenatal diagnosis.

*Keywords:* PGD, PGS, PGSS, embryo biopsy, trophoctoderm biopsy.

### Introduction

The term Reprogenetics was proposed to designate the combination of two types of approaches, reproductive technology and genetic methods. This combination emerged with the advent of *in vitro* fertilization. The most important issue is the use of preimplantational diagnosis in routine *in vitro* fertilization (IVF) prior to embryo transfer. Another important topic is prenatal diagnosis. The purpose of preimplantation genetic diagnosis (PGD) is to avoid the transmission of genetic disease in the offspring of couples with increased risk, similar to conventional prenatal diagnosis (PND). However, with this procedure arises the possibility of its use for tailored babies with certain genetic characteristics. Many ethicists argue that the purpose of PGD is eugenics, but such a purpose should be condemned if it were to be imposed by the state. The inherent desire of every parent is to give birth to a healthy and happy child.

Preimplantation diagnosis has existed since IVF use began, first with morphological observations and then with specific genetic analyses of embryos produced *in vitro*. PGD has an advantage over PND because it facilitates a

pregnancy under conditions in which a couple would otherwise have a great risk. PGD essentially consists of several steps: 1) ovarian superstimulation, 2) aspiration of ovarian follicles, 3) oocyte retrieval, 4) intracytoplasmic injection of oocytes with processed sperm, 5) *in vitro* culture of fertilized oocytes until D5-6, blastomere biopsy on D3 or trophoctoderm biopsy on D5, 6) genetic testing and 6) the transfer of a genetically normal embryo. If the blastocyst is not transferred to a receptive uterus until the 5th or 6th day, it loses the ability to produce an embryo. To preserve this possibility, it must be vitrified for later transfer.

The first preimplantation diagnosis was performed in 1989 for sex selection due to an X-linked disease. Currently, there are an estimated 10,000 children who were born after preimplantational biopsies. Preimplantation diagnosis is requested by geneticists and reproductive specialists. Usually, geneticists request PGD because one or both members of the couple have an increased genetic risk for passing a particular genetic disease to their offspring. Additionally, reproductive specialists request embryo aneuploidy screening (PGS) to assure a euploid embryo transfer, even when the parents have normal karyotypes. As in PGD or PGS, only unaffected embryos are transferred, and both methods can thus avoid the decision to interrupt the pregnancy due to a genetic problem; this is an important difference with conventional prenatal diagnosis.

## Indications of PGD

Indications are similar to conventional PND with regard to 1) genetic risks with monogenic or chromosomal causes, 2) major predisposition to tumors, 3) non-genetic risks or 4) selection of the best embryos in IVF laboratories.

As PGD or PGS involves both an IVF or intracytoplasmic sperm injection (ICSI) procedure and a genetic study, it is mandatory to predict the number of unaffected embryos obtainable for transfer prior to realizing the procedure. The number depends on the embryogenic potential of the fertilized oocytes and the implicated risk according to the genetic disorder. The embryogenic potential depends mainly on the woman's age and the absence of factors that facilitate the production of incompetent gametes. Generally, when a woman is younger than 35 years and the male produces good quality sperm, the embryogenic potential is approximately 50%. The embryogenic potential decreases when a woman's age increases or when sperm is of inferior quality. However, the genetic risk depends on the type of disorder (recessive, dominant, sex-linked) or if the disorder is chromosomal. Table 1 shows the estimated number of embryos needed to have the chance to transfer some unaffected embryos, based on the reasoning of PGD.

## Recessive Monogenic Disorders

Examples of recessive disorders are congenital disorders such as cystic fibrosis, Tay-Sachs, and thalassemia, which involve two mutated chromosomes from each healthy carrier parent. When the disorder is molecularly characterized, the mutation may be analyzed in cells removed from a cleavage embryo or blastocyst. Minisequencing is the method of choice. However, when the mutation is not known, it might be determined by a linkage study.

In cases where the mutation has not been identified in one of the parents, the use of polymorphic markers linked to the gene of interest could help to provide a better diagnosis and allow to have more transferable embryos; otherwise, embryos carrying the known mutation would be considered

as affected when they could be healthy carriers. Today, with the availability of SNParrays, the characterization of individual mutations is no longer needed.

## Dominant Monogenic Disorders

Examples of autosomal dominant disorders are myotonic dystrophy, fascio-scapular-humeral dystrophy, retinoblastoma, Von Hippel Lindau, MEN I and II, Huntington's disease, osteogenesis imperfecta, and achondroplasia.

When the patient has a "*de novo* mutation" it is necessary to sequence the entire gene to identify the mutation. Once the mutation has been characterized, this sequence can be targeted in the cells removed from the embryo. In contrast, when there are several affected members in the family, PGD can also be addressed with polymorphic markers linked to the respective gene.

Usually, Huntington's disease develops late in life or when the offspring are of child-bearing age. Many of them do not want to perform the genetic study because they do not want to know their genetic status in advance, but they want to make sure that their children do not have the mutation. Unlike PND, PGD for Huntington's disease avoids disclosure of the status of the carrier of the mutation.

It is well known that people with certain genetic disorders live in communities, such as mute communities for congenital deafness or persons with achondroplastic dwarfism, and that these couples desire PGD to increase their likelihood of having similarly affected offspring. This is a situation in which it is difficult to satisfy the parents because the medical team cannot help them.

## Sex Linked Disorders

X-linked disorders are transmitted by the healthy carrier mothers to their sons, while the affected males transmit the condition to their grandchildren through their healthy carrier daughters but not through their sons. When the mutation is characterized, it is recommended to perform PGD by minisequencing the mutation. Some reprogeneticists

**Table 1** - Prediction of the number of transferable unaffected embryos according to the reason for PGD.

Disorders	Affected embryos	Unaffected embryos	Ongoing embryos	Transferable embryos
Autosomal recessive (AR)	1/4	3/4	1/2	3/8
Autosomal dominant (AD)	1/2	1/2	1/2	1/4
X-linked recessive (XLR)	1/4	3/4	1/2	3/8
HLA	3/4 (no histoidentical)	1/4 (histoidentical)	1/2	1/8
AR+HLA	1/4 y 3/4	3/4 x 1/4	1/2	3/32
AD+HLA	1/2 y 3/4	1/2 x 1/4	1/2	1/16
RLX	1/4 y 3/4	3/4 x 1/4	1/2	3/32
Reciprocal translocation	4/5	1/5	1/2	1/10
Robertsonian translocation	3/4	1/4	1/2	1/8

carry out embryo sexing to avoid the birth of males, in such cases, but as was mentioned, this is not recommended.

Examples of recessive X-linked diseases are hemophilia, Fragile X, and Duchenne muscular dystrophy.

In contrast, dominant X-linked diseases are transmitted by affected women to 50% of their daughters and sons, but affected males do not transmit it to their sons. Examples of diseases linked dominant X are Rett syndrome, incontinentia pigmenti, pseudohyperparathyroidism, and vitamin D-resistant rickets.

As an example of Y-linked disorders, there are some AZF region microdeleted in the long arm of the Y-chromosome. In this case, the only option to avoid transmission to the offspring is female sex selection.

### Chromosomal Disorders

This category mainly includes carriers of balanced chromosomal rearrangements, such as reciprocal translocations, Robertsonian translocations, inversions and some cryptic deletion-duplication abnormalities. Most of the numerous anomalies in autosomal chromosomes are lethal and rarely reach adulthood, except Down syndrome. Women affected with Down syndrome are fertile and have a 50% risk of transmitting the condition, while affected males are sterile. In contrast, the numerous chromosomal abnormalities of the sex chromosomes allow individuals to reach adulthood, but some cause sterility and other types of infertility or sterility alone. Women that are 47, XXX are generally fertile, and they have a 50% of risk to have daughters with the same condition and males with 47, XXY. The 47, XYY males who are fertile have no risk of transmitting the YY condition because one of these chromosomes is excluded from the meiotic sexual body. However, the XXY males who produce sperm in fact have mosaic gonads, and only the XY spermatogonial cells undergo meiosis (Sciurano *et al.*, 2009). Prior to the advent of the Comparative Genomic Hybridization array (aCGH), these PGDs were addressed by FISH using probes of the chromosomes involved. Today, the best tool for chromosomal PGDs is molecular karyotyping or aCGH. The carriers of reciprocal translocations, both male and female, have a theoretical risk of 80% to produce abnormal gametes because the meiotic quadrivalent is segregated. Only alternate segregation produces balanced gametes; instead, adjacent 1, adjacent 2, 3:1 and 4:0 segregations are all abnormal. The real risk evaluated in sperm, oocytes and cleavage embryos on day 3 agrees with the theoretical risk. The carriers of Robertsonian translocations also have a theoretical risk of 75% because the meiotic trivalent is segregated (alternate, adjacent 1, adjacent 2 and 3:0), but the real risk observed in gametes and cleavage embryos is approximately 30%. It is much lower than the theoretical risk. The same occurs with the carriers of peri/paracentric chromosome inversions. In fact, both Robertsonian translocations and chromosome inversions are considered to be benevolent rearrangements

compared to reciprocal translocations (Coco *et al.*, 2005). Cryptic duplications or deletions have a theoretical risk of 50%, but there are not enough published reports to estimate the empirical risk of carriers of such anomalies.

The frequency of balanced chromosome rearrangements is 0.2% in control population of newborn, while it is 0.6% in infertile couples, 3.2% in those with recurrent failed IVF procedures, 9.2% in couples with recurrent miscarriages, 3.1% in men who require ICSI, and a similar figure for their partners (Peschka *et al.*, 1999; Stern *et al.*, 1999; Gekas *et al.*, 2001; Clementini *et al.*, 2005; Mozdarani *et al.*, 2008). Therefore, it is advisable to always carry out karyotyping of the couple prior to an IVF/ICSI procedure.

While the carriers of balanced chromosome rearrangements would be ideal candidates for pre-implantation genetic diagnosis, one has to consider that the only couples that can take advantage are those in which women respond very well to ovarian stimulation and are under the age of 40 years.

### PGD for Rh Blood Group Typing

PGD can also be indicated in women who are Rh negative and are highly sensitized with antibodies against Rh factor. If Rh genotyping in the male shows that he is heterozygous, it is feasible to perform a PGD to avoid possible erythroblastosis fetalis and intrauterine blood exchange transfusion. PGD has also been used in women sensitized by other blood factors, such as the Kell/Celentano group.

### PGD for Human Leukocyte Antigen HLA Typing

It is known that persons affected with certain genetic or acquired disorders require an HLA-compatible bone marrow transplant to survive. Due to the existence of public international banks of bone marrow or umbilical cord blood, the majority of those in need may find donors. When it is impossible to find a compatible bone marrow and the couple is still young and wants to have another child, the couple might use PGD for HLA typing to have an HLA-compatible child that can save the life of a sick sibling. PGD is performed using single tandem repeat polymorphisms (STRs) linked to HLA that map to chromosome 6. The purpose of PGD is to select the embryos with the same haplotype of the person who needs the transplant. These types of PGD are a real challenge because the probability of finding an HLA-compatible unaffected embryo is one in every 10 embryos studied (see Table 1). Socially, this type of PGD is known as PGD for having a child that serves as a "medication baby" as well as for "a la carte" designed babies, both of which are pejorative terms. If the best option is the latter one after an exhaustive international donor search, I consider it correct to attempt to have a child who can save the life of a sibling that will die if he/she does not receive a compatible bone marrow transplant.

## Indications of Preimplantation Genetic Screening (PGS)

PGS could have the same indications as PND, if one assumes that the chromosomal constitution found in polar body I, in a blastomere removed on day 3, or in several cells of the trophoblast on day 5 represent the constitution of the future embryo. However, today, there is evidence that this is not always true. Taking into account that the majority of miscarriages in the first trimester are caused by aneuploidies, and the rate of aneuploid oocytes increases with advancing age and is increased in males with oligoasthenoteratozoospermia (AOT), the ideal candidates for PGS would be as follows: 1) advanced maternal age, 2) couples with recurrent miscarriages, 3) couples with repeated IVF failures, and 4) severe male infertility. It could also be used to select a single euploid embryo for transfer, especially to avoid multiple pregnancies or to restrict the number of embryos to vitrify.

### Advanced Maternal Age

Women at an advanced age have a greater chance of having aneuploid pregnancies because they have increased rates of producing aneuploid oocytes. Oocytes are always the same age as the woman. However, in males, sperm are produced every 65-75 days. Therefore, it might be said that sperm are not the same age as the male. The prolonged arrest of oocytes at meiotic prophase I mainly contributes to aneuploidy due to the decline in competence of the cytoplasm of the oocyte. The number and distribution of chiasmata during prophase I as the weak centromeric cohesion may be the main factor that predisposes aneuploidy that is inherent to age. In fact, the principal cause of oocyte aneuploidy is the precocious separation of sister chromatids rather than classic non-disjunction (Chiang *et al.*, 2012). In the male, the expected sperm aneuploidy rate is between 0.5 and 1% because the sperm is not the age of the male, but if the sperm is not ejaculated for prolonged periods, it could have a high rate of DNA fragmentation, which is also responsible for abnormal fertilization. Competent oocytes from young women can repair the DNA fragmentation of the sperm, but the oocytes from older women cannot. Therefore, women of advanced age have higher probabilities of having abnormal pregnancies that might end in miscarriage or in a malformed newborn. Most of these embryos are lost during pre or post implantation stages, while a minority come to term. That is why the possibility of miscarriage also increases with the age of the woman (see Table 2).

It is well recognized that most autosomic aneuploidies in live newborns are *de novo* or inherent to maternal age, while most sex chromosome aneuploidies are of paternal origin independent of paternal age, or are associated with poor sperm quality (Hassold *et al.*, 1984; Jacobs and Hassold, 1995). The majority of males with a normal

**Table 2** - Pregnancy loss rate and maternal age.

Maternal age	Down's risk	All chromosome risks	Miscarriages rate
20	1/1667	1/526	8
25	1/1200	1/476	10
30	1/952	1/385	12
35	1/378	1/192	16
40	1/106	1/66	40
45	1/30	1/21	60

karyotype and a normal spermiogram have a 0.5 to 1% sperm aneuploidy rate, while the rate for oocytes is much higher, between 20 and 50%, mainly depending on the age of the woman (Hultén *et al.*, 2005). However, the rate of sperm aneuploidy in males with OAT and a normal karyotype is much greater than that observed in males with normozoospermia (Coco, *et al.*, 2000; Colagero *et al.*, 2001; Rubio, *et al.*, 2001; Burello *et al.*, 2003). Although women more than 37 years old and males with OAT could be ideal candidates to benefit from PGS, most of them fail due to the low probability for producing euploid embryos. The first randomized controlled clinical trials (RCT) for PGS in patients with advanced maternal age were promising due to the lower miscarriage rate and higher take-home baby rate achieved in the group that underwent PGS (Munné *et al.*, 2006). However, other clinical trials emerged without differences in the findings between both groups, with and without PGS, and others showed even worse results in the studied group with PGS. (Staessen *et al.*, 2004, 2008; Stevens *et al.*, 2004; Jansen *et al.*, 2008; Mastenbroek *et al.*, 2007; Hardarson *et al.*, 2008; Schoolcraft *et al.*, 2009). Most of those studies were performed with biopsies on D3 and FISH, first enumerating five chromosomes and later 7, 9 and 12 chromosomes. Three arguments were used to explain these poor performances: a) limitation of the technique to enumerate all chromosomes, b) lower implantation rates after the removal of one or two blastomeres, which means a loss of embryonic mass of between 12.5% to 25%, and c) discarding supposed aneuploid embryos that might have been self-corrected and lead to a normal pregnancy. Currently, we have much hope with comparative genomic hybridization arrays (aCGH), which could solve the first and second limitations because they allow the study of all 24 chromosomes, and because the cells extracted correspond to trophectoderm cells and not to the stem cells of the inner cellular mass of the blastocyst (ICM). The third and last limitation mentioned remains unanswered until we know the rate of false positives and negatives for this method. While we are performing randomized controlled trials (RCTs) similar to those carried out with PGS by FISH, we will continue, of course, without knowing the clinical value of PGS.

The first clinical trial with aCGH in blastocysts was performed by Yang *et al.* (2012). The authors, worked with patients with good prognoses, and these showed an increased pregnancy rate in patients who had received the best euploid blastocyst compared to those without aCGH (70.9% vs. 45.8%). The same authors (Yang *et al.*, 2013), also working with patients with good prognoses, documented a better implantation rate in a group with aCGH (65%) vs. a group without aCGH (33%). The authors also showed that the rate of spontaneous miscarriage was significantly lower in the group screened with an array (0%) vs. controls (16.7%).

Schoolcraft and Katz-Jaffe (2013), working with patients over 35 years old, also showed a better ongoing pregnancy rate (60.8%) in the group that had received devitrified euploid blastocysts compared to the control group (40.9%) that had received blastocysts without aCGH. A clinical trial of quite different design was published by Scott *et al.* (2013). These authors carried out the transfer of the two best embryos, whereby one was biopsied on D3 or D5 in accordance with the routine protocol for patients with good prognosis, but without knowing the outcome of an array before the embryo transfer. The authors found that 48.2% of the euploid blastocysts achieved pregnancy, while 93.5% of the aneuploid blastocysts did not achieve implantation. In contrast, the transfer on D3 showed that 29.2% of the euploid embryos achieved pregnancy, while 98.1% of the aneuploid embryos failed to do so. These results clearly reinforce the need to continue with this type of clinical trial in patients indicated for an IVF procedure, especially those of poor prognosis due to advanced age. The authors also showed that the implantation rate decreases nearly 50% when the biopsy is performed on D3 with respect to non-biopsied controls (30.4% vs. 50.0%). However, the implantation rate was not modified by the trophoctoderm biopsy (51.0% vs. 54.0%). Therefore, such biopsy done on D5 has no deleterious effect compared to the one on D3. Another RCT (Forman *et al.*, 2013) showed that the cumulative rate of take-home babies was similar when transferring the best euploid single blastocyst (69%) or the two best blastocysts without aCGH (72%), with a 47% multiple pregnancy rate in the group transferred with the two best blastocysts. The authors documented an aneuploid blastocyst rate of 31%, with 21% of women younger than 35 years and 56% older than 40 years. Almost half of the newborns were twins in the transfer group with two blastocysts. The rate of preterm, low birth weight and greater number of days required in neonatal intensive care was also twice that of the group with a single blastocyst transfer.

### Recurrent Pregnancy Loss (RPL)

Usually, RPL is defined as two or more consecutive pregnancies lost before 20 weeks of gestation. Different cytogenetic studies of miscarriages in the first trimester of

pregnancy show that aneuploidy rates varied between 50% and 80% (Strom *et al.*, 1992).

Additionally, it has been documented that couples with RPL produce more aneuploid embryos than those who have not had RPL (Pellicer *et al.*, 1999). According to some authors, PGS does not improve the rate of pregnancy in RPL, but increases the chance of birth at term (Platteau *et al.*, 2005).

### Recurrent IVF Failure (RIF)

RIF is usually defined as the failure of three or more IVF attempts with good quality embryo transfer. Some authors argue that these couples produce more embryos with aneuploidies (Hodes-Wertz *et al.*, 2012). However, there is no evidence that PGS improves the rate of pregnancy or live IVF births.

### Severe Male Factors

As mentioned above, the rate of aneuploidy in spermatozoa from fertile males with a normal spermiogram is much lower than that observed in oocytes, and aneuploidy also does not increase with age in men.

The study of the chromosomal complement of the sperm has been feasible since Rudak *et al.* (1978) published the possibility of fertilizing oocytes from hamsters with human sperm. Later on, the hamster test was replaced by FISH on semen, a technique that is much less complex than Rudak's technique. Although FISH is ideal for enumerating all chromosomes, it has the limitation of the number of chromosome probes used per hybridization round, which is fundamental for the small size of the sperm nucleus, because the number recommended is no more than three probes per round of hybridization. Rives *et al.* (1998) performed FISH for all chromosomes in semen samples from four semen donors. They found uniformity in the percentage of autosome disomies, which varied between 0.1 and 0.5%. This finding encouraged several authors to evaluate the chromosome complement of spermatozoa by performing FISH on semen samples with a few chromosome probes and to estimate the rate of aneuploid sperm using a simple mathematical calculation that assumes that the remaining chromosomes (*i.e.*, those not studied) would behave similarly. In a study conducted with 10 voluntary donors of semen in our laboratory during the year 2000, we found that the percentage of sperm aneuploidy ranged from 4.2% to 14.3%, with an average value of  $10.1\% \pm 3.8$ . In contrast, when we studied patients with oligoastheno-teratozoospermia (OAT), the frequency of aneuploidies varied from 4 to 83% with an average value of  $25.8\% \pm 8.4$  ( $n = 53$ ). We also found that sperm aneuploidies increased with the severity of OAT. We have extended the study to 10 patients with OAT who used the ICSI procedure. FISH in semen was performed with the same sample used in the ICSI procedure, and we found that the percentage of sperm aneuploidies

was higher in the group that had not achieved pregnancy (Coco *et al.*, 2000). These findings put in evidence the importance of the genetic risk assessment before the ICSI procedure to predict the chance of success. Now, with the possibility of PGD and lower costs, FISH is no longer used to assess sperm. It should be noted that the chances of selecting an euploid embryo mainly depend of the number of embryos produced during the procedure. When it is suspected that the couple has a major chromosomal risk due to advanced maternal age or severe male factors, it is mandatory to inform them of the low chance of achieving a pregnancy with the PGS procedure, unless the couple produces many embryos that provide one or two euploid embryos apt for transfer (Harper and Sengupta, 2012).

### Sex Selection (PGSS)

The selection of the gender of the future child is a wish that a majority of people have when they plan to have offspring. However, for most people, it is not viewed positively unless sex-linked diseases or other important concerns exist. Today, sex selection is a feasible reality that can be satisfied without ethical or legal disadvantages if the selection was carried out when the pregnancy is not established. Before the advent of PGD, there were no attempts to perform a prenatal diagnosis for sex selection, for diseases of late onset in life or for the predisposition to suffer certain tumors. While it is reasonable to state that the bioethics status of a preimplantation embryo is not equal to that of an embryo-fetus, there are, nonetheless, those who consider that already a fertilized oocyte has the status of a person, and for these, PGD would not be an option. Nonetheless, if we take into account the number of PGSS procedures performed worldwide as a proportion of the total number of requests for sex selection, it reasonable to assume that many of these reconsidered their concepts after being informed of the procedure.

Table 3 provides a list of the different motivations for PGDs from 54,589 cycles registered during data collection I-XIV for PGD by the ESHRE Consortium (Traeger Synodinos *et al.*, 2013).

### Biopsy Techniques

There are several types of recognized biopsies: polar bodies, blastomeres on D3, trophoctoderm on D5/D6 and, more recently, attempts to perform blastocentesis in blastocysts on D5/D6, as a new type of noninvasive embryo biopsy based on the presence of cells and DNA in the blastocoelic cavity. Actually, all of them are invasive and involve some risk of loss of the embryo. Working with a simple cell is not easy and may yield no results. In this regard, the biopsy of blastocysts is most suitable. Blastocentesis is by now a new hope for less invasiveness. While none of these methods assures the proper constitution of the future embryo, they minimize the risk of the disorder that is

**Table 3** - Different reasons for PGD from the I-XIV ESHRE PGD Consortium data collection.

Reason for PGD	N° PGD cycles	Percentage
Monogenic	11.084	20.3%
Chromosomal	8.104	14.8%
Sex selection for monogenic X-linked	1.603	2.9%
Social sexing	765	1.4%
PGS	33.033	60.6%
Total	54.589	100%

being investigated. The best result was obtained via the biopsy of blastomeres on D3 with fresh transfer on the same day of the biopsy, or transfer on D4/D5. To remove one or two cells from the preimplantation embryo it is first necessary to perforate the zona pellucida. The perforation of the zona pellucida can be done by several methods: A) mechanically, by cutting through the pellucida with a micropipette; B) chemically, by dissolving part of the pellucida with an acid solution; or C) by laser, through modulating a laser beam via the optical system of a microscope. Prior to biopsy, the preimplantation embryos can be placed in a suitable medium to loosen the cell junctions at room temperature. Then, the embryos are placed in separate microdrops composed of the medium for biopsy under oil and labeled. It is not convenient to have more than two pre-embryos in a dish to minimize their time out of the incubator. Using a micromanipulator/microscope setup, the oocyte or the pre-embryo that will be biopsied is placed in the center of the field and focused at a 400X magnification. The embryo or oocyte is fastened with a micropipette holder. The zona pellucida is perforated, and the polar bodies (PBI/PBII), or blastomeres are removed gently with an appropriate micropipette. When the biopsy is performed on D5, the zona pellucida is perforated on day 3 to facilitate the hatching of the blastocyst and to easily remove some cells of the trophectoderm. The cells will have to be collected according to the protocol of the genetic study indicated. If the indication is a FISH study, the removed cells are fixed on a slide, but if the indication is a PCR assay, the removed cells are collected in a small tube.

### Biopsy of Polar Bodies (PB I/PBII)

The biopsy of the first PB prior to the fertilization of the oocyte evaluates the result of the first meiotic division. Because errors can also occur during the second division of the oocyte, it is necessary to also study the second PB to avoid misdiagnosis. The second division of the oocyte is completed when the sperm penetrates the oocyte. Therefore, the biopsy of the second PB is performed once the ovum has been fertilized. As PB biopsies do not allow the evaluation of male meiotic errors and/or errors that occur after fertilization of the egg, the biopsy of blastomeres is more preferred because it allows the assessment of both pa-

rental contributions and/or errors during cleavage. Polar body biopsy is only useful when women have a major risk of transmission of monogenetic diseases or aneuploidies inherent at the maternal age. As was mentioned above, to avoid misdiagnoses, both polar bodies should always be biopsied. When the purpose is to evaluate aneuploidies, both biopsies can be performed simultaneously. Instead, when the question is a monogenetic disease, it is necessary to perform the biopsy in a sequential way. The biopsy of the first polar body prior to fertilization only indicates errors during the first meiotic division and/or whether the oocyte carries the same maternal mutation, always assuming that an interchange did not occur in the locus where the mutation maps to. In countries where embryo biopsy is prohibited, the pre-implantation genetic diagnosis can only be performed by biopsy of polar body I because the second polar body appears after the fertilization of the oocyte. Therefore, the biopsy of the second polar body would have the same connotation as the embryo biopsy.

PBI biopsy to assess aneuploidies is not optimal because there are other possibilities for segregation during the second division, without assuming that the first meiosis was normal or abnormal. Figure 1 shows the different possibilities of the second division after a normal first meiotic division, where two of three possibilities are abnormal. Figure 2 shows the different possibilities of the second division after a non-disjunction occurred during the first division; one of the six possibilities corresponds to an aneuploid rescue.

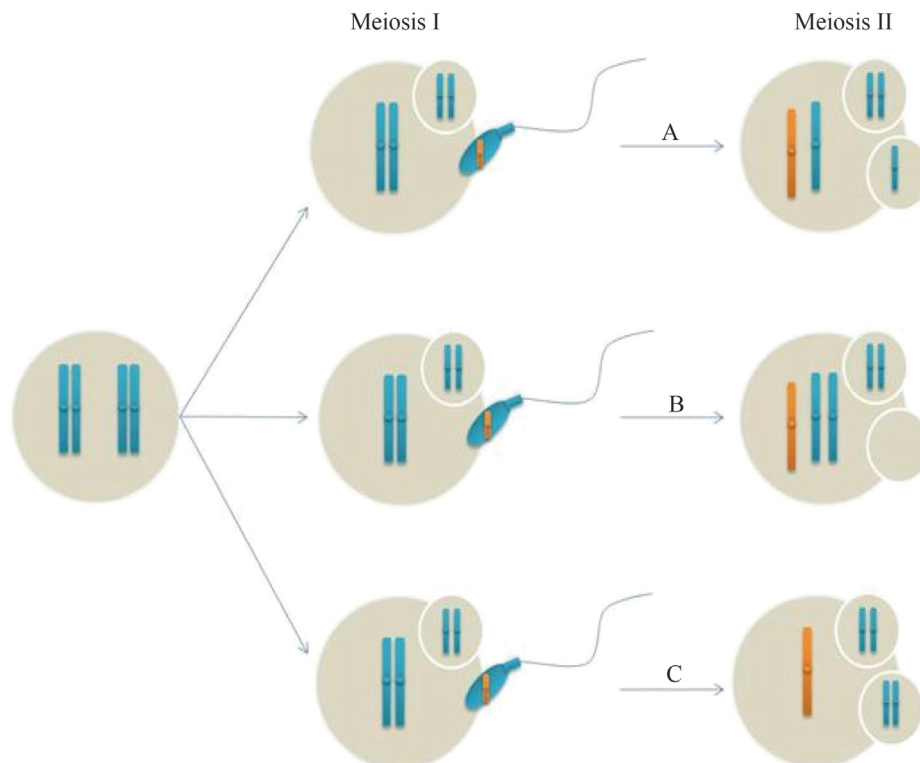
Figure 3 shows the different possibilities during the second division after a premature separation of sister chromatids in the first meiotic division; two of six possibilities correspond to an aneuploidy rescue.

Polar body biopsy is also not ideal for monogenic diseases due to the possibility of crossing over. Figure 4 A shows a normal segregation without crossing over, while Figure 4 B shows a segregation after a crossing over event at the level of a mutated gene that is able to produce normal and abnormal oocytes.

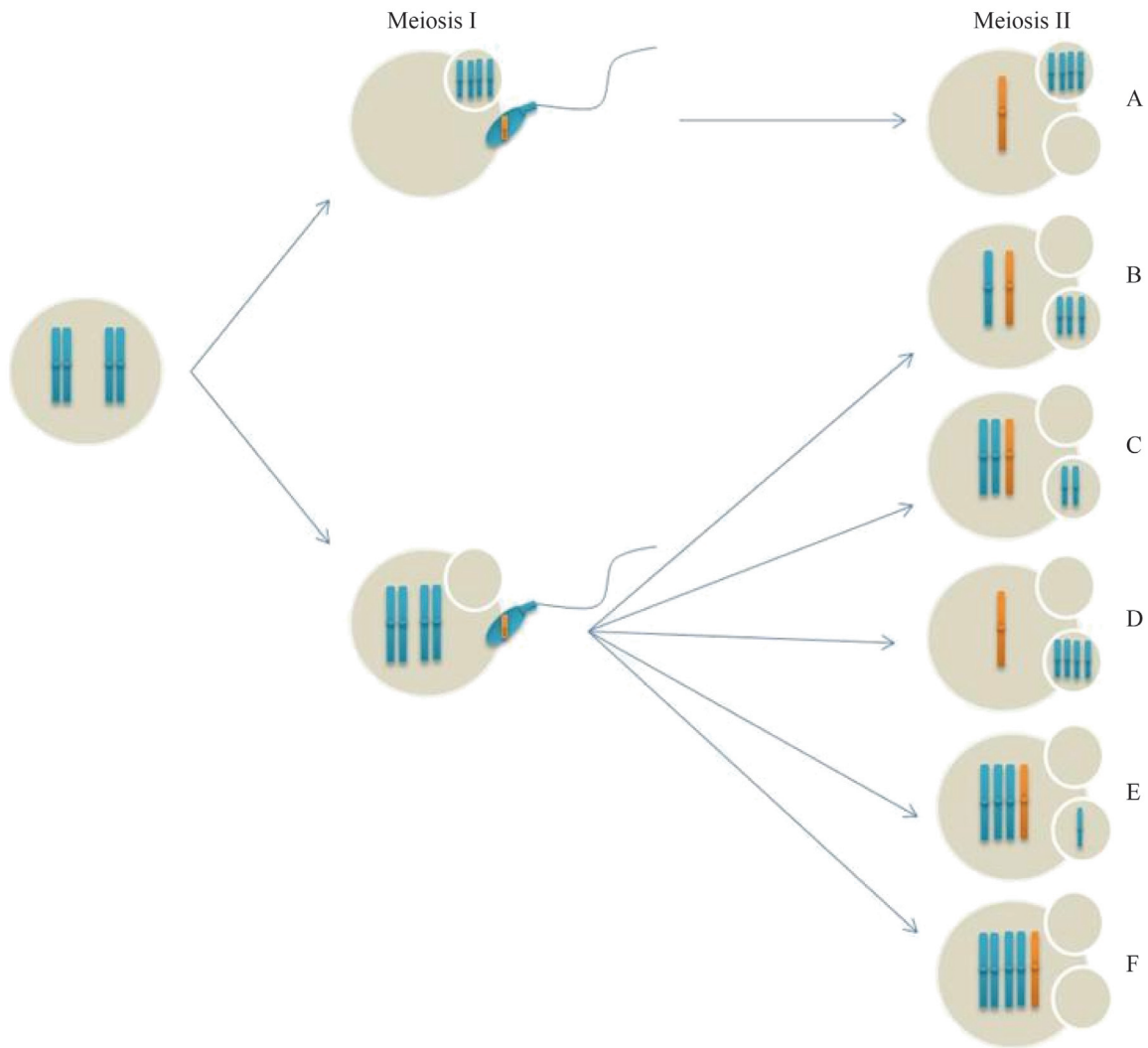
In spite of the disadvantages indicated according to the ESHRE Consortium, 16% of the biopsies done correspond to those of polar bodies. Kuliev and Verlinsky (2004) studied more than 8,000 oocytes from women older than 35 years with FISH for chromosomes 13, 15, 16, 21 and 22 found that more than 50% had aneuploidy. Recently, a pilot study of the ESHRE PGD Consortium using aCGH in polar bodies from women over 40 years showed an aneuploidy rate of 75% (Geraedts, 2013)

### Biopsy of Blastomeres in Cleaved Embryos on D3

This is the type of biopsy used to remove one or two cells in embryos with more than six cells on D3. Embryologists have acquired great skill in performing this technique, but there is evidence that it decreases the rate of implantation. FISH or PCR analysis in a simple cell is a real challenge for specialists and for patients. It is not easy to work



**Figure 1** - Possibilities of segregation during the second division from a normal oocyte. One of the three possibilities is normal (A).



**Figure 2** - Possibilities of segregation during second division from an abnormal oocyte by non-disjunction. One of the six possibilities is normal (B).

with a single molecule of DNA for chromosomal and genetic tests. However, this was the methodology used in the last 20 years. Indeed, 80% of the PGDs recorded by the ESHRE Consortium were D3 blastomere biopsies. During that time, fresh transfer was used to avoid cryopreservation. At first, the protocol mostly used for PGD/PGS was transfer on the same day of the biopsy. Improvement of sequential culture media allowed the prolonging of *in vitro* development until the fifth day, and transferring only those that reached the blastocyst stage. Biopsy on D3 achieved a clinical pregnancy rate of 18.7% and a take-home baby rate of 14.7%, with a misdiagnosis between 5 and 10%, according to records I-XIV of the ESHRE Consortium.

### Blastocyst Biopsy

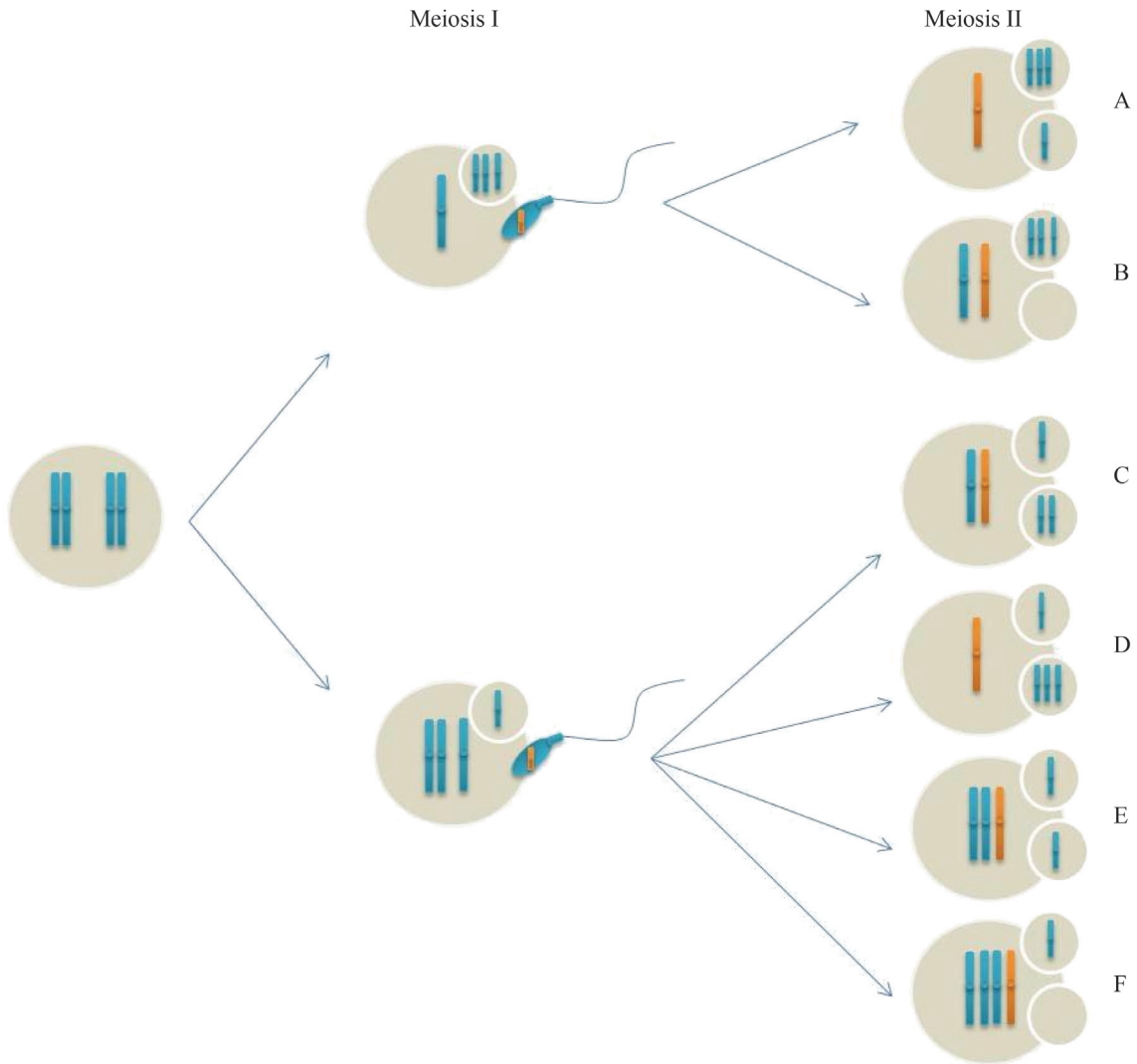
The blastocyst is the highest degree of development that an embryo can reach *in vitro*, and it is characterized by three elements: the inner cell mass, the outer cell layer or

trophoblast and the blastocoel. The blastocyst begins to form on the fifth day and is completed on the sixth.

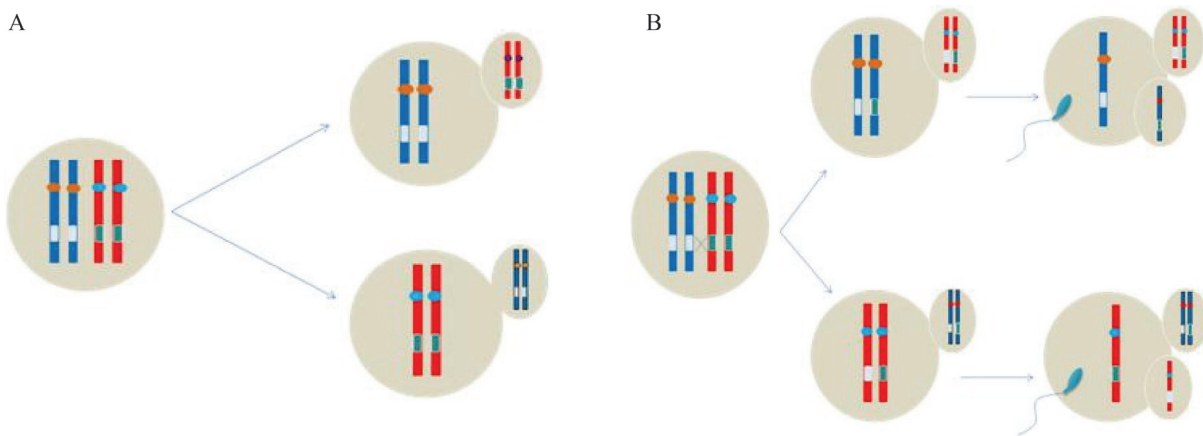
A blastocyst usually has more than 100 cells. The majority will form the placenta, the chorionic villus and other extraembryonic structures. Only a small percentage of the ICM will differentiate into the embryo proper after implantation of the embryo in the endometrium. Therefore, the trophoblast biopsy procedure is considered equivalent to the puncture of a chorionic villus, with the same limitations of not corresponding to the constitution of the embryo *per se* due to the possibility of mosaicism.

Once the decision to perform a trophoblast biopsy is taken, it is preferable to perform the transfer in a deferred cycle, because not all blastocysts are obtained on D5, and the genetic studies demand time. This decision is very important because it allows better organization of the genetic laboratory assays. At present, there is sufficient evidence that the deferred transfer to the stimulated cycle has its advantages in terms of implantation, ongoing pregnancy and





**Figure 3** - Possibilities of segregation during second division from an abnormal oocyte by early separation of sister chromatids. Two of the six possibilities are normal (B and C).



**Figure 4** - Segregation with and without cross-over. (A) Normal segregation without cross-over at the level of the mutated gene. The oocyte will always have the opposite chromosome constitution to that of polar body I. (B) Normal segregation with a cross-over at the level of the mutated gene. In contrast, when exchange occurs, the egg may or may not have the mutation.

lower risk of genetic and epigenetic alterations (Papanikolaou *et al.*, 2006; Maheshwari *et al.*, 2012; Shapiro *et al.*, 2012, Roque *et al.*, 2013). In 2011, our team took the decision to switch from the blastomere biopsy to the trophectoderm biopsy procedure with deferred transfer to the stimulated cycle. The results were much better, and importantly, the entire team worked in a more comfortable, friendly, healthy and efficient environment (Coco *et al.*, 2012).

## Genetic Testing

There are three fundamental techniques in a PGD program: FISH, PCR and aCGH. Handyside (2013) recently published the different diagnostic tests available to carry out PGD/PGS, along with their pros and contras.

## Considerations of PGD/PGS

Not all people who want to perform PGD are able to do so. There are two fundamental requisites: the couple should be fertile and it should have the genetic characterization of the disorder which is intended to be diagnosed. This last point is very important because couples assume that they will have a normal healthy child, whereas it is only possible to offer to minimize the risk for the disease for which they have a great risk of transmitting to their offspring. As there is a small risk of misdiagnosis due to the existence of mosaicism or limitations of the techniques used, couples should always be offered the possibility of an amniocentesis to double check the results. It should be remembered that all the other prenatal diagnoses, both the non-invasive prenatal test (NIPT) and the chorionic villi sample, as well as PGD are screening methods because these analyses are based on cells from the trophectoderm. The procedure should always be explained in detail during the entire treatment to ensure that couples are informed about the potential risks in the short, medium and long term.

Because the majority of patients for PGD are advised by a geneticist, the patients are usually more informed about the risk at birth or during pregnancy, from the end of the first trimester and beginning of the second, which are completely different from the risk during the preimplantation phase, particularly with regard to the risks of aneuploidies and abnormal segregations in carriers of chromosomal rearrangements. During preimplantation development, the risks are much higher. To benefit patients with a more predictable PGD, doctors should ensure that the couple produces a sufficient quantity of embryos to obtain non-affected embryos for transfer.

With respect to PGS, it can be said that the ideal candidates have the following characteristics: women of advanced maternal age, couples with recurrent miscarriages and recurrent IVF failures, and men with severe male factors. However, the usefulness of PGS is still controversial.

For PGS by FISH the results first were very promising, but later on discouraging and, finally, undesirable because this method produces effects contrary to those expected. These became evident when studying biopsies taken on D3. We have now begun to investigate biopsies taken on D5 as these provide more cells to study and permit to apply different diagnostic methodologies that are more robust than FISH. The use of blastocyst biopsy is very promising, as happened at the beginning with FISH with biopsy on D3. Although it is undisputed that the CGH array is superior to FISH, there are doubts about the benefit of these arrays in the group of patients who are more likely to produce aneuploid embryos. Women of advanced age have a higher risk of producing aneuploid oocytes. Additionally, such women respond poorly to ovarian stimulation and thus produce only few blastocysts. If the existence of a male factor is added, the prognosis is even more discouraging. The meiotic risk of the couple based on the woman's age and the existence of male factors should be used to estimate the number of blastocysts required to obtain at least one euploid blastocyst apt for transfer. For women of advanced age more oocytes are needed to have a chance of finding euploid blastocysts, but they produce much fewer oocytes than young women. An alternative would be the collection of blastocysts in various cycles of ovarian stimulation instead of only one, though there are insufficient, primarily anecdotal data to conclude that this may be a solution (Mondadori *et al.*, 2012). Harton *et al.* (2013) recently reported that selective transfer of euploid embryos showed that implantation and pregnancy rates were not significantly different between reproductively younger and older patients of up to 42 years of age. Nevertheless, information on more well-designed clinical trials is needed to know if the embryos from older women are really harmed or not by biopsies, because the poor quality of the embryos might make them more vulnerable to the procedure.

Therefore, for biopsies on D3 or D5, one must assume that the result found in the removed cells corresponds to the condition of the rest of the embryo. However, this is only true if errors do not occur after fertilization. In contrast, when an error occurs after fertilization, the probability to detect it depends on the cleavage stage and the day that the biopsy is carried out. If the biopsy is performed on D3 and the error occurred in the first division, there is a 50% possibility of detection; but when it occurred during the second division, the chance drops to 25%, and during the third division, the probability is only 12.5%. If the biopsy is performed on D5 and the errors have occurred in the first three divisions, there is a chance of detection, but it is also true that a newly originated trisomy might be detected in one of the isolated cells of the trophoblast. One trisomic cell among 5 to 10 removed cells is already detectable with the current diagnostic tools, and if the trisomy is viable, it would surely be confined to the placenta, without any implication for the chromosome constitution of the embryo.

Patients should understand that the constitution of the trophoctoderm is not always coincident with that of the ICM because different possibilities of chromosomal constitution can exist: a) the trophoctoderm and the ICM are homogeneously normal or abnormal, b) the trophoctoderm may be abnormal but the ICM may be normal, (c) the trophoctoderm may be normal but the ICM may be abnormal, d) the trophoctoderm may be a mosaic and the ICM may be normal or abnormal, and e) both the trophoctoderm and ICM may be mosaic.

If clinical trials are done on a group with PGS and another one without PGS and the euploid embryos are always transferred, one can never know the rate of false positives and negatives that may PGS produce. This is not a minor detail, and it is relevant, especially when couples produce one or two blastocysts, and the array indicates that they are abnormal. Should we advise discarding them? Obviously, it is necessary to have better designed clinical trials to obtain the correct answer. If we are to follow the same type of clinical trials as carried out on PGS by FISH, we are likely to make the same mistakes, and we are perhaps eliminating the last chance for older couples to be parents of their genetic children. Today, there is evidence that blastocyst biopsy does not harm the implantation process, so we should offer it to patients who require IVF/ICSI to participate in a clinical trial. The patients must accept that the best blastocyst be biopsied without knowing the result of aCGH. I personally consider that this type of clinical trial will allow us to know the behavior of the anomalies detected in the trophoctoderm. The classical randomized clinical trials include two groups of patients, studied and unstudied, with subsequent re-analysis of the blastocysts that were not transferred because they had been diagnosed as aneuploids with the purpose of verifying the existence of mosaics; these are, in my opinion, very expensive for an unrecoverable blastocyst. Johnson *et al.* (2010) reported a correspondence of 96.1% between karyotypes of the trophoctoderm and ICM when they re-examined 51 blastocysts diagnosed as aneuploids. However other authors (Liu *et al.*, 2012) who re-examine 13 blastocysts diagnosed as aneuploids found a high rate of mosaics in the trophoctoderm, and in four cases mosaics were not present in the ICM because the aCGH was normal. The establishment, characterization and differentiation of a karyotypically normal human embryonic stem cell line from a blastocyst diagnosed as chromosome 21 trisomy was recently reported (Mandal *et al.*, 2013). Such a finding could be due to a self-correction of the aneuploidy or to the existence of a mosaicism of the trophoctoderm, as well as due to a recent error that occurred in only one cell among the cells removed during trophoctoderm biopsy.

## Main Conclusions

PGD is an alternative to prenatal diagnosis for patients with increased genetic risk in their offspring. It is a

prerequisite that the couple should be fertile or that their infertility can be reverted by IVF. Preimplantation diagnosis has a great advantage over conventional prenatal diagnosis because it can avoid possible genetic miscarriage. However, it has the disadvantage of being expensive, and fertile couples have to undergo a highly complex treatment, as do infertile couples. When the couple is genetically infertile it is mandatory to perform PGD to avoid the inherent risk to the offspring.

Although PGS is indicated for advanced maternal age, recurrent miscarriages, or for severe male factors, the benefits of its use are not yet clear, especially for patients with normal karyotypes. It is true that these patients have a higher risk of producing aneuploid zygotes, but it is also true that most homogeneous aneuploidies are lethal, on the order of 99%, and that the majority die before implantation or during embryo-fetal development (Hassold and Hunt, 2001). Unlike partial aneuploidies, almost from abnormal segregation of carriers of balanced rearrangements, the pregnancy may continue to term and birth a malformed child. Therefore, the above-mentioned patients should use PGD, especially if they are infertile.

The experts' committees of the main scientific societies for reproductive medicine support the idea that there is still no evidence that establishes PGS as beneficial for those groups of patients who would be the ideal candidates for PGS (Hardarson *et al.*, 2008). As these groups of patients produce fewer normal embryos for transfer, they should be informed that the pregnancy rate after PGS could be lower compared to IVF without PGS.

Among the different types of existing biopsies, it is possible to conclude that the polar body biopsy has very low diagnostic efficiency. Blastomere biopsy on D3 decreases the rate of implantation, and between 10 and 20% of the embryos studied may not give results due to the limitations of the assays performed on only one or two DNA molecules. The rate of misdiagnosis according to the data of the ESHRE PGD Consortium is between 3-10%.

Thus far, there is no information on the increase of malformed children after PGD/PGS. However, one cannot rule out a potential risk for genetic diseases with any of the IVF procedures. The procedures IVF / ICSI / PGD should be considered experimental until one can see what happens to the grandchildren born post assisted reproduction technologies (ART). Trophoctoderm biopsy might be considered less invasive than blastomeric biopsy. In fact, the most recent work by Scott *et al.* (2013) demonstrated that it does not affect the implantation rate. The removal of more cells always has the advantage of obtaining a result with genetic testing, favorable or not, in addition to permitting the realization of any of the available genetic diagnostic methods. However, PGS has the disadvantage of discarding aneuploid blastocysts that might undergo self-correction or be confined to the placenta. Improvement in culture media for embryos, incubators with low concentration of oxygen and

vitrification permits to implement blastocyst biopsy. This has the advantage of performing the biopsy in an embryo that has reached the maximum developmental degree in the laboratory and can be transferred to the uterus in its optimal state of development. Also, there is a cost reduction because only embryos that reach this stage are biopsied and studied. The reduced invasiveness of the biopsy, the larger number of cells removed and the programming of the studies on several weekdays are responsible for its good acceptance for teamwork. Our PGD program has used blastocyst biopsy with transfer in an unstimulated cycle since 2011 (Coco *et al.*, 2012). Such decisions allow older women to produce several blastocysts in several cycles of ovarian stimulation, and they minimize the risk of miscarriage while transferring euploid blastocysts (Mondadori *et al.*, 2012). Today there is no doubt that the pregnancy rate with transfer in the unstimulated cycle is much higher than that in the stimulated cycle (Shapiro *et al.*, 2012; Roque *et al.*, 2013). Additionally, a recent systematic review and meta-analysis of 11 studies showed better obstetric and perinatal outcomes with cryopreserved embryos *vs.* non-cryopreserved ones (Davies *et al.*, 2012; Maheshwari *et al.*, 2012). The improved results are most likely due to the better embryo-endometrial synchronization in a natural or more physiologically acceptable cycle (Haouzi *et al.*, 2010).

All the biopsies that can be realized in the *in vitro* preimplantational stage are invasive, and they have the value of screening, rather than a diagnostic value. Therefore, there should always be more benefits than risks. Most likely, the aspiration of the blastocoel is indeed less invasive. Because all embryo transfers in the future will probably be done with devitrified blastocysts in non-stimulated cycles, and because blastocysts with induced collapse are better vitrified, the aspirated fluid might be kept for PGS using new generation sequencing with a much lower cost compared to the current method. If all blastocyst fluids are chromosomally analyzed after transfer, we would have the ideal clinical observational essay to determine the rate of false positives and negatives of the PGS and to know more about the biological behavior of chromosomal anomalies during the *in vitro* preimplantational stage.

There are no doubts that reprogenetic developments have attained huge achievements during recent years and that the final beneficiaries are persons/patients who struggle with their difficulty to create families. All this progress benefits them greatly by providing information based on which they have to take very important decisions in their lives.

There are three conditions that have to be fulfilled to make these decisions sustainable for life: 1) clear and neutral information, 2) a healthy and productive doctor-patient relationship, and 3) psychological coaching to allow patients to cope with a genetic condition and to avoid transferring it to their offspring.

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