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Preimplantation genetic testing: current status

Description générale, indications et résultats des tests génétique préimplantatoires

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Abstract. Preimplantation genetic testing (PGT) is a procedure that involves the removal of 1 or 2 polar bodies (PB) from the oocyte or one blastomere or some trophectoderm cells from an embryo in order to test, prior to implantation, if the oocyte or embryo has alterations in the genome sequence or in its chromosome's structure or number. Thousands of children have already been born following PGT, without finding a significant increase in the prevalence of congenital malformations, compared to that in the general population. The major improvement in PGT has recently been achieved through the application of next generation sequencing (NGS), coupled with blastocyst biopsy and vitrification. According to some authors; this combination allows to perform an embryo transfer in a subsequent unstimulated cycle, resulting in statistically significant increased implantation and pregnancy rates, and a reduction of the spontaneous abortion rate. However, there are still contradictory opinions about the benefit of the application of these techniques. For this reason, it makes recommendable counseling of couples considering PGT, including genetic counseling, discussion of the risks associated with IVF, embryo biopsy, extended embryo culture, and discussion of the limitations of PGT, including the risk for misdiagnosis. The present revision gives a current vision of the utility and limitations of PGT and also an overview of the procedures involved in the technique.

Key words: preimplantation genetic testing (PGT), comprehensive chromosome screening (CCS), Next generation sequencing (NGS), monogenic disease, aneuploidy

Résumé. Le test génétique préimplantatoire (PGT) est une procédure qui consiste à retirer un ou deux corps polaires (PB) de l'ovocyte ou d'un blastomère ou de certaines cellules du trophectoderme de l'embryon afin de vérifier, avant l'implantation, si l'ovocyte ou l'embryon a altérations de la séquence du génome ou de la structure ou du nombre de ses chromosomes. Des milliers d'enfants sont déjà nés à la suite de la TPP, sans que la prévalence des malformations congénitales ait augmenté de manière significative par rapport à celle de la population en général. L'amélioration majeure du TCP a récemment été réalisée grâce à l'application du séquençage de nouvelle génération (NGS), associée à la biopsie du blastocyste et à la vitrification. Selon certains auteurs; Cette combinaison permet d'effectuer un transfert d'embryons au cours d'un cycle non stimulé ultérieur, ce qui entraîne une augmentation statistiquement significative des taux d'implantation et de grossesse et une réduction du taux d'avortement spontané. Cependant, il existe encore des opinions contradictoires sur les avantages de l'application de ces techniques. Pour cette raison, il recommande des conseils aux couples qui envisagent le TCP, y compris un conseil génétique, une discussion sur les risques associés à la FIV, une biopsie d'embryon, une culture embryonnaire étendue et un débat sur les limites du TCP, y compris le risque d'erreur de diagnostic. La présente révision donne une vision actuelle de l'utilité et des limites du TCP, ainsi qu'un aperçu des procédures impliquées dans cette technique.

Mots clés : tests génétiques préimplantatoires (PGT), dépistage complet des chromosomes (CSC), séquençage de nouvelle génération (NGS), maladie monogénique, aneuploïdie

In vitro fertilization (IVF) is a well-established reproductive technique used in couples for the treatment of infertility [1] and its goal is to achieve the delivery of a healthy baby through the transfer of a single euploid embryo. The presence of abnormalities in the number of chromosomes (aneuploidy) is the major source of adverse outcomes in human reproduction. Aneuploidy is a common feature in IVF and arise primarily due to the inheritance of maternally-derived aneuploidies. The incidence of aneuploid conceptions is strongly correlated with maternal age rising exponentially during the decade before the onset of menopause [2].

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Unfortunately, a high proportion of embryos are aneuploid, and the transfer of this kind of embryos is associated with high miscarriage rates and decreased implantation and live birth rates [3]. To try to bypass the presence of aneuploid embryos, reproductive specialists have traditionally transferred more than one embryo with the aim of achieving at least one single live birth [4]. This practice has been associated with a high rate of multiple pregnancies, which carries several risks to the health of the mother and also the foetus [3]. Morphologic evaluation of the embryos remains the gold standard and most commonly used method for embryo selection, but this type of selection carries many limits and has been associated with conflicting reproductive results [3, 5].

The most biologically plausible and promising means of embryo selection remains the assessment of the genetic component of the embryo following embryo biopsy, a process known as preimplantation genetic testing (PGT) [3, 6], also known as preimplantation genetic diagnosis (PGD) and screening (PGS). These terms, PGD and PGS, have now been replaced by preimplantation genetic testing PGT, according the International Glossary on Infertility and Fertility Care [7]. Although after the application of PGT a prenatal test is always recommended, PGT is considered by some patients an early type of prenatal diagnostic testing performed on the embryo *in vitro* prior to its transfer to the uterus, therefore, they often choose PGT to try to avoid traditional prenatal testing (chorionic villus sampling or amniocentesis) and subsequent termination of a pregnancy of an affected fetus or, at least, to try to minimize the risk of having these complications.

PGT can be applied for the detection of aneuploidies (PGT-A), structural rearrangements (PGT-SR) and also for monogenic diseases (PGT-M) involving also PGT for diseases with genetic predisposition and preimplantation testing for nongenetic conditions, such as HLA typing. It allows couples carrying genetic diseases to have an unaffected child. Nowadays, PGT is an established clinical procedure in reproductive medicine and genetic practices [8], with thousands of apparently healthy children born, suggesting that PGT is a safe and reliable procedure with no significant incurred adverse effects. Although during the past 28-year experience, the PGT technology has been significantly improved, it still requires refinement in its every component [9].

PGT-A by fluorescence *in situ* hybridization (FISH) for a limited number of chromosomes was widely applied for almost two decades. However, this technique lacked sensitivity and was far from being comprehensive regarding the coverage of all the chromosome complement [10]. PGT-A by FISH seemed to reduce the livebirth rate in women with advanced maternal age instead of improving it, and because of that, PGT-A using FISH was under debate [11].

Comprehensive chromosome screening (CCS), allows for the analysis of the whole chromosome complement and has been developed and used recently in PGT-A cycles [12]. This technique has been applied in biopsies of polar bodies (PB) and also of cleavage-stage and blastocyst stage embryos [3, 8]. CCS can be achieved with the use of different genetic platforms, including metaphase comparative genomic hybridization (mCGH), array comparative genomic hybridization (aCGH), single nucleotide polymorphism (SNP) microarray, quantitative polymerase chain reaction (qPCR) and, more recently, next-generation sequencing (NGS) [3, 12].

On the other hand, some authors have claimed that the reported improved efficacy and outcomes of PGT are related to various factors [13], including the favorably selected patients, whose embryos have reached the blastocyst stage, thus, excluding elderly and those with decrease ovarian reserve [13].

PGT should call for a proper randomized controlled trial, aiming to further evaluate the cumulative live birth rates (LBRs) following a single oocyte retrieval, utilizing all fresh and frozen embryos [13].

Indications

Evaluation of a couple's reproductive history and adequate genetic counselling should be part of the preliminary evaluation for all PGT patients, particularly for couples at high genetic risk due to monogenic diseases or chromosome structural abnormalities [8]. An informative visit is always recommended to explain accurately the procedure and the possible results.

The basic indications for recommending a PGT could be divided into two groups:

Monogenic diseases

In its original applications PGT was primarily used for mendelian disorders. Mendelian disorders are single-gene defects often defined by describing their basic pedigree patterns: autosomal dominant, autosomal recessive, X-linked (recessive or dominant), and Y-linked [14]. Common mendelian disorders in which PGT is usually applied include cystic fibrosis, b-thalassemia, sickle cell disease, myotonic dystrophy, Huntington disease, fragile X syndrome, and spinal muscular atrophy among other disorders [14, 15]. More recently, part of PGT cases include HLA typing in addition to monogenic testing, which aids treatment strategies for a living sibling or other relative. One example of this is a PGT for Fanconi anemia [14].

Numerical and structural chromosomal abnormalities

Another indication for PGT is the presence of aneuploidies such as polyploidies, monosomies and trisomies. They are basically due to advanced maternal age (women

Table I. Main indications to perform a PGT

Type of PGT (old name)	Indications
PGT-A (PGS)	Advanced maternal age, 38 years or more, women who have suffered two or more spontaneous abortions, couples with previous gestation with chromosomal abnormalities, implantation failures and alterations of meiosis in testicular biopsy.
PGT-SR (PGD)	Karyotypes with structural anomalies: reciprocal translocations, Robertsonian translocations, inversions and deletions
PGT-M (PGD)	Monogenic diseases: Both patients, one of them or their family antecedents with a disease located in a gene

over 38 years old) and are the cause of repeated abortions and implantation failures. Structural chromosomal abnormalities which include translocations, inversions, deletions, and other rearrangements in the chromosomes (*table 1*) are also possible findings in spontaneous miscarriages and in affected fetuses, although overwhelmingly most are numeric [16]. Although structural alterations represent a small number of miscarriages, they represent a majority of PGT cases done for chromosomal abnormalities [14, 15].

In Spain, the latest data collected by the National Registry of Assisted Human Reproduction Activity in 2016, shows that the main indication to perform a PGT is advanced maternal age, repeat abortions, cytogenetic diseases, and finally, molecular diseases [17].

Biopsy procedure

PGT is a procedure that requires the removal of one or more cells from the embryo in order to have sufficient genetic material for the diagnosis (*table 2*). Originally PGT was done biopsying single blastomeres from a cell-stage preimplantation embryo (*figure 1*) or testing female gametes after removing polar bodies (1PB and 2PB) (*figure 2*), extruded after the maturation and fertilization of the oocytes. However, a different approach has currently shifted to blastocyst biopsy followed by blastocyst vitrification, providing also a possibility for testing on a number of cells, and transfer of the embryos in a subsequent non-stimulated cycle. There are evidences indicating improved implantation and pregnancy rates, with a corresponding reduction of spontaneous abortions [13].

Embryo biopsy at the blastocyst stage that has currently become the method of choice (*figure 3*), allows to test for both maternally and paternally derived conditions. Although the potential effect of the biopsy on embryo viability cannot be excluded, available data shows no evidence for significant detrimental effect of the blastocyst biopsy procedures. It is generally accepted that trophectoderm biopsy has less impact on embryo viability than cleavage-stage biopsy [18]. This is because even though more cells are removed during trophectoderm biopsy, it represents a smaller percentage of embryo mass and, by definition, trophectoderm biopsy removes only trophectoderm cells and not cells that have any fetal fate [18].

DNA amplification

There are many methods of DNA amplification, which likely represents the most critical element in the success of CCS. The most common method, whole genome amplification (WGA), can itself be performed using many commercially available kits [10]. There are different factors to take into account when choosing which WGA method is the best to use. For example, when the objective is accurate genotyping of a multiple displacement amplification (MDA)-based approach may be more accurate than PCR-based methods [19]. Methods such as karyomapping [20] and parental support [21] have used MDA-based WGA as they rely upon genotype information to make diagnoses. In contrast, PCR-based WGA methods have demonstrated superior performance for copy number-based analyses such as array comparative genomic hybridization (CGH) [22], quantitative single nucleotide polymorphism (SNP) array methods [23], and NGS [24, 25].

In PGT performed for aneuploidy and translocations, there are no restrictions on the technique of insemination. Conversely, for PGT-M where PCR will be applied, intracytoplasmic sperm injection (ICSI) is recommended in order to avoid sperm contamination. For the same reason, the removal of corona cells should be especially careful and exhaustive. *Table 3* shows a summary guideline for good practice in PGT-M.

Methods of genetic diagnosis

The methods of analysis for PGT currently used allow for the analysis of all the chromosomes; that is to say, they allow to perform comprehensive chromosome screening (CCS).

CGH arrays

CGH arrays have been identified as a robust and accessible diagnostic approach to assess 24-chromosome aneuploidy, and consequently IVF programs moved toward CCS using CGH arrays when the technique was developed [26]. DNA amplification from a single cell or

Table 2. Differences between the biopsy methods used for preimplantation genetic diagnosis. Adapted from Chen et al. [54].

Stage of biopsy	Advantages	Disadvantages and limitations
Oocyte/Zygote (polar body)	No effect on subsequent embryo development Enough time to perform analysis prior to transfer	Only one or two cells available for analysis Only maternal message obtained DNA liable to degenerate
Cleavage stage	Diagnosis of maternal and paternal inherited disease Possibility of sex determination Up to two cells available for analysis	Limited time for analysis High incidence of chromosomal mosaicism
Blastocyst	Fewer number of embryos to be biopsied and fewer specimens to process Three or more cells per embryo available to overcome allele dropout Less problem of mosaicism	Occasions when embryos failing to blastocysts Need for cryopreservation most of the time

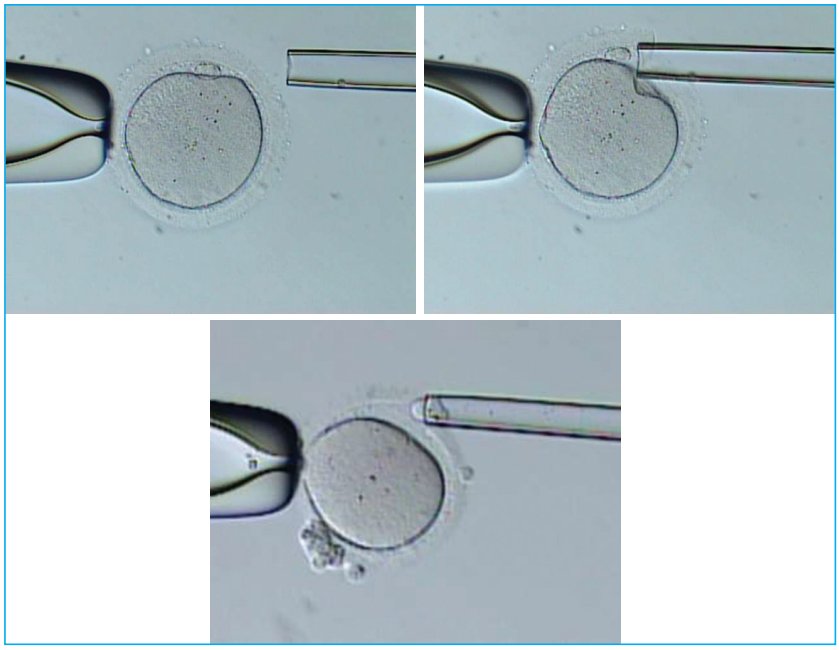

Figure 1. 1PB biopsy using PZD.

4–5 cells is performed, followed by DNA labeling with Cy3 and Cy5 fluorophores and co-hybridization onto the arrays for 4–12 h. Fluorescence intensity is detected using a laser scanner and specific software is used for data processing. This platform was first validated by reanalyzing the same embryos with fluorescence *in situ* hybridization (FISH), confirming its high efficiency for aneuploidy detection [12]. Therefore, CCS with CGH arrays can be accurately applied at different embryo biopsy stages, and currently, blastocyst biopsy would be the most common approach, with some groups also describing successful results with day 3 biopsies.

Single nucleotide polymorphism (SNP) arrays

SNP arrays allow for both genotyping and copy number predictions at thousands of positions in the genome. Some SNP array-based CCS methods rely upon the genotypes alone [20, 21], whereas others involve quantitative analysis of copy number assignments [23]. Typically, the

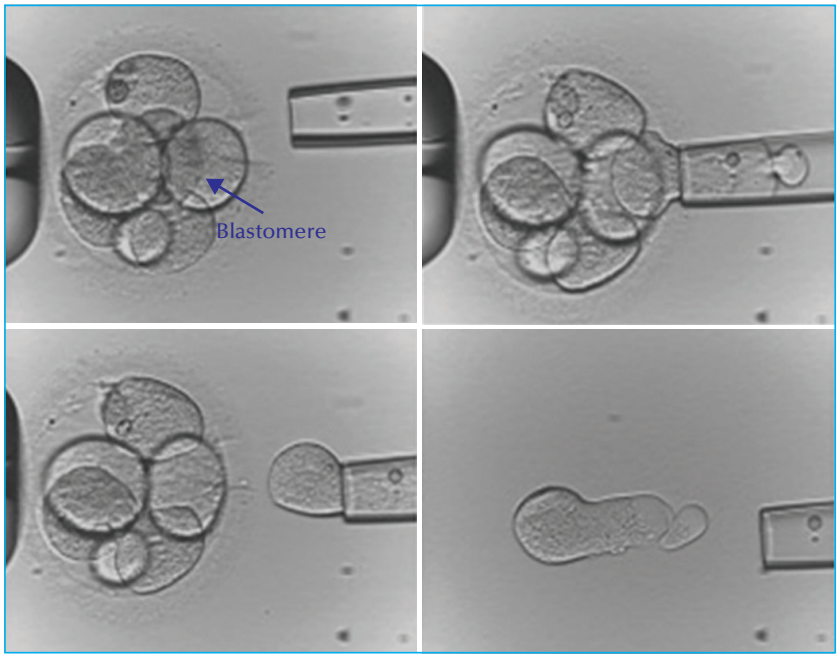

Figure 2. Day 3 biopsy using laser.

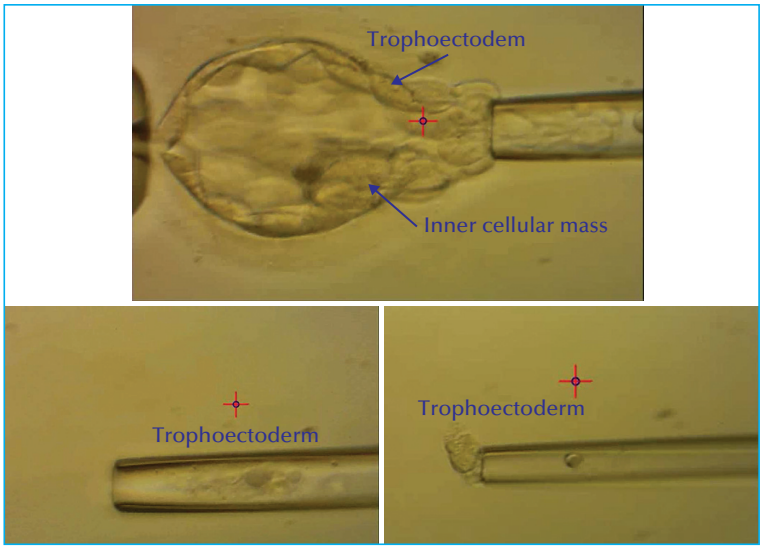

Figure 3. Blastocyst biopsy using laser.

signals at each position are compared between the embryo biopsy and those obtained from known normal samples in order to identify possible imbalances in the embryo. One advantage of SNP arrays is the ability to characterize copy number neutral events, such as recombination sites [20], uniparental disomy [27], parental origin of aneuploidy [20] and balanced translocations [28]. However, like array CGH, disadvantages include the time to obtain a result and the expense of the procedure, both of which have been overcome by the development of qPCR-based CCS [29].

qPCR

The reason why qPCR is faster and cheaper is the elimination of the need of WGA. Instead, a multiplex PCR reaction is performed to pre-amplify 96 copy number neutral positions in the genome, four *per* chromosome. Each of the 96 positions are then quantified in individual reactions using TaqMan primers and fluorescent target sequence specific probes on a 384 well plate, followed by normalization to previous data from known normal samples. The process can be completed in 4 hours, requires the least hands on time, is easily automatable, involves relatively

Table 3. Guidelines for good practice in PGT-M [55].

PGT-M set-up///Before initiating hormonal stimulation and PGT-M for single-gene disorders, a work-up should be performed to establish the feasibility of the assay and the experimental conditions.
PCR for single-gene disorders
(i) Confirmation of the detected mutations in patients' DNA.
(ii) Identification of closely linked markers, if available, to be included in the reaction by studying family members.
Definition of minimal requirements exists
(iii) Availability of exogenous DNA-free reagents.
(iv) Availability of a dedicated, fully equipped area to PGT-M.
(v) Identification of the appropriate positive and negative controls.
(vi) Test validation on single cells (e.g. buccal cells, lymphocytes, discarded blastomeres).
Recommended guidelines
• amplification efficiency not lower than 90%
• use of markers for allelic dropout (ADO) detection are recommended given that ADO cannot be lower than 10%
• exogenous DNA contamination no greater than 5%. Use of markers indicating DNA contamination is recommended.

low technology equipment and is one-third the cost of array-based methods. Another advantage is the ease by which additional primers can be incorporated in order to characterize single gene disorders [10], small duplications and deletions [28], mitochondrial disorders [30] or unbalanced translocations [31].

Next generation sequencing

Perhaps, the most ideal combination of throughput, cost and capability is afforded by NGS-based CCS [24, 25]. The amplified DNA undergoes massively parallel sequencing in order to count the number of sequences 'reads' which align to each of the 24 chromosomes. The read counts are then normalized to data from known normal samples in order to define the copy numbers present in the embryo biopsy. Reduced cost per embryo is provided by the opportunity to perform molecular barcoding, which allows multiple samples to be sequenced in parallel in the same reaction and then segregated back out to the original sample using standard bioinformatics methods [32].

NGS is much more sensitive than array CGH for detecting sub-chromosomal abnormalities and mosaicism. Also, there are different NGS platforms, which may affect the detection rate of mosaicism. The most widely used platform is based on Illumina's Variseq NGS strategy, representing a high resolution NGS, detecting 20–80% mosaicism in blastocyst samples, which could improve the accuracy of PGT-A.

Mosaicism

Mosaicism in cleavage-stage embryos refers to the presence of two or more cell lines with a different chromosome content within the same embryo. This phenomenon can involve the presence of cells with different types of aneuploidy in the absence of any normal cells or a mixture of euploid and abnormal cells [33]. Mosaic embryos are the consequence of errors in chromosome segregation occurring during mitotic divisions. Although is a common phenomenon, the exact prevalence is still unknown [18].

The frequency of embryonic mosaicism reported in the literature varies greatly. The explanation for these differences is based on the combination of unsuitable fixation techniques, the controversy in the criteria used to classify an embryo as abnormal [34], the bias in the type of material tested, and the iatrogenic effect influenced by culture conditions (temperature, pH, media. . .) [33].

The method with greatest power to detect mosaic embryos is hr-NGS. However, there are some technical limitations that prevent to completely validate this method. Studies using appropriate methods and conditions provide consistent frequencies of mosaicism around 30% in both cleavage-stage embryos [35] and blastocysts [36]. These frequencies are maintained across all maternal ages [33].

It has been demonstrated that mosaic embryos, implant less frequent and miscarry more often, in comparison to euploid blastocysts. However, some mosaic blastocysts can reach term [37] and should not be considered in the same category as those that are completely aneuploid.

Mosaic embryos may be differentiated according to the percentage of abnormal cells in the biopsy specimen, the chromosomes involved, and the types of abnormalities, and appropriate genetic counseling should be offered to couples prior to transfer any mosaic embryo. As reported by Fragouli et al. [37] embryos with several chromosomes affected by mosaic aneuploidy have significantly lower ongoing implantation rates (6%) than any other class of mosaic embryo. In contrast, embryos with a mosaic segmental abnormality had similar capacity to implant in comparison to euploid embryos. Blastocysts with 40%–80% aneuploid cells in the biopsy sample were associated with an ongoing pregnancy rate of 22%, and those with <40% abnormal cells resulted in a 56% ongoing pregnancy rate [37].

Studies using preimplantation genetic testing

Preimplantation genetic testing for aneuploidies

PGT for aneuploidies (PGT-A) has currently shifted almost completely to the next-generation technologies, including array-CGH and NGS [9].

The application of next-generation technologies represents a major breakthrough in PGT, with tens of thousands of cases performed annually, with the purpose to improve the ART practice, particularly for couples of advanced reproductive ages [38]. However, the application of NGS has also revealed mosaicism and sub-chromosomal variations, such as segmental aneuploidies, not detected by FISH, the biological and practical significance of which is still not clear. Detection of mosaicism depends on the sensitivity of NGS platform applied, which detects much higher rate of mosaicism than array CGH. The fact that the overall mosaicism prevalence did not show a relationship with maternal age may have indicated that a significant proportion of mosaicism is either artifactual and of no clinical relevance, or simply transitional without affecting the embryo viability, or may be the consequence of de generative processes in the embryos prior to embryo arrest. This might actually have been the major reason for the controversy on the usefulness of PGT-A performed at the cleavage stage [9].

The value of PGT-A as a universal screening test for all IVF patients has yet to be determined. Some studies reported here provide important perspectives on the value of 24-chromosome testing, demonstrating higher birth rates after aneuploidy testing and eSET in the primary embryo transfer of favorable-prognosis patients, suggesting the potential for this testing to increase eSET utilization and further decrease the incidence of multiple gestations. However, these studies have important limitations and there are still questions about appropriate patient selections and testing platforms [18].

Preimplantation genetic testing for structural rearrangements

Balanced translocations may result in infertility, recurrent miscarriage, or the birth of a child with congenital anomalies as a result of inheriting an unbalanced chromosome rearrangement. Because many carriers of balanced translocations have a poor chance of having an unaffected pregnancy, PGT for structural rearrangements (PGT-SR) has a clear advantage over the traditional prenatal diagnosis in assisting these couples to establish an unaffected pregnancy and deliver a child free from unbalanced translocation [9]. There is poorer clinical outcome in reciprocal than Robertsonian translocations. Without PGT, it may take years until the translocation carriers could get an unaffected offspring, so the current PGT recommendations include chromosomal rearrangements as one of the main indications for PGT [9, 39].

Of special practical significance is the ability to distinguish between normal and balanced embryos, increasingly requested by the carriers of translocations. The most recent approach to achieve this is based on a specially designed NGS technology, called mate pair sequencing (MPS), involving a high depth sequencing to define the exact breakpoints, required for designing specific primers that allow distinguishing normal from carrier embryos [40].

The application of the above techniques resulted in significant improvement of reproductive outcome of PGT-SR, with the accumulated experience of hundreds of PGT-SR cycles, presently demonstrating significant improvement of pregnancy rate and at least a fourfold reduction of spontaneous abortions in these couples, compared to their experience before PGT-SR [9].

Preimplantation genetic testing for monogenic disorders

Monogenic disorders are caused by pathogenic variation in a single gene. Initial preimplantation testing for monogenic disorders in the 1980s focused on simple DNA amplification techniques to determine the gender of the embryo in order to perform sex selection for X-linked disorders [41]. A few years later, the same group performed preimplantation genetic diagnosis (PGD) for cystic fibrosis using a targeted PCR reaction on a biopsy from a cleavage stage embryo [42]. Technological advances have led to an explosion in the number of couples who are otherwise fertile and present for care at an IVF center specifically for PGT for monogenic disorders (PGT-M) testing to rule out a family mutation. PGT-M is now commonly available for disorders found on routine (cystic fibrosis, fragile X syndrome, and spinal muscular atrophy) and expanded carrier screening panels, as well as inherited cancer genes, adult-onset neuromuscular disorders (Huntington's disease and amyotrophic lateral sclerosis), and human leukocyte antigen (HLA) matching. Findings from whole-exome sequencing are becoming increasingly popular, typically when the testing was performed on an affected child [43].

One of the more commonly used methods for PGT-M involves WGA followed by PCR for short tandem repeats (STRs). STRs are highly polymorphic and found throughout the entire genome, creating an excellent tool for linkage analysis, which is often the foundation of PGT-M. One limitation of this approach is the risk of recombination when the STR markers that are nearest to the disease locus are still several megabases away and the mutation cannot be directly detected; however, laboratories utilizing this method report that they are often performing sequencing on an aliquot of the WGA product to look for the mutation,

which is unfortunately associated with a significant risk of allele dropout. Recombination between STRs and disease loci can also lead to a misdiagnosis [44].

PGT-M can also be performed using informative SNPs for linkage. SNPs are denser across the genome than STRs, so the method can be widely applicable. Typically, laboratories utilizing a SNP-based approach use either a SNP microarray (often referred to as karyomapping) [45] or TaqMan genotyping probes designed for informative SNPs near the disease locus [46]. Karyomapping arrays contain approximately 280,000 SNPs across the genome, and the same arrays used to determine linkage in family members can also be used to generate the diagnosis for embryos, thus streamlining the ability to perform PGT-M on a single standard platform without needing to design custom probes for each family. This nearly eliminates the need to work up a family, and there is little delay between case initiation and the IVF cycle to generate embryos. Because the SNP microarray is run on every embryo, this testing type can be quite costly. The method is also limited to using a linkage-based approach, so family member participation is required, and the mutation cannot be directly targeted. There are also regions of the genome where homology can interfere with accuracy or the SNP probe density may be low (e.g., the SMN1 gene). An inconclusive rate of approximately 10% has been reported for karyomapping PGD [45].

PCR and day 3 embryo biopsy are still widely used methods for PGT in Europe [15]. However, the labour-intensive design and extensive preclinical optimization and validation required for the 'locus-by-locus' gene/patient-specific multiplex PCR approaches are not time- and cost-effective in the private sector. Karyomapping provides an example of genome-wide linkage based analysis of any familial SGD within the regions of the genomes covered by the SNP loci used without the need for patient-specific tests [20]. Many centers have now moved toward techniques that allow combination of PGT-M and PGT-A on whole-genome amplified DNA from trophectoderm cells biopsied at the blastocyst stage. This double genetic analysis is extensively used in several major centers for reproductive medicine [46, 47]. By combining PGT with the concomitant detection of chromosome aneuploidies, the pregnancy rate per transfer is significantly improved to 50–68% [47] despite a reduced proportion of transferable embryos. However, for many PGT centers this strategy may represent additional complications, costs and logistical challenges, including the need for embryo cryopreservation. Furthermore, the capacity of a single trophectoderm biopsy at the blastocyst stage to reliably determine whether an embryo can be discarded or transferred, as well as the techniques used to detect aneuploidy and mosaicism, have recently been severely questioned [21]. There is thus considerable concern about the clinical utilization of PGT-A.

Registered activity regarding the use of preimplantation genetic testing

In USA, according to the last report published in 2015 [18], 5% of the treatments with fresh nondonor cycles were performed with PGD/PGS.

In Europe, according to the European Society of Human Reproduction and Embryology (ESHRE) PGD consortium data collection in 2014 [48], PGT activities were reported from 22 countries (20 in 2013, 19 in 2012). The number of treatment cycles was 15,894 (2.05% of all ART treatments), which compared to 2013 represents a drastic rise in treatment numbers (+6,103). These involved 13,460 fresh cycles and 2,434 thawings, resulting in 6,269 fresh and 2,021 frozen embryo transfers (FET). In total, 2,538 pregnancies (42.5% per transfer) and 2,024 deliveries (32.3% per transfer) resulted from fresh cycles. Corresponding figures for FET were 801 (41.8% per transfer) and 619 (30.8% per transfer). The main contributor was Spain with 5,242 cycles [48].

According to this report, in 2014 in France, 90,434 ART treatments were performed, 1,039 (1.2%) with PGT. In other countries such as Spain, the number of cycles with PGT has increased and in 2016 it represents 5.8% of the total cycles initiated [17].

Most published studies comparing a strategy of PGT-A with morphologically assessed embryos have reported a higher implantation rate per embryo using PGT-A, but insufficient data has been presented to evaluate the clinical and cost-effectiveness of PGT-A in the clinical setting [49]. There are other important benefits to PGT-A, fewer clinical losses, less time to achieve an ongoing pregnancy, and reduced number of embryos transferred *per* transfer, resulting in the near elimination of multiple gestation [2]. Thousands of children have already been born following PGT, with no increase in the prevalence of congenital malformations observed, compared to that in the general population [50].

Limitations and controversies

The application of new higher resolution technologies, together with the shift of the biopsy procedures from cleavage stage to blastocyst and the application of vitrification techniques, has contributed greatly to the improvement of PGT reproductive outcomes. However, there are still remaining limitations that require the development of additional methods to further improve effectiveness and reliability of these techniques.

Opponents of PGT remark the incorrect diagnosis of aneuploid embryos due to false positive results. As blastomeres divide at very high rates, particularly in the trophoectoderm, the risk of mitotic errors and mosaic aneuploidy increases, even if the inner cell mass remains

euploid. That would result in discarding embryos that would otherwise lead to normal births [2].

Furthermore, the additional manipulation of embryos and the potential damage of biopsy procedure that is required to obtain the cells is inherently traumatic and decreases the live birth potential of those embryos judged to be normal [2].

Future approaches

New alternatives to embryo biopsy

A few recent attempts have been reported to investigate the feasibility of performing PGT using DNA obtained from blastocoel or spent culture media [9, 51]. While aspiration of blastocoel fluid (blastocentesis) is less invasive than the trophectoderm biopsy, it is still not completely noninvasive and its accuracy still has to be demonstrated [9]. Sampling of spent culture media seems to be a more reasonable approach to noninvasive PGT [9, 51], by analogy to NIPT, which uses cell-free DNA from maternal circulation. However, data are too preliminary, with many limitations and problems. Most recent studies involved testing nuclear and mitochondrial DNA in spent embryo culture media or blastocoel fluid do not provide enough reliable diagnostic rates to justify their use in clinical PGT treatments and concluded that DNA from culture media cannot be used for PGT because of contamination with DNA from other origins, such as cumulus cells [52]. However, recent data of Magli et al [53] proposes that the timing of blastocoel fluid aspiration is a factor possibly affecting the amplification outcome. Thus, blastocentesis could be an additional tool aimed at contributing to the knowledge of early embryogenesis but more research is required to investigate the feasibility of PGT without biopsy.

New markers

What is clear is that not all euploid blastocysts result in a healthy newborn. Many fails to implant and progress, indicating that additional factors are important to the reproductive potential of the embryo. Determining the role of the embryonic 'omics' or noninvasive predictions obtained by using the media, cumulus cells or time-lapse imaging, and identifying biomarkers is the next challenge. Furthermore, appropriately designed studies to develop new biomarkers of reproductive potential should include control over the presence of aneuploidy within cases and controls, as this is one of the most well proven factors influencing the reproductive potential of the embryo.

Of special importance will be the development of universal PGT in a single test, which could allow for simultaneous testing for multiple disorders, presenting not only at birth but also in older life, for which no pre-symptomatic diagnosis and/or treatment are available [20].

Monitorization

Many investigators have advocated for an increased monitoring of the PGT cycles and the long-term health outcomes of the children born from these technologies, but there is no consensus on how this monitoring ought to be done. Some scientists have advocated for government involvement and legislation. In the United States, there is little legislation, restriction, or monitoring of PGT use and application; however, the American Society for Reproductive Medicine (ASRM) provides reasonable practice guidelines for the use of PGT. The ASRM recommends thorough counseling of couples considering PGT, including genetic counseling, discussion of the risks associated with IVF, embryo biopsy, extended embryo culture, and discussion of the limitations of PGT, including the risk for misdiagnosis [24].

In conclusion, although PGT is a well-established technique which is currently applied in IVF clinics, future technical improvements of the existing techniques in combination with new approaches are expected to improve not only the knowledge that professionals have of the embryos but also the information that is given to our patients. A strict control of the results and a well-designed monitoring system of the newborns is also mandatory to be able to offer PGT properly to the appropriate group of patients.

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