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Preimplantation genetic screening: does it help or hinder IVF treatment and what is the role of the embryo?

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Abstract Despite an ongoing debate over its efficacy, preimplantation genetic screening (PGS) is increasingly being used to detect numerical chromosomal abnormalities in embryos to improve implantation rates after IVF. The main indications for the use of PGS in IVF treatments include advanced maternal age, repeated implantation failure, and recurrent pregnancy loss. The success of PGS is highly dependent on technical competence, embryo culture quality, and the presence of mosaicism in preimplantation embryos. Today, cleavage stage biopsy is the most commonly used method for screening preimplantation embryos for aneuploidy. However, blastocyst biopsy is rapidly becoming the more preferred method due to a decreased likelihood of mosaicism and an increase in the amount of DNA available for testing. Instead of using 9 to 12 chromosome FISH, a 24 chromosome detection by aCGH or SNP microarray will be used. Thus, it is advised that before attempting to perform PGS and expecting any benefit, extended embryo culture towards day 5/6 should be established and proven and the clinical staff should demonstrate competence with routine competency assessments. A properly designed randomized control trial is needed to test the potential benefits of these new developments.

Keywords Preimplantation genetic screening · Aneuploidy · In vitro fertilization · Embryo quality · Embryo biopsy

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Capsule Using PGS to improve live birth rates in IVF treatments may be hindered by factors such as a(n) unqualified technical staff, less than optimal culture media, use of 2-cell biopsy of cleavage-stage embryos, or misdiagnosis (due to mosaicism), and thus hinder IVF treatments.

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Terminologies: PGD, PGD-AS and PGS

Genetic testing of preimplantation embryos was initially performed to diagnose patients who were known to carry sex-linked disorders [1], monogenetic disorders [2–4], or chromosomal structural abnormalities [5]. This was referred to as preimplantation genetic diagnosis (PGD). Over time, genetic testing has evolved so that is it now also used to screen for numerical chromosomal abnormalities [6]. Thus, the term preimplantation genetic diagnosis for aneuploidy testing (PGD-AS) or preimplantation genetic screening (PGS) was adopted to differentiate this new use for patients with no known genetic disorder from the original diagnostic use for patients with known indications. In this paper, we will use the term PGS. More recently, PGD has been used to identify late-onset diseases [7] and to test for HLA compatibility [8].

Chromosomal abnormality

There are two types of chromosomal abnormalities: numerical and structural. In regards to numerical abnormalities, the addition or deletion of an entire chromosome is called aneuploidy, and the addition or deletion of an entire set of chromosomes is referred to as polyploidy and haploidy, respectively. Aneuploidy occurs in approximately 20% of cleavage-stage human embryos [9]. It also occurs in 45% of cleavage-stage embryos taken from patients with advanced maternal age (AMA; >36 years) [10]. Polyploidy and haploidy occur much less frequently (in 7% and 3% cleavage-stage embryos, respectively) [10]. Embryos classified as abnormal on day 3 reached the blastocyst stage at a 40% rate if they were trisomic (having a third copy of a particular chromosome), 21% if polyploid (containing more than 2 sets of chromosomes) and 0% if haploid or monosomic (having one less than the diploid number of chromosomes)—this was true for all chromosomes except for chromosome X or 21 [11]. More recent studies using comparative genomic hybridization (CGH) indicate that the trisomy to monosomy ratio is 60:40 [12].

Structural chromosomal abnormalities can occur spontaneously or as a result of external forces such as radiation. Numerical and structural abnormalities can be present concurrently.

Embryos can also be mosaic, that is, they contain several cell lines with different chromosome complements. Mosaicism may have originated and persisted as a result of the low expression of certain cell cycle checkpoint genes during the first cell divisions in the early developing embryo. During this period, maternal transcripts control the cell cycle until the cleavage stage, which is where the embryonic genome takes over. It is believed that the once the embryonic genome becomes fully active, it can overcome mosaicism by permitting normal cells to proliferate and inhibit abnormal cells from mitotic activities [13].

We will focus primarily on numerical chromosomal abnormalities in the current review. Aneuploidy can persist through implantation and may result in a live birth for trisomy 21 (Down syndrome), trisomy 13 (Patau syndrome), trisomy 18 (Edwards syndrome), monosomy X (Turner syndrome), trisomy XXY (Klinefelter syndrome), and other gonosomal constitutive aneuploidies. At the cleavage stage, aneuploidies affect chromosomes 15, 16, 21 and 22 most frequently and chromosomes X and Y least frequently [14].

What is the prevalence of PGS in the clinical setting?

The European Society of Human Reproduction and Embryology (ESHRE) PGD Consortium collects PGD data from a small number of clinics worldwide [15]. According to this organization, 66% of the PGD cycles were used as aneuploidy screening, or PGS. Although the precise number of clinics practicing PGS worldwide is unknown, there is evidence of a growing trend [15, 16].

Evolution of PGS: from sex selection to embryo quality

Initially, the aim of PGD was to detect embryos at risk for inheriting monogenic diseases from the parents. PGD was first used for gender determination in couples who were carriers of sex-linked disorders. In that case, DNA amplification was performed followed by PCR for a specific repeated gene sequence on the Y-chromosome [1]. PCR was found to be less effective in determining gender since it produces qualitative and not quantitative data, meaning the test results could confirm the sex by highlighting the presence or absence of a specific repeated gene sequence unique to the Y-chromosome without providing further information on the existence of an X or Y chromosome. To overcome this disadvantage, fluorescent in situ hybridization (FISH) was substituted [17].

The use of FISH with X and Yprobes was later expanded to include somatic chromosomes. In 1993, Munne et al. used the FISH procedure to detect chromosomal abnormalities for five chromosomes and thus performed the first cases of PGS [17]. It is well known that chromosomal abnormalities play a major role in failed embryo implantation after in vitro fertilization (IVF) treatment. Thus, it was hypothesized that identifying such preimplantation embryos would improve implantation rates. PGS was used to increase implantation rates and live births while reducing spontaneous abortions ("treatment benefit") by determining the aneuploidy status of the embryos ("diagnostic benefit") in patients with no known genetic disorder.

Munne et al. 1995 [18] and Verlinsky et al. 1996 [19] performed PGS using FISH in first and second polar bodies (PBs). However, the use of FISH with cleavage-stage embryos became more commonplace except in areas where policies prohibited such activity. The first clinical studies on cleavage-stage embryos showed increases in implantation and ongoing pregnancy rates as well as and decreases in spontaneous abortion rates [6, 20–24]. Even so, its use is still considered controversial as investigations to validate the use of PGS have yielded contradicting results.

As media culture permitted the growth of embryos to the blastocyst stage, PGS evolved to include trophectoderm cell biopsy [25]. Here, several trophectoderm cells are removed and screened for aneuploidy using FISH or CGH instead of one or two blastomeres with cleavage-stage biopsy [25, 26]. McArthur et al. 2005 reported the first routine use of blastocyst biopsy with FISH in human preimplantation embryos to produce successful pregnancies and live births.

Most recently, in assisted reproductive technology, there has been growing support towards elective single embryo transfer to decrease the number of high order births, which is considered to be the principal risk factor in IVF [27–32]. IVF babies are more likely to be born prematurely—due mostly to multiple implantations of embryos—and tend to have a greater risk of low birth weight, developmental delays, cerebral palsy and congenital malformations [33, 34]. The chances of prematurity increases significantly with multiple embryo transfer, which is commonly used to improve implantation rates in infertile couples with a poor prognosis. Single embryo transfer is ideal for preventing multiple births if a good quality embryo with a high chance for implantation can be identified. A high quality embryo is most likely to bring about an ongoing pregnancy and ultimately a healthy, live birth [35].

Indications for PGS

The main indications for the use of PGS in IVF treatments include AMA, repeated implantation failure (RIF), and recurrent pregnancy loss (RPL). It is well known that the rate of chromosome abnormalities is higher in patients with AMA and RPL. Also, PGS has been used in women with previous trisomic conceptions [36] who have partners with male factor infertility [37–39], and in egg donors [40]. Today, the use of PGS for healthy patients with no indications for the purpose of improving IVF outcomes is on the rise.

Contributions to chromosomal abnormality—maternal meiotic abnormalities

Currently, there are two theories that attempt to explain the cause of maternal aneuploidy: the two hit theory and the production line theory. Both are discussed in a publication by Jones (2008). The production line theory suggests that the oldest oocytes within the ovary are the first to mature, and those that mature later in life may be of lower quality perhaps as the result of a combination of negative environmental factors exposed over her lifetime and age-related insults. No evidence currently exists to support the production line theory [41]. Yet, there are studies to support the two-hit theory which suggests that oocyte aneuploidy results from two “hits” that must occur during meiotic division. The first hit occurs during fetal development when oocytes undergo meiosis I and homologous chromosome pairs do not recombine or poorly recombine. The second hit occurs during adulthood when the oocytes complete meiosis and fails to detect the recombination errors [41].

Oliver et al. 2008 reported that aneuploidy, such as trisomy 21, is mainly caused by nondisjunction in maternal meiosis I [42]. His study suggested that normal disjunction during meiosis I occurred predictably in the same location on chromosome 21—the center of 21q—and deviations from it increased the likelihood of a chromosomal 21 nondisjunction. He proposed that it is associated with the sister chromatid cohesion complex. Either it was been too distally located from the exchange site and preventing appropriate orientation of homologues on the spindle or its distance weakened the cohesion and compromised the integrity of the chiasma [42].

Maternal meiotic abnormalities maybe caused by one of two mechanisms—meiotic nondisjunction (MND) or premature separation of sister chromatids (PSSC) during maternal meiosis I and II [43–46]. As a result of MND, 100% of the gametes are aneuploidy versus 50% in PSSC. The mechanism that is the leading cause is not entirely agreed upon. An analysis of polar body I found a higher number of hyperhaploidy polar bodies, suggesting that PSSC is the main source [46]. This study was further supported by Vialard et al. 2006 who applied FISH analysis to the first polar bodies in women with AMA undergoing intracytoplasmic sperm injection (ICSI). The results showed that PSSC was more prevalent than MND [47]. Another study of oocytes that remained unfertilized after ICSI demonstrated that PSSC occurred more often than nondisjunction. Aneuploidy oocytes caused by PSSC were characterized by a replacement of one chromosome or two chromatids, or the presence of an additional chromatid [48]. A study of oocytes and polar bodies using CGH for chromosome analysis confirmed the presence of chromosomal abnormalities in oocytes that had originated from either MND or PSSC, but the authors were unable to substantiate which one was more prevalent [49]. Nonetheless, all these findings should be interpreted with caution because PSSC has also been shown to be more common in oocytes aged in culture media as opposed to in vivo [50].

Contributions to chromosomal abnormality—paternal factors

Centrosome anomalies (abnormal or more than one centrosome produce multipolar spindles) resulting in chaotic mosaics were most likely of paternal origin [51–53]. Indeed Obasaju et al. 1999 showed high rates of chaotic mosaics in patients with partners that had male factor infertility. This incident disappeared in the following cycle after patients used sperm donation [54]. Another recent hypothesis by Leduc et al. 2008 suggested that alterations in the steps of chromatin remodeling or the DNA repair mechanism in elongating sperm during spermiogenesis are vulnerable to DNA fragmentation and continue to persist because spermatids lack a repair

mechanism, which ultimately leads to infertility [55]. These theories could explain why the incidence of aneuploidy in embryos fertilized by men with severe male factor infertility is high.

Post meiotic abnormalities

Post meiotic abnormality rates are constant regardless of maternal age. Such abnormalities affect approximately 30% of embryos and increase with dysmorphism [10, 56, 57]. Because aneuploidy increases with maternal age, post-meiotic abnormalities are more common than aneuploidy in younger women whereas aneuploidy is more common in women with AMA. It is also interesting to note that an embryo can be affected by multiple abnormal cell divisions at different developmental stages. The result is an embryo with complex abnormalities such as inclusion of two or more varieties of aneuploidy cell lines [58].

Five types of mosaic have been described [58]: non-disjunction, endoreplication, chaotic, anaphase lag, poly-ploid/diploid embryos. Polyploid/diploid embryos have the lowest rate of cells with abnormalities, and polyploid cells seem to be a culture artifact. For example, one group of researchers found that the rate of polyploid embryos decreased substantially between 1995 and 2000 simply because they analyzed the embryos 1 day earlier before they arrested. Arrested embryos tend to become polyploid [56, 57]. Chaotic embryos, as their name indicates, have undergone random mitotic divisions and each cell is usually different than the others, indicating spindle and centrosome impairment. They tend to be 100% abnormal. In total, Colls et al. 2007 [24] reported that the majority of the cells in chaotic embryos were abnormal. The implications for PGD are that only 5% to 7% of embryos will be misclassified.

Researchers once believed that an aneuploidy of mitotic origin was never passed on to the fetus and never resulted in a fetal or newborn chromosomal abnormality. Based on mCGH data on day 3 embryos, mitotic aneuploidy incidences should range between 15% and 30% [59, 60]. Meiotic abnormalities can lead to a newborn with a chromosomal defect depending on which chromosomes persist to the blastocyst stage and further develop into a fetus.

Correction mechanisms

Approximately 50% of chromatid events appear to self-resolve, and so it has been hypothesized that a meiotic I abnormality could be self-corrected by a meiotic II error [49]. It is important to note that PCCS may resolve itself in the oocyte and not the embryo. If a correction mechanism proves to exist, MII should not be considered abnormal until it has been fertilized since the chromatid can still end up in the right place.

Another misconception about self-correction was highlighted in the study by Munne et al. 2005 [61]. In that study, the normal cells of the inner cell mass (ICM) and the chromosomally abnormal cells were cultured in a monolayer to produce stem cells. Some of these abnormal culture cells showed partial or complete normalization. It has been very difficult to produce long-lasting aneuploid stem cells for this reason. This, however, should not be confused with in vivo self-correction of abnormal embryos [61].

Self-correction is believed to be involved in uniparental disomy (UPD), a condition where an embryo has balanced chromosomes but contains a set of chromosomes that belong to only one parent. This could be caused either by the combination of a nulisomic and disomic gametes for the same chromosome pair combining, or by a trisomic zygote or embryo losing one of the extra chromosomes or by a monosomic rescue. The occurrence of UDP, however, is extremely low compared to the high rate of aneuploidies detected at the cleavage stage. In cases of UPD, the effect on the embryo itself is usually minimal unless combined with other gene defects. Almost all uniparental disomic chromosomes rarely survive postnatal, and thus, few cases have been reported [62, 63].

In regards to self-correction mechanisms, it is reasonable to assume that during blastocyst development the embryo experiences a stringent self-correction probably based on cell cycle checkpoint control. In fact, it has been shown that loss of embryo viability due to chromosomal mosaicism is caused by the activation of a spatially- and temporally-controlled p-53 independent apoptotic mechanism and is not the result of a failure to progress through mitosis in the mouse model [64]. These determinant mechanisms that control programmed cell death have been documented in the blastocyst stage embryo but not in the early cleavage stage embryo [65]. For this reason, genetic problems, such as aneuploidy, are likely to have a negative effect as preimplantation development continues. Moreover, in order to form a blastocyst, the embryo must successfully undertake the first cellular differentiation and the critical epigenetic modification that underline this process [formation of the trophectoderm (TE) and inner cell mass (ICM)]; a subtle process that may be hampered by the inappropriate gene expression that inevitably accompanies aneuploidy. In the case of mosaic embryos, aneuploid cells could arrest development in favor of euploid ones [66]. In addition, self-correction of a meiotic or mitotic derived aneuploidy could happen as a consequence of a secondary mitotic error. From a molecular point of view two main mechanisms for the induction of mitotic chromosome errors are known: mitotic non-disjunction (MND), anaphase lagging (AL). Special situations occur when cells displaying single trisomy or

monosomy are affected by MND involving only that specific chromosome which then corrects one daughter cell to normal disomy and makes the other tetrasomic or nullisomic. These situations are known as trisomic zygote rescue (TZR) and monosomic zygote rescue (MZR) respectively, and are characterized by uniparental disomy (UPD) and are the mechanism behind some imprinting defect syndromes [67] or placental mosaicism [67]. AL concerns two or multiple chromosomes and/or chromatids simultaneously, a random mixture of losses of the different chromosomes among the daughter cells can be expected, resulting in a few unchanged daughter cells [68]. Also the special situation of TZR can occur, when AL corrects a cell with single trisomy to two normal daughter cells or one normal and one original daughter cell [69]. Finally, data from newborn with imprinting disorders due to UPD support the presence of self-correction mechanism. However, they are not a good model to estimate the incidence of self-correction because they are rare and highly chromosome specific. The actual incidence of self-correction is very difficult to estimate accurately in human preimplantation embryos. The sequential chromosomal analysis of day-3 and day-5 stage is biased by the presence of mosaicism that could explain the inconsistencies between the two developmental stages. Moreover, rescued cells could allocate randomly in the resulting blastocyst. Consequently, addressing its real incidence is very prohibitive. In one study by Li et al. 2005, a comparison of sequential chromosomal data on the two developmental stages, day-3 and subsequently on day-5 with both stages tested with five-chromosome FISH, demonstrated that 40% of aneuploid embryos on day-3 are euploid on day-5 [13]. In another study by Munne et al. 2005, 145 embryos were diagnosed as abnormal on day-3 by FISH but only 55 embryos developed further to the blastocyst stage. These 55 embryos were further cultured to day 12 and re-analysis via nine-chromosome FISH on day-6 and day-12 demonstrated chromosome self-normalization in 18 embryos [61]. A similar study supported this finding. Barbash-Hazan et al. 2008 re-analyzed abnormal day 3 (both analysis tested with eight-chromosome FISH) and of the 83 abnormal day-3 embryos, 27 embryos (32.6%) underwent self-normalization [70]. More interestingly, 41% of the abnormal embryos diagnosed as trisomic underwent trisomic rescue (which is the loss of a chromosome in trisomic cells) [70]. More recently, a study by Northrop et al. 2010 has demonstrated similar results with re-analysis of blastocyst-stage embryos using SNP microarray-based 24 chromosome aneuploidy screening [71]. In this study, day-3 embryos were evaluated by nine-chromosome FISH technique and the abnormal embryos were determined to be either monosomic, disomic, or complex aneuploid. Then, they were cultured to the blastocyst stage [71]. Re-analysis of these embryos at the blastocyst stage with SNP microarray-based 24 chromosome aneuploidy screening revealed 65% of the monosomic, 47% of the trisomic, and 63% of the complex aneuploid embryos were euploid at the blastocyst stage [71]. This study found a significant number of euploid blastocyst-stage embryos that were previously diagnosed as aneuploid with nine-chromosome FISH technique on day 3 [71].

Part of these presumed self corrections in many PGS studies including the ones mentioned in this review may be due to false positive data on day 3 (mild mosaicism or FISH pitfalls) or false negative at the blastocyst stage. However, a study by Fragouli et al. 2008 demonstrated consistent results between FISH and CGH in ten out of 12 blastocyst stage embryos that were analyzed with both techniques [72]. The supposed self corrections may also be due to monosomic or trisomic rescue as demonstrated in some studies [61, 70, 71]. This important aspect of preimplantation embryo development needs to be further addressed to prevent possible erroneous disposal of euploid blastocysts that were previously diagnosed as abnormal via FISH at the cleavage stage [71].

Diagnostic challenges

There are many challenges facing IVF clinicians who use PGS as a strategy for improving IVF outcomes. Here we discuss a few of them: (1) patient factors, (2) possible procedural risks, (3) strategies, technology and techniques involved in PGS. We emphasis strategies, technology and techniques to highlight how these parameters directly influence PGS results and ultimately IVF outcomes.

Patient factor Infertile patients with potential indications for PGS tend to have multiple conditions that may contribute to infertility. Some conditions that can further complicate the diagnosis include polycystic ovarian syndrome (PCOS), endometriosis, and diminished ovarian reserve. Patients with PCOS tend to have a large number of follicles and oocytes but lower quality embryos. Thus, the incidence of aneuploidy in these patients is suspected to be high. Embryos of women with PCOS have suboptimal development, which makes them more sensitive and not ideal candidates for biopsy and extended culture. A retrospective study in one private PGD lab found no correlation between PCOS and a high aneuploidy rate [73]. It is important to note that this study used PGS-FISH to determine euploid/aneuploid status with an error rate for false-negative (for aneuploidy) at 4.1% [36, 73]. Similarly, patients with endometriosis, another common cause of infertility, frequently have an inadequate number of oocytes

and poor embryo quality and therefore tend to have implantation and receptivity problems. Thus, a higher incident of aneuploidy can be detected in some of these patients [74]. In patients with diminished ovarian reserve, the follicle/oocyte pool has been emptied and thus, the remaining oocytes generally are of low quality and tend to contain chromosome abnormalities. Patients with this condition generally require high doses of follicle stimulating hormone (FSH) for ovarian stimulation which may also affect the quality of ovaries, follicles, oocytes and endometrium. In a recent study by Massie et al. 2011, a comparative karyotype analysis of the products of conception between patients with spontaneous conception and patients who underwent ovarian stimulation demonstrated that the latter group did not have a higher incidence of aneuploidy [75]. This suggests that ovarian stimulation with exogenous FSH was not likely to result in an increased risk for embryonic aneuploidy [75].

Possible procedural risk It is currently not known whether PGS procedures negatively impact the developing human embryo and if so, how, especially in later developmental stages. Time-lapse imaging comparing the development of mouse embryos with and without blastomeres demonstrated key differences in the speed of growth, frequency of contraction and expansion, diameter of contraction and expansion, and hatching of the blastocyst from the zona pellucida. Mouse embryos that underwent blastomere biopsy had a slower growth pattern. Hatching occurred at the site where the blastomere was removed and did result in a hernia-like appearance [76]. Also, expansion and contraction occurred more frequently in the smaller embryos. It is not known if similar events occur in human embryos and if so, what effects it will have on the developing embryo and offspring.

Ovarian stimulation The rate of aneuploidy in IVF has been shown to be influenced by the type of ovarian stimulation used (mild vs. conventional) and the inclusion of luteinizing hormone (LH) supplementation. In ICSI, the rate of aneuploidy is influenced by delayed embryo fertilization. A randomized control trial comparing conventional and mild ovarian stimulation found that the number of retrieved oocytes was higher in patients given conventional ovarian stimulation but that a higher proportion of chromosomally abnormal embryos were derived from those oocytes [77]. In contrast, a lower number of oocytes were retrieved with mild ovarian stimulation but a larger number of the embryos were derived from these oocytes were normal. Therefore, the type of ovarian stimulation may influence the quality and quantity of the oocytes available for fertilization, which is possibly in an inverse relationship [77]. This random control trial (RCT) was prematurely terminated, thus, future studies will be required to confirm the findings.

Ovarian stimulation with exogenous LH-containing gondadotropin was also shown to be associated with a higher diploidy rate than FSH in IVF patients with no known history of RIF, RM, or severe male factor infertility [78]. But in a subsequent study by the same group, the results indicated that there was no significant difference in diploidy between patients undergoing ovarian stimulation with FSH or combination FSH/LH-containing gonadrotopin [79]. Further studies comparing differing levels of LH-containing gonadotropin by patient age may explain the difference in previous results since controversies surrounding LH-levels have been documented [80–84].

In another interesting study following in vitro maturation, IVF (with or without the use of ICSI) was more likely to result in an aneuploid embryo than the use of in vivo maturation followed by ICSI [85]. Hormone stimulation can exert various influences on chromosomes, which can make the task of identifying a cause of aneuploidy even more challenging. And thus, patients may be subjected to "IVF-induced" (due to the ovarian stimulation) aneuploidy.

Strategy, technology and technique Strategy, technology and techniques for performing PGS vary between IVF centers. However, no study has looked at the PGS strategies that are currently used for each subpopulation of patients. Should the same PGS strategies be used for a patient with RIF and one with no known cause of infertility? For example, should different sets of probes be used for the various subgroups of patients [86]?

Bielanska et al. 2002 compared aneuploidy rates for three different sets of probe mixtures to determine which combination was most efficient in determining postzygotic chromosomal abnormalities. Although all three had sets of probe mixtures demonstrating a similar (~50%) rates of aneuploidy, this result was likely due to the presence of a large proportion of chaotic embryos that had no chromosomal specificity [86].

Two other interesting questions arise. First, should the choice of cleavage- or blastocyst stage biopsy depend on the most likely cause of aneuploidy for a particular subgroup of patients in order to maximize use of polar body analysis to minimize harm to the embryo? And second, for patients who elect single embryo transfer, is the blastocyst stage biopsy the best PGS strategy in terms of efficacy?

The current technical options presently in use for PGS have limitations, which may influence the result of embryo implantation and pregnancy success rates. Furthermore, it can be challenging to diagnose aneuploidy using currently available technology since it requires a highly skilled clinician who can be precise and minimize technical errors. Indeed, only one or two blastomeres are available at a time,

and the timeframe for testing prior to implantation is limited if we are to implant fresh embryos. Certain technical options can influence IVF outcomes. These include assisted opening of the zona pellucida, blastomere biopsy, one versus two blastomere removal, blastomere fixation [87], and FISH versus CGH.

The zona pellucida can be opened mechanically or chemically (with acidified Tyrode) or with a laser. Few studies have examined the differences between these techniques and their effects on the early developing embryo. Studies that have compared the use of chemical acidified Tyrode and laser ablation have found no differences in terms of damage to the embryo, implantation rates and pregnancy rates [88–90]. In mouse embryos that have undergone blastomere biopsy, hatching occurred at the site where the blastomere was removed and did not exhibit a hernia-like appearance [76]. It is not known if the similar events occur in human embryos and if so, whether or not they have any negative influence on their development.

Three major biopsy methods used for PGS include polar body polar bodies, cleavage stage, and blastocyst biopsy. Polar body biopsy involves the removal of one or two polar body(ies) and is commonly used where legislation prohibits embryo biopsy such as Germany, Austria, Switzerland and Italy [91]. Polar body biopsy limits the analysis to maternal causes of aneuploidy and is an indirect method of screening for aneuploid embryos. Although the procedure is non-invasive to the embryo, it might have negative effects on the developing embryo. If oocyte maturation to the metaphase II stage is completed just before the PB biopsy, it may result in inadvertent damage to the meiotic spindle of the oocyte (or even a complete enucleation)—with all its negative consequences. Additionally, it is believed that the presence of the two polar bodies during fertilization serves (or correlates) to orient the developing embryo. Initially, the two polar bodies are next to each other immediately after mitosis during fertilization. The two polar bodies then migrate to opposite ends providing axes of symmetry for cell determination. Next, the farthest cell from the second polar body differentiates and secretes hCG [92]. This signaling event further determines the blastomeres that will become the inner cell mass and the trophectoderm [93–95]. Premature removal of the polar bodies may disorient the patterning of embryo development and result in negative effects to the embryo at later developmental stages if the embryo does not undergo developmental arrest. It is important to note that this was observed in animal models and has not been shown in human embryos.

Today, cleavage stage biopsy is the most commonly used method for screening preimplantation embryos for aneuploidy [16, 96]. It involves the removal of one or two blastomeres from a 6- or 8-cell embryo on day 3. Approximately 90% of all reported PGD/PGS cycles involve cleavage stage biopsy [97]. However, a recent report by ESHRE suggests that there is movement away from cleavage stage biopsy towards polar body biopsy and trophectoderm biopsy [98]. The major reasons may be due to the high degree of mosaicism of cleavage stage embryos [99] or lack of evidence to support its use [100–105].

Blastocyst biopsy is rapidly becoming the more preferred biopsy method for aneuploidy screening. It was initially performed in an Australian private IVF clinic where researchers would remove trophectoderm cells—the procedure resulted in a live birth [25]. Biopsy at the blastocyst stage has been demonstrated to be more desirable since embryos at this stage have a smaller risk of aneuploidy (38.8%) than embryo biopsy at the cleavage stage (51%) [72]. This is likely due to the fact that mosaic embryos (mitotic origin of chromosomal abnormalities) have a higher proportion of aneuploid cells on day 2/3 and will not develop to the blastocyst stage (thus part of the "aneuploid embryos de-select"). If embryos with a moderate/low level of mosaicism will "correct" when developing to the blastocyst stage, then a higher proportion of embryos will be chromosomally normal. In a preliminary prospective RCT study by Scott et al. 2010, the results of a 24-chromosome aneuploidy screening of a blastocyst stage embryo with fresh transfer demonstrated a significant improvement (12 of 13; 92%) as compared to controls (9 of 15; 60%) in clinical pregnancy rates [169].

Normally, one or two blastomeres are removed during cleavage stage biopsy. A delicate balance exists between the risk for misdiagnosis due to mosaicism with the removal of one blastomere and a risk of injury to embryonic development with the removal of two blastomeres [106, 107]. An evaluation that compared the effect of one-cell blastomere biopsy with that of two-cell blastomere biopsy on clinical outcomes found no differences in live birth rates [106]. Moreover, a prospective study by Goossens et al. 2008 documented an improvement in the efficiency of diagnosis for amplification-based PGS with the two-cell blastomere biopsy (96.4%) compared with the one-cell biopsy (88.6%) [108]. The same study found no increase in the efficiency or accuracy of diagnosis with FISH PGS [108]. The ESHRE PGD consortium guidelines for amplification-based PGS recommends using only one-cell biopsy of cleavage stage embryos [109]. For FISH PGS, the ESHRE PGD consortium does not recommend biopsy of cleavage stage embryos. Instead, they recommend using a polar body or blastocyst biopsy with FISH [110] because studies have shown no adverse affects on either embryo implantation or development to term [25, 111–113].

The importance of selecting the cell/blastomere to be removed from a day-3 cleavage stage embryo for biopsy has not been thoroughly discussed. Although completely overlooked, the "selection" is critical. Not only is the

"presence" or "absence" of a visible single nucleus important [108], but also the size, orientation, shape and relative volume of the blastomere. Relative to other cells within the embryo, significantly larger or smaller cells than the "typical" average size cell in that embryo is likely to be chromosomally abnormal. A follow-up of the embryo may indicate that those cells have been excluded from further development within the embryo.

The FISH technique is limited in that it can analyze only a small number of chromosomes at a time. However, studies have demonstrated that it has no significant affect on the results for determining aneuploidy since any chromosome can be involved. Thus any combination of probes carries the same chance of diagnosis [24]. A recent study by DeUgarte et al. 2006 re-examined the accuracy of the FISH technique for predicting chromosomal abnormalities in day 3 embryos and found that it is a good enough technique. Of the 198 embryos diagnosed as abnormal by FISH on day 3, 164 of them were confirmed to be abnormal on day 4 or 5, for a positive predictive value of 83% [114], which is a measure of the performance of FISH to correctly diagnose numerical chromosomal abnormality in day 3 embryos. These results, however, should not be considered conclusive [114]. Furthermore, a number of enhancements have been made to the FISH technique. Sequential FISH is the completion of multiple rounds of FISH. The difference is focused on using peptide nucleic acid (PNA) probes instead of DNA probes in multiple sequential rounds of FISH with a lower temperature to minimize loss of signal. The last round of FISH uses the conventional techniques including DNA probes and a higher temperature [115]. Another FISH enhancement called cenM-FISH is a 24-color centromere specific technique that analyzes the entire set of chromosomes and origin of the aneuploidy; it reveals whether the abnormality originated from nondisjunction or premature division of sister chromatids.

FISH, however, still has limitations, including cell fixation and overlapping signals [116]. Once the blastomere is removed, it must be fixed prior to cell examination. A study by Velilla et al. 2002 compared three types of blastomere fixation methods including (2) the acetic acid/methanol, (2) Tween 20, and (3) a combination of acetic acid/methanol and Tween 20 [87]. The results indicated that the most optimal blastomere fixation method was the combination of acetic acid/methanol and Tween 20 as it offered a reasonably good nuclear quality, was easier to learn and required fewer technical skills [87].

Other improvements made to FISH that have shown promising results include double-labeling, which confirms the results using two labels [117] and 3D FISH staining, which does not require cytoplasmic removal [118].

Emerging technologies such as comparative genomic hybridization (CGH) and microarrays have the potential to revolutionize PGS techniques and provide more efficacious results to further improve implantation rates and ongoing pregnancy rates resulting in a healthy, live newborn [72, 119, 120]. The major advantage of CGH is that it can analyze the entire chromosome set without the need for cell fixation, unlike other techniques such as conventional karyotyping [121], spectral karyotyping [122], and multiplex fluorescence in situ hybridization [116, 123, 124]. Because cell fixation is not required, CGH does not limit analysis to metaphase chromosomes and thus cells in any cell cycle phase may be analyzed. This is also true for the FISH technique. CGH testing on polar body one and metaphase II oocytes has been shown to offer equal or better PGS results than FISH techniques within an acceptable timeframe conducive for ART procedures. Furthermore, CGH may be able to detect whole chromosome abnormalities as well as unbalanced translocations at least 10–20 Mb in size [124]. A comparison of CGH and FISH by concurrent removal of two blastomeres from day 3 embryos confirmed the reliability of CGH [125, 126]. Furthermore, CGH analysis for 30% to 33% of embryo aneuploidies would not have been detected using FISH [124, 126]. A preliminary study for the use of CGH with trophectoderm biopsy (69%) has shown a significant increase in implantation rates compared to the control group (45%) [26]. The major limiting factors of CGH-trophectoderm biopsy is the length of time (4 days) and familiarity with molecular genetics and cytogenetics. However, vitrification techniques—a fast freezing method with less negative effects than the conventional slow freezing—used in conjunction with CGH is a promising solution to the limitation.

Another solution is the use of CGH with microarrays. In recent studies, array comparative genomic hybridization (aCGH) was completed within 10 to 18 h instead of 4 days [119, 127]. However, a recent investigation looking to decrease the hybridization time of aCGH has shown that the modified approach may be applied to the cleavage stage preimplantation embryos successfully, thereby avoiding cryopreservation [128]. A validation study of aCGH by Gutierrez-Mateo et al. 2010 demonstrated that aCGH detects 42% more abnormalities and 13% more abnormal embryos than the standard 12-probe FISH method [129]. The investigators believe that aCGH is now considered fully validated for clinical use.

Underway is another promising technique that can produce a comprehensive 24-chromosome analysis similar to aCGH called single nucleotide polymorphism (SNP) microarrays. SNP microarray technology has been shown to be capable of detecting aneuploidy and monogenetic disorders [130] and provide genotype data to produce a unique DNA fingerprint for each embryo [119, 131, 132]. Validation studies have been underway and show promising results.

Complementary options

Accuracy in the diagnosis of aneuploidy using PGS-FISH varies between IVF clinics. Increasing the accuracy rate of tests may help to improve IVF success rates. One strategy is to combine PGS-FISH with a number of complementary options that are inexpensive, quick, reliable and perhaps, noninvasive. For example, the selection of gametes or embryos based on morphology and developmental characteristics is a simple, fast and relatively inexpensive initial step—an ideal complementary option to PGS-FISH. In this regard, the location of the PGS laboratory may be important factor. Is the diagnosis performed "in house" or are the fixed cells sent to an outside PGS laboratory? Although sending fixed materials to a reference PGS center is usually more cost effective, "in house" PGS-FISH does provide some unique benefits, including immediate embryo re-biopsy (if needed, due to a lack of a nucleus from the cell that first was removed). It may also permit direct communication/consultation between the embryologist and PGS staff on individual embryo quality and other parameters that are important to know when FISH analyses are performed.

Sperm parameters A low sperm quality has been correlated with a higher incidence of embryo aneuploidy [133]. Here, we discuss some specific sperm parameters that are simple and inexpensive to assess, and when combined with PGS-FISH technique, will improve the success of fertility.

A higher incidence of chromosomal abnormalities has been demonstrated in the sperms of patients with oligozoospermia ($<20\times10^6$ / ml sperm concentration). From these patients were also shown to produce embryos with a higher incidence of chromosomal abnormalities than normal. Younger women were not included in the study so as to remove the effects of AMA. Using PGS-FISH in couples with a high sperm aneuploidy rate has been shown to improve fertility results [134]. Therefore, the study by Sanchez-Castro et. al. 2009 recommends the use of aneuploidy screening via FISH in sperms from oligozoospermic patients [133, 134]. Perhaps a study comparing the combination of PGS-FISH in sperm and embryos versus PGS-FISH in embryos only in an oligozoospermic patient group will result in a significant benefit for clinical application.

Another more involved method of analyzing the sperm genome is the development and study of an androgenetic embryo. One sister blastomere of a 2-cell haploid androgenetic embryo is produced by enucleating of oocytes at telophase II with ICSI. The androgenetic embryo would have the exact same sperm genome for assessment prior to fertilization [135]. Although this approach is unique and highly informative, it is likely to remain a research tool for the coming years.

Similarly, patients presenting with azoospermia—no sperm in the concentrated semen after centrifugation—have a higher incidence of creating aneuploidy embryos in IVF cycles. Within this patient population, no significant difference was found between non-obstructive azoospermia and obstructive azoospermia. Although we would expect to find a lower incidence of embryo aneuploidy in "obstructive male-factor" patients, a few studies have shown that both azoospermic patient populations were at very high risk for chromosomal abnormalities and would benefit from PGS-FISH on sperm extracted from testicular sperm extraction (TESE) operations [38, 39].

Besides sperm concentration, sperm morphology has also been linked to a higher incidence of embryo aneuploidy [136]. Interestingly, a study comparing teratozoospermic patients with normal sperm producing patients found that the aneuploidy rates for such conditions as monosomy, trisomy, abnormal complex, and abnormal sex chromosomes were similar in both groups. Despite the lack of differences in aneuploidy rates, the IVF outcomes for the two groups of patients (teratozoospermic and normozoospermic) were very different. Pregnancy rates and implantation rates were significantly lower in the teratozoospermic patient group. Teratozoospermic patients whose sperms present with large, elongated heads are the more common abnormal sperm morphology associated with embryo aneuploidy [137]. Examining sperm morphology is a simple and inexpensive method to include alongside PGS-FISH for infertile, teratozoospermic patients.

Pronuclear morphology Several studies have demonstrated a correlation between pronuclear morphology and the embryo chromosomal complement. Pronuclear morphology is a quick, easy and reliable means for detecting haploploidy, triploidy and other polyploidy (abnormal number of an entire set of chromosome). This early examination into the fate of the embryo prior to PGS has been in practice and is a proven strategy [138–141]. Edwards and Beard 1997 was the first to theorize that the orientation of the polar body is important for cleavage development [142]. Interruption to the polar body orientation may result in uneven division of the chromosomal and an aneuploid embryo. The position of the polar bodies [143] thus may play a role in further embryo development or alternatively, it may only be just an indicator of the cell cleavage planes, without the negative effects (this question needs to be clarified in future studies).

Embryo morphology/development Embryonic morphology varies between embryos during early development. The difference may be used to determine which embryos to diagnose as abnormal, especially in cases where there PGS-FISH results are unclear. A combined effort of morphology screening and PGS-FISH may produce better diagnostic

results for determining the embryo's eventual chromosomal fate [144]. It is imperative to identify precisely which characteristic of embryo morphology to examine within the 2-cell, 4-cell and 8-cell stage that presents the most accurate picture for the future of the embryo chromosome complement. A scoring criterion for embryo morphology at day two was developed by Holte et al. 2007 from a medical evidence-based perspective. It demonstrates the predictive power for some of the variables chosen such as blastomere size, symmetry of cleavage, occurrence of blastomeres with a visible single nucleus, degree of fragmentation, and cleavage rate [145]. Of the five variables, the cleavage rates had the highest predictive value. Four blastomeres are most ideal in embryos during the day 2 stage. Anything more or less correlates with a higher aneuploidy rate [145].

However, the 8-cell stage is a better stage for predicting embryo aneuploidy on day 3 of embryo development. During this stage, eight blastomeres are most ideal. An even number of blastomeres is superior to an odd number up to eight blastomeres. Embryos with nine blastomeres were more ideal than those with ten. When cleavage rates were compared, neither fast nor slow cleavage rates demonstrated a more positive outcome [146]. Fragmentation at the 8-cell stage on day 3 has been observed to be a potentially good indicator of chromosome normality in women with AMA [147].

The way the nuclear material is organized within an embryo at the 6- to 8-cell stage can provide clues to the aneuploidy status. A high density of nuclear material centrally located in the cell is most desired as opposed to a low-density of gene material in the peripheral region of the nucleus. However, localization of nuclear material does not indicate whether the embryo is morphologically normal or abnormal, only whether euploidy (normal number of chromosomes) or aneuploidy is present [148].

Examining an 8-cell stage embryo for abnormal cleavage rates, an abnormal number of blastomeres, and abnormal localization of nuclear material has been demonstrated to show a high correlation to aneuploidy. However, more prospective randomized control trials with combination morphology assessment and PGS-FISH should be completed to determine whether it is more advantageous than PGS-FISH alone. This would be a convenient and complementary option since the lab personnel is already working on the embryo at the 8-cell stage. A quick and inexpensive morphological examine prior to blastomere removal would not add a significant amount to the time and labor yet may yield a significantly more reliable diagnostic result.

Combination techniques (cenM-FISH, PB1-CGH) Magli et al. 2004 examined embryo viability after subjecting the embryos to a polar body biopsy (I and II) following oocyte maturation [113]. Subsequently, the same embryo underwent a blastomere biopsy on day 3. It was determined that there was no significant difference between the single biopsy control group and a cohort of embryos undergoing double biopsy methods [113]. Double biopsy methods may be used to confirm results, improve diagnostic results and/or lower misdiagnosis rates. However, a two-cell biopsy is more likely to damage an embryo or negatively affect its future development. More studies are needed to confirm the preliminary findings.

Other innovative non-invasive procedures such as proteomics (the study of the protein structure and function of a set of proteins in a cell), metabolomics (the study of a set of low molecular weight substrates and by-products of enzymatic reactions that have a direct effect on the phenotype of the cell), and transcriptomics (study of the set of mRNAs or "transcripts" of one cell or a group of similar cells) are techniques that when combined with PGS-FISH could be used to confirm and/or improve diagnostic results. These techniques must be evaluated first before they could potentially replace PGS or provide an additional source of testing information. However, it will take some time before these techniques can be applied. More studies pertaining to early embryo development and its by-products are required before unique biomarkers can be identified to distinguish between high potential (normal, high-quality) versus low potential (abnormal, low quality) embryos for successful implantation and a healthy, live birth.

Challenges to studying the role of the embryo

Standard definitions The definition of AMA, RIF, and RM are inconsistent between different studies. For example, an AMA could be >35, >37, or >38 years of age. RIF can be defined as 3 or more implantation failures; sometimes the phrase "with good quality embryos" is added. RM can mean 2+, 3+ or 4+ previous miscarriages; sometimes the word, "consecutive" miscarriages is included in the definition [149].

Severe male factor (SMF) infertility also has many definitions. The definitions can vary to include some of the following patient populations: azoospermia, severe oligoasthenotetrazoospermia, macrocephalic head, Klinefelter syndrome, males whose semen analysis do not meet WHO criteria, testicular sperm extraction patients, altered male meiosis, altered FISH results, non-obstructive azoospermia, Y chromosome deletion and immature spermatids.

Because these definitions do vary widely, the ESHRE recognizes the need for consistency for the purpose of information gathering and data analysis, to be addressed in the future [149]. Meta-analysis of studies becomes a challenge with inconsistent definitions. Thus, comparisons

may lead to inaccurate assessments. Recently, the ESHRE PGD consortium published a best practices guideline that includes the definitions for AMA (>36 completed years), RIF (≥3 embryo transfers with high-quality embryos or ≥10 embryos in multiple transfers) and RM (≥3 miscarriages) [150]. These definitions are not absolute and will vary as determined by individual clinics.

Competent skills training, certification and regulation and quality control Transitioning PGS from research into clinical practice requires the community of physicians and geneticists in reproductive medicine to prove its efficacy, benefits and effects on embryos. In addition, quality standards must be met and labs must be proficient. A comparison between several factors including technology, number of cells biopsied and study design from several studies was undertaken to explain the conflicting results of various studies. It was found that the difference in technology between the studies accounted for most of the differences in study results. In particular, (1) too many cells were biopsied, (2) inadequate probes were used, and (3) suboptimal fixation technology was applied [106]. Currently, no formal system exists to govern quality control and ensure lab proficiency [151]. For example, no certification exists in the U. S for the lab technicians performing embryo biopsy—a technique requiring a highly skilled lab personnel [15]. This is critical for maintaining the highest standards of patient care. Organizations such as the ESHRE PGD Consortium and the International Working Group on Preimplantation Genetics are in the best positions to provide strict procedural outlines to avoid sample mislabeling, mix up, and contamination as well as to insure skill proficiency with internal and external proficiency testing [152, 153]. The ESHRE PGD Consortium has published a set of guidelines is to assist IVF clinics in providing quality medical services and laboratory practices for their patients based on results of current research studies [154].

IVF laboratory set up, embryo culture conditions A highly significant contributor to a successful PGD program is the general IVF laboratory set-up, the quality of embryo culture conditions, and the experience with extended embryo culture. Any potential benefit from day-5 embryo biopsy combined with FISH analysis can only be expected if embryo culture is performed in a laboratory that maintains high-quality embryo culture conditions, especially in regards to extended culture. Otherwise, in laboratories where extended culture is not established or in those with poor experience, PGS-FISH cannot provide any benefit. It is not only the quality of the biopsy (which can impact embryo development depending on the experience of the operator) but also the "quality of the extended embryo culture." That is important since it can also dramatically influence PGS-FISH results. For example, Rubio et al. 2009 criticized two studies, one that [155] reported relatively low blastocyst rates per biopsied embryo and another one [156] that showed incredibly high miscarriage rates after PGS, clearly indicating that the embryos had been damage during the procedure. This suggests that laboratories with difficulties in with prolonged embryo culture should choose early embryo transfer rather than day 5 blastocyst transfer [157]. Also, chromosomally normal embryos may arrest in a sub-optimal culture condition before reaching the blastocyst stage, and thus any "theoretical" advantage of selecting chromosomally normal embryos will be lost if the embryos degenerate after biopsy or arrest during (and due to) the extended culture. Internal analysis of data from more than 1,000 cases of PGS in the span of 10 years has clearly demonstrated that there is a benefit of using PGS testing during this period as it leads to a large proportion of PGS "normal" embryos (FISH evaluation on 9 chromosomes). The strongest correlation with improvement was found in the establishment of an improved embryo culture system (starting from day 0 to day 5)—the PGS-FISH technique itself did not change in the same period. Thus, it should be clearly advised that before attempting to perform PGS (and expecting to see any benefit from it), extended embryo culture to day 5 or day 6 should be established.

The stage of embryo biopsy is shifting from day 3 to day 5. Instead of one to two blastomere(s), three to six trophectoderm cells are removed. Instead of using 9- to 12- chromosome FISH, a 24-chromosome detection by microarray is used. The combination of these is resulting in improved diagnostic accuracy and improved "treatment benefit" (higher pregnancy/implantation rates). The main reason is that embryos are developing to the blastocyst stage with a lower incidence of mosaicism. Low-quality embryos with post-zygotic/mitotic chromosome errors will not develop to the blastocyst stage. Thus, screening all chromosomes helps to eliminate meiotic originated chromosome errors with high accuracy. Another advantage is the larger initial amount of DNA that is available for analysis from blastocyst stage embryos. While these new developments may benefit a number of patients, at the same time, there will be other couples who will not be able to take advantage of these improvements, especially if they have a low number of high-quality embryos that cannot develop to the blastocyst stage (which again, may depend also on the culture system). However, we will need more studies from well-designed randomized trials to test the potential benefits of these new developments.

The proposed "indications" for PGS vary, but based on the purpose, it can be categorized as a "treatment option"—meaning the aim is to improve implantation/pregnancy rates through embryo selection (e.g., in a young patient with a

large number of high-quality embryos). It can also be categorized as a "diagnostic option" where obtaining test results is the primary aim (e.g., in a patient with AMA and/or few high-quality embryos). It can also be categorized as both "treatment" and "diagnostic" (e.g., a young patient with a large number of good quality embryos with repeated failed prior cycles). To put this in a different aspect, with an optimal embryo culture system, few embryos (per patient) are needed to obtain benefits ("treatment option") from PGS. This obviously varies from one IVF center to the other. Therefore, each IVF lab has to establish its own "threshold" level where the benefit of PGS may start. This level cannot be applied "universally" to other centers.

Ethical considerations Ethical considerations pertaining to PGS include the moral status of the embryo, embryo freezing, embryo loss during cryopreservation, disposal or donation of unused embryos, abortion rates and parental interest and decisions [158]. The moral status of the embryo is the most important because of the legal, social, and other ethical implications involved. There are three distinctions to the moral status of the embryo. The embryo may be considered to have no moral status, full moral status, or no moral status [159]. For example, an embryo with full moral status has the same rights as a human being, and so, any biopsy or destruction to the embryo is considered illegal in some countries and punishable by law. Hence, the availability for unused embryos to be used in research studies is limited by each country's ethical considerations for the embryo.

Economics The cost of an IVF cycle alone is already expensive without PGS services. There are rarely any cost-effective studies for IVF only compared to IVF/PGS. The basic cost for IVF with monitoring and medications per cycle was estimated to be $6233 [160], $9226 [161], and $25,700 [162] in years 1995, 2001, and 2008, respectively. Patients with PGS indications usually require multiple IVF cycles. PGS is not covered by insurance in most countries and can add $1000 to $2500 to routine IVF costs [162]. Furthermore, it does not replace prenatal diagnostic procedures, such as amniocentesis and chorionic villus sampling, which is often recommended alongside PGS procedures for confirmation. It therefore makes sense to ensure the efficacy of PGS so that the benefits are proven to outweigh the additional cost in terms of money, time and risk to the mother and baby. A cost comparison of IVF alone ($68,026) versus IVF with PGS for patients ages 38 to 40 years demonstrated a significantly higher cost with IVF/PGS ($118,713), but there were no differences in cost for patients over 40 years of age [162]. This does not take into account indirect costs such as the physical pain from the procedure and emotional burden for the couple.

Conclusions

Clinical relevance A report by the American College of Obstetricians and Gynecologists (ACOG) released in 2009 concerning PGS does not support the use of PGS for AMA, recurrent unexplained miscarriage and recurrent implantation failures. The committee also does not support the use of PGS to improve IVF success rates and considers it possibly detrimental. Moreover, the ACOG committee advises PGS activities be limited to research studies [163]. Recently, the ESHRE PGD Consortium steering committee issued a statement stating that current evidence does not support the routine use of PGS-FISH on cleavage stage embryos of AMA patients to improve live birth rates in IVF treatments [164]. In the future, PGS-FISH may be replaced by comprehensive chromosomal tests such as aCGH and SNP microarrays once these techniques have been thoroughly tested in multicenter, randomized control trials and the cost and limitations have been addressed appropriately.

Efficacy of PGS: the debate The efficacy of PGS was questioned soon after the first randomized control trial found that PGS-FISH did not improve implantation rates for AMA patients [165]. Since then, additional randomized control studies have had the same results (a lack of support for PGS to directly improve implantation rates and pregnancy rates in older patients who are otherwise healthy) [103, 156, 166, 167]. The biggest challenge to the debate appears to be the lack of randomized control testing using optimal PGS procedures and techniques [168] with the appropriate sample size of a subgroup of patients. According to Munne et al. 2009, a threshold error rate for PGD/PGS should be defined, and IVF clinics with error rates above the threshold should declare their procedures experimental [168]. The question remains as to whether or not we should try to improve our experimental techniques, to perform more randomized control studies and obtain more reliable data. A recent systematic review and meta-analysis on ten randomized trials by two independent reviewers that assessed the efficacy of PGS concluded that additional trials should not be performed to assess the efficacy of PGS. The current set of research data is powerful enough to unequivocally determine that PGS does not work and should not be routinely used [104]. Furthermore, technical expertise is critical but the failure of PGS in randomized control trials to demonstrate an advantage cannot be attributed exclusively to a lack of technical competence or mosaicism. If PGS can be effectively applied by few, then its application is very limiting [105]. However, many of the studies fail to show a benefit for PGS, and worse yet some even found decreased results. In PGS studies with detrimental results, the typical day of embryo transfer is day 2 or day 3 and not day 5. This

also means that the same center(s) may have limited experience with day 5 embryo transfer and consequently with extended embryo culture. Clearly, if extended embryo culture is not well-established, one cannot benefit from embryo biopsy and PGS—as embryo biopsy is performed on day 3 and extended culture to day 5 has to be performed (which was the case in all these studies).

Consequently, even if PGS techniques are found to be effective, they would not improve outcomes if embryos lose viability (fully or partially) due to suboptimal extended culture conditions. In reality, embryo culture quality is the true clue to understanding the conflicting experience reported with PGS. It is important to note that not only does the culture conditions beyond day 3 effect embryo development (and viability), but also early culture conditions (day 0 to day 3), even if this is more difficult to recognize. Because embryo development in the early stages (day 2 and day 3) is not necessarily very different when optimal versus less optimal culture conditions are used, noticeable differences may required the embryo to be cultured further to day 5 before differences can be recognized it. Thus, an unrecognized suboptimal early culture will "pre-determine" an impaired development in extended culture (independent of the quality of the extended culture). In addition, it may also lead to a higher incidence of mosaic embryos on day 3 resulting in a potential loss of benefit from PGS testing.

Summary

- To date, PGS is the only commonly available testing procedure that can provide potentially useful information—in addition to morphology—on embryo quality assessment.
- The technical execution of biopsy requires a high level of training. PGS testing can be applied at different stages including: PB from MII-oocyte/2PN-zygote stage, blastomere from day 3 (possibly day 2) and trophectoderm cells from blastocyst stage embryos. Day 3 of cleavage stage embryos is the most typical, but biopsy of day 5/6 blastocyst stage embryos is also becoming more common.
- The benefit of PGS-FISH has been strongly debated (only when it is used as "treatment option"—but not as a "diagnostic" option) to the extent that PGS may lead to decreased pregnancy and implantation rates. These contradicting findings may be explained, at least in part, by differences in patient selection (indications), technicalities of embryo biopsy (1 cell versus 2 cells from day 3 embryo; type of assisted hatching, experience), and technicalities of fixation and FISH handling (as well many other related aspects of "gentle" embryo transfer of biopsied embryos, besides others). However, a majorly overlooked aspect of these different findings is the quality of embryo culture. The more advanced the culture system (and the more experience the technicians in working with cultured systems), the more benefit that can be obtained, and controversially, the less optimal the embryo culture, the less benefit that can be obtained.
- A high incidence of mosaicism of human embryos associated with embryo biopsy at the cleavage stage, which was an unexpected finding, may also contribute to the challenges of assessing PGS outcomes. Technological improvements such as the shift towards the use CGH and SNP microarrays can help meet some of these challenges.

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