PREIMPLANTATION GENETIC TESTING

Chromosome abnormalities in human embryos

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Abstract

Aneuploidy is a frequent event in human embryos, and its incidence is higher in oocytes and embryos from women of advanced maternal age. Aneuploidy may also be a contributing factor in infertile populations, such as couples with recurrent miscarriages, repetitive implantation failure, or male infertility. For these reasons, preimplantation genetic testing for aneuploidy (PGT-A) has been proposed to prevent miscarriages and increase live birth rates in infertile couples undergoing *in vitro* fertilisation. Next-generation sequencing is currently being applied for the detection of aneuploidies in human embryos, including whole chromosome aneuploidies, segmental aneuploidies, uniform, and mosaic aneuploidies. More recently, this technology has been incorporated for the analysis of the cell-free DNA secreted by the embryo to the culture media. Chromosome abnormalities mostly originate in female meiosis. Recombination between homologous chromosomes is a critical event that occurs in the foetal ovary. The importance of altered recombination pertains to paternally as well as maternally derived trisomies, but as most aneuploidy arises during oogenesis, the female is at greater risk. For males, sperm concentration is associated with a higher risk of aneuploid sperm and thus aneuploid embryos. Mitosis errors can occur at all stages of early embryo development that result in chromosomally distinct cell populations. The clinical impact of mosaicism depends on the mosaicism type, location, and number of aneuploid cells. Transfer of mosaic embryos has been proposed when no euploid embryos are available in the PGT-A cycle.

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Introduction

Aneuploidy is the most common chromosomal abnormality observed in human embryos. Trisomic and monosomic embryos account for at least 10% of human pregnancies, and that incidence may exceed 50% for women nearing the end of their reproductive lifespan (Nagaoka *et al.* 2012, Soler *et al.* 2017). Further, aneuploidy rates are higher in oocytes and embryos from women of advanced maternal age (AMA) (Rubio *et al.* 2019b), probably stemming from meiotic recombination defects exacerbated by age (Herbert *et al.* 2015). Recent studies of humans and model organisms have shed new light on the complexity of meiotic defects, providing evidence that age-related increase in errors in human females is not attributable to a single factor but to an interplay of unique features of oogenesis and a host of endogenous and exogenous factors (Nagaoka *et al.* 2012). Age-related defects result in higher aneuploidy rates in offspring and increased spontaneous abortions, thereby reducing ongoing implantation rates. Aneuploidy may also be a contributing factor in other

infertile populations, such as couples with recurrent miscarriages (Sugiura-Ogasawara *et al.* 2012) or repetitive implantation failure (Margalioth *et al.* 2006). In male infertility, an increase in sperm chromosomal abnormalities due to impairment of the meiotic process has been described (Silber *et al.* 2003, Rodrigo *et al.* 2011). Additionally, a higher incidence of aneuploidy has been described in miscarriages of couples undergoing intracytoplasmic sperm injection (ICSI) because of male infertility (Kim *et al.* 2010, Campos-Galindo *et al.* 2015).

Preimplantation genetic testing for aneuploidy

Aneuploidy rates are high in human embryos and they increase with advancing female age (Frasnias *et al.* 2014, Rubio *et al.* 2019a). For this reason, aneuploidy testing in embryo biopsies known as preimplantation genetic testing for aneuploidy (PGT-A), was incorporated in IVF programs with the aim to improve pregnancy rates per transfer and to decrease miscarriage rates in infertile couples. However, the potential benefits of PGT-A were questioned after

the publication of several randomized controlled trials (RCT) using fluorescence in situ hybridisation (FISH) and cleavage-stage biopsies, in which no improvement in live birth rates were observed (reviewed by Mastenbroek *et al.* 2011). Later on, another RCT using FISH in patients with advanced maternal age (AMA) between 41 and 44 years of age showed increased live birth rates per patient using PGT-A and increased cumulative ongoing pregnancy rates (Rubio *et al.* 2013). The discrepancy with the previous studies could be due to differences in the FISH technique as well as the age range, with younger women included in the previous ones. In the same publication, the authors did not find a benefit with PGT-A for repetitive implantation failure, despite live birth rates per patient were 20 points higher in the PGT-A group (Rubio *et al.* 2013).

One of the main limitations of FISH was the limited number of chromosomes that could be analysed in a single biopsy. Later on, new technologies emerged that allowed the analysis of the 23 chromosome pairs, such as SNP arrays (Treff *et al.* 2010), quantitative PCR (qPCR) (Treff *et al.* 2012), array comparative genome hybridisation (aCGH) (Gutierrez-Mateo *et al.* 2011), and next-generation sequencing (NGS) (Wells *et al.* 2014) (Fig. 1). NGS was also linked to improvements in clinical outcomes compared to aCGH for couples undergoing single embryo transfer (SET) (Friedenthal *et al.* 2018).

With the introduction of NGS in the PGT-A field, new types of chromosomal abnormalities can be detected in trophoctoderm (TE) biopsies, with the identification of different levels of mosaicism. However, the diagnosis of mosaicism requires several considerations. The accuracy

of NGS results depends on one hand on the quality of the sequencing results, and a proper validation is required for each platform and protocol to define the level of mosaicism that can be detected with a balance between sensitivity and specificity (Goodrich *et al.* 2016). On the hand, there are biological differences among biopsies related to the number of cells biopsied and their integrity. Also, subjectivity among different observers can be a source of variability in the diagnosis of mosaicism. Therefore, there is a need for objective evaluation of sequencing data and the development of specific algorithms to interpret the different levels of mosaicism (Goodrich *et al.* 2016). In addition, there has been a number of publications comparing the clinical outcome after the transfer of euploid and mosaic embryos, suggesting lower implantation and higher miscarriage rates with the transfer of mosaic embryos, with poorer prognosis with higher mosaicism levels (Greco *et al.* 2015, Fragouli *et al.* 2017, Munné *et al.* 2017, 2019a,b, Spinella *et al.* 2018, Victor *et al.* 2019).

In the first studies published in the PGT-A field, the main indications were to increase implantation and pregnancy rates and to decrease miscarriage rates and the risk of aneuploid offspring. However, in more recent publications, the time to conceive, the number of transfers required, and the associated cost are considered important benefits of PGT-A compared to conventional IVF (Neal *et al.* 2018, Somigliana *et al.* 2019).

Currently, most PGT-A programs are based on TE biopsy, vitrification, and deferred transfer. Coates *et al.* compared the results of deferred cycles with fresh transfers and found similar implantation rate per

Figure 1 Evolution of PGT-A technology from day-blastomere biopsy using FISH technology to trophoctoderm biopsy and NGS technology.

transferred embryo in deferred cycles (75% vs 67%), and significantly higher ongoing pregnancy rates (80% vs 61%) and live birth rates (77% vs 59%) in deferred cycles compared to fresh transfers (Coates *et al.* 2017).

Natsuaki and Dimler (2018) recently addressed the safety of embryo biopsy in a meta-analysis and systematic review of all reports investigating neonatal outcomes after PGT for standard IVF up to 9 years of age. They included 18 studies for childhood outcomes, which entailed anthropometric, psychomotor, cognitive, behavioural, and family functioning data. Regardless of the biopsy stage and method, PGT did not impact neonatal and postnatal outcomes, indicating the safety of approaches used to date. Further, He *et al.* (2019) reviewed neonatal outcomes in children born from cryopreserved blastocysts with and without TE biopsy and concluded that TE biopsy does not add additional risk.

Non-invasive PGT-A (niPGT-A)

More recently, a new perspective in the field of PGT-A has emerged – the non-invasive analysis of the cell-free DNA (cfDNA) secreted by the embryo to the culture media (Fig. 2). After initial demonstration of embryonic cfDNA in spent blastocyst media (SBM) and the possibility of identifying chromosome copy number (Shamonki *et al.* 2016), several studies have explored concordance between cfDNA in SBM and DNA from the human embryo obtained from polar bodies (PB) (Feichtinger *et al.* 2017), TE biopsies (Xu *et al.* 2016,

Ho *et al.* 2018, Vera-Rodriguez *et al.* 2018, Yeung *et al.* 2019, Rubio *et al.* 2019b), and whole blastocysts (Huang *et al.* 2019).

In these studies, informative rates for cfDNA in terms of successful amplification and interpretable NGS results ranged 80–100%. Concordance rates varied from 3.4 to 85.7% for PB and TE biopsies (Shamonki *et al.* 2016, Xu *et al.* 2016, Ho *et al.* 2018, Vera-Rodriguez *et al.* 2018, Yeung *et al.* 2019) and increased up to 93.8% when compared with whole blastocysts, suggesting that niPGT-A could be more representative of embryo chromosomal status than TE biopsies (Huang *et al.* 2019). Since the first proof-of-concept paper (Shamonki *et al.* 2016), technology development and new IVF culture protocols have markedly increased concordance rates between TE and SBM. Nevertheless, in all these studies, embryos were previously manipulated in some way, with assisted hatching, biopsy, or vitrification. However, another study achieved a high concordance rate (84%) on day 6 blastocysts without any previous manipulation (Rubio *et al.* 2019b).

Further, another study compared results from a combination of blastocoel fluid and SBM analysis with TE and with whole blastocysts and found higher concordance rates between media and whole-blastocyst embryos than between TE and whole blastocysts, pointing out that blastocoel fluid and SBM could be more representative of 'true' embryo chromosomal content than TE (Kuznyetsov *et al.* 2018). Another study indicated similar results clinically, with decreased miscarriage rates when transferred blastocysts had

* NGS: Next Generation Sequencing

Figure 2 Schematic comparison of aneuploidy analysis in blastocyst, with trophectoderm biopsy and with the analysis of the embryonic cell-free DNA in the spent blastocyst medium.

euploid TE and euploid SBM compared with euploid TE and aneuploid SBM (Rubio *et al.* 2019b).

The origin of the embryonic cfDNA is uncertain, a recent study (Rubio *et al.* 2020) has found similar concordance rates of the cfDNA with TE biopsy and with the inner cell mass biopsy (87.5 and 84.5%, respectively). In another publication, it was suggested that the cfDNA could be more representative of the chromosome content of the full blastocyst than the TE biopsy, due to the high concordance rates observed with the whole blastocyst (Huang *et al.* 2019). However, the reply to this article argued that TE cells should be the main source for the cfDNA secreted to the medium, since TE cells are in direct contact with the medium and they are higher in number compared to the inner cell mass (Gleicher & Barad 2019).

A non-invasive approach to assess the chromosomal status of embryos could have several important advantages compared to current invasive PGT-A with TE biopsy. Such advantages include avoiding invasiveness and potential embryo harm, as well as extending the feasibility of PGT-A in a larger number of clinics and increasing its accessibility to a wider population of patients by minimising laboratory and personnel expenses. Therefore, this approach would be accessible worldwide. However, further clinical studies and more basic research are needed to fully address remaining questions related to the origin of cfDNA, its representativeness of the full embryo chromosome content, and its potential clinical applications to improve IVF outcomes.

Aneuploidy of maternal origin

Chromosome abnormalities mostly originate in female meiosis (Hassold & Hunt 2009). Recombination between homologous chromosomes is a critical event that occurs in the foetal ovary (Gruhn *et al.* 2013). The importance of altered recombination pertains to paternally as well as maternally derived trisomies, but as most aneuploidy arises during oogenesis, the female is at greater risk. Therefore, either more recombination errors are made in females or these errors are more efficiently culled in males (Hassold & Hunt 2009). Immunofluorescence has made it possible to examine crossover-associated proteins in pachytene spermatocytes and oocytes to test these alternatives. Strikingly, almost all chromosomes in males are joined by at least one crossover, although the same does not apply to females. Indeed, >10% of all human oocytes contain at least one 'crossover-less' bivalent. Because half of all such bivalents are expected to result in aneuploidy, the stage appears to be set for meiotic errors from the beginning of oogenesis (reviewed by Nagaoka *et al.* 2012).

Female age has a strong impact in the incidence of aneuploidy, and this is the most important contributing factor for the high incidence of aneuploidies in women

of advanced age (Franasiak *et al.* 2014, Rubio *et al.* 2019a). Fertility declines as women age, owing to both a diminished ovarian reserve and impaired oocyte quality that leads to an increase in embryo aneuploidy in humans (Gruhn *et al.* 2019). In this recent study, the authors proposed that chromosomal errors originating in oocytes determine the curve of natural fertility in humans in women from 9 to 43 years. In the same study, it was described that whole-chromosome nondisjunction events are preferentially associated with increased aneuploidy in young women (>20 years), whereas centromeric and more extensive cohesion loss limit fertility as women age (≥ 33 years).

Different models have been proposed to explain the effect of female age on aneuploidy. One of the earliest models of the maternal-age effect in mammals was the 'production-line model' with two key assumptions: first, that oocytes entering first meiosis were the first ovulated; and second, that the first to enter meiosis have more recombination events (crossovers) than those that enter meiosis later in foetal life (Herderson *et al.* 1968). However, a later molecular cytogenetic study of second-trimester human foetal ovaries directly examined the number and distribution of crossover-associated proteins in prophase-stage oocytes, showing similar recombination levels between oocytes entering meiosis early in foetal life and those entering late in foetal life. This study concluded that the production-line model is not the basis for the maternal-age effect on trisomy on humans (Rowsey *et al.* 2014).

Another source of chromosome segregation errors and aneuploidy in oocytes from older women is premature loss of centromeric cohesion (Herbert *et al.* 2015, Zielinska *et al.* 2015, Lagirand-Cantaloube *et al.* 2017). More lately, female age has been proposed to impact interactions between spindle microtubules and kinetochores as drivers of chromosome segregation in three mammalian species: mice, pig and humans. Centromeric chromatin recompacts with AMA, and kinetochores built on decompacted centromeres frequently lose their integrity and fragment into multiple lobes. The partial cohesin loss that occurs in oocytes with AMA is enough to trigger centromere decompaction and kinetochore fragmentation. Fragmented kinetochores are frequently abnormally attached to spindle microtubules, suggesting that kinetochore fragmentation could contribute to the maternal-age effect in mammalian eggs (Zielinska *et al.* 2019).

Due to all the previously described evidence that female age is the main contributor to embryo aneuploidy, this was the first group of patients in which PGT-A was proposed. A study of PB biopsies reported that the rate of mis-segregation for most clinically relevant aneuploidies (chromosomes 13, 16, and 18) increased from 20 to 60% in women with age range between 35 and 43 years of age (Kuliev *et al.* 2011). At cleavage stage, four RCTs were published for women of AMA

using FISH for a limited number of chromosomes. Three indicated that PGT-A offered no benefit (reviewed by [Mastenbroek et al. 2011](#)). However, these studies have been criticized by several authors who argue that the methodologies had some important pitfalls, including patient inclusion criteria, embryo biopsy procedures, embryo culture conditions, and type of genetic analyses performed. The fourth study in women of 41–44 years of age showed a significant increase in live birth rates with PGT-A compared to a conventional blastocyst transfer (32.3% vs 15.5%; $P=0.0099$) ([Rubio et al. 2013](#)). Later, another RCT study using aCGH showed similar cumulative pregnancy rates, but confirmed higher live birth rates per transfer and decreased miscarriage rates and number of transfers needed to achieve an ongoing pregnancy with PGT-A in women 38–41 years of age ([Rubio et al. 2017](#)).

More recently, an RCT using NGS and TE biopsy showed a potential benefit of PGT-A in a subgroup of women aged 35–40 years who had two or more embryos that could be biopsied, but this was not significant when analysed by intention to treat ([Munné et al. 2019b](#)). Further, a retrospective observational study reported the results of clinical, gestational, and neonatal outcomes for women of AMA after 2 years follow-up. The study included a control group of 2538 couples undergoing 2905 egg collections, 308 couples undergoing PGT-A, and 106 couples in a drop-out group who consented to PGT-A but withdrew due to poor embryological outcomes. The authors concluded that PGT-A improves clinical outcomes, particularly by reducing pregnancy loss and chromosomally abnormal pregnancy for patients of AMA, with no major impact on cumulative live birth rate per egg retrieval ([Sacchi et al. 2019](#)).

Finally, with the introduction of NGS, PGT-A cost is becoming increasingly affordable and enables embryo chromosome analysis in IVF at lower cost. Comparison of the cost-effectiveness between two IVF treatment strategies, serial transfer of all available blastocysts without genetic testing (first fresh transfer and subsequent frozen-thawed transfer), and systematic use of genetic testing (TE biopsy, freeze-all, and frozen-thawed transfers of euploid blastocysts) demonstrates the cost-effectiveness of PGT-A with increased female age and number of available blastocysts ([Neal et al. 2018](#), [Somigliana et al. 2019](#)).

Sperm contribution to embryo aneuploidies

Sperm aneuploidy has been described as a contributing factor for embryo aneuploidy ([Rodrigo et al. 2010](#), [Daughtry & Chavez 2016](#)). Aneuploid and diploid sperm can be originated by meiotic errors in the synapsis, recombination, or DNA repair mechanisms ([Nicklas et al. 1997](#), [Vendrell et al. 1999](#), [Egozcue et al. 2000a,b](#)). With the introduction of ICSI, prenatal follow-up revealed an increased incidence of de novo sex chromosome

aneuploidies and structural rearrangements that were attributed mostly to sperm quality ([Van Opstal et al. 1997](#)). Since then, sperm aneuploidy has been associated with lower pregnancy and implantation rates and to higher miscarriage rates after ICSI ([Rubio et al. 2001](#), [Petit et al. 2005](#), [Nicolopoulos et al. 2008](#), [Sarrate et al. 2010](#)).

FISH technology in decondensed sperm heads was employed for the analysis of sperm aneuploidies. FISH studies on sperm over the past 20 years have shown a higher incidence of sperm aneuploidies in infertile males compared to the fertile population ([Bernardini et al. 1998](#), [Aran et al. 1999](#), [Pang et al. 1999](#), [Ushijima et al. 2000](#), [Ramasamy et al. 2015](#), [Rodrigo et al. 2019](#)). FISH analysis of sperm has been applied mainly to patients with impaired sperm parameters (low concentration, impaired motility or abnormal morphology), and to couples with a clinical history of recurrent miscarriage or repetitive implantation failure ([In't Veld et al. 1997](#), [Pang et al. 1999](#), [Rubio et al. 2001](#), [2009](#), [Martin et al. 2003](#), [Rodrigo et al. 2004](#), [2019](#), [Petit et al. 2005](#), [Vialard et al. 2008](#), [Sarrate et al. 2010](#), [Ramasamy et al. 2015](#)).

The clinical outcome of couples with a normal sperm FISH was similar with conventional IVF/ICSI or PGT-A treatments. However, in treatments with abnormal sperm FISH, PGT-A with the transfer of euploid embryos offered better clinical outcomes ([Rodrigo et al. 2019](#)). Similarly, two small studies including 29 and 56 patients supported an association between total sperm aneuploidy rate and clinical pregnancy, with lower clinical pregnancy rates in patients with higher sperm aneuploidy ([Petit et al. 2005](#), [Nicolopoulos et al. 2008](#)).

Although most embryo chromosomal abnormalities miscarry or do not implant, several studies have reported ongoing pregnancies in which the fathers have increased sperm chromosomal abnormalities associated with the chromosomal abnormalities observed in their children. [Blanco et al. \(1998\)](#) described a high incidence of sperm with disomy for chromosome 21 in two men (0.75 and 0.78%) with children with Down syndrome, with a paternal origin of the extra chromosome 21. Similar reports in couples who miscarry or have children with aneuploidies for sex chromosomes, such as Turner syndrome or Klinefelter syndrome, have described high incidence of sperm with aneuploidies for sex chromosomes ranging 0.20–24.7% after sperm FISH analysis ([Martínez-Pasarell et al. 1999](#), [Lowe et al. 2001](#), [Eskenazi et al. 2002](#), [Tang et al. 2004](#)).

Regarding sperm concentration and aneuploidy risk, the first studies were performed in testicular biopsies to assess the meiotic process. Oligozoospermic males showed a higher incidence of abnormal chromosome pairing ([Vendrell et al. 1999](#), [Egozcue et al. 2000b](#)). Then, FISH studies in ejaculated sperm revealed an association of aneuploid and diploid sperm with oligozoospermia ([Rubio et al. 2001](#), [Martin et al. 2003](#), [Nagvenkar et al.](#)

2005, Durak Aras *et al.* 2012, Rodrigo *et al.* 2019). This correlation has also been observed in testicular sperm from azoospermic males, mainly those with non-obstructive azoospermia, where up to 42% of men have an abnormal FISH results (Rodrigo *et al.* 2011).

However, the correlation regarding sperm motility or morphology is not as clear. Regarding sperm motility, no clear correlation of isolated sperm motility and aneuploidy was found (Samura *et al.* 1997, Zeyneloglu *et al.* 2000, Sarrate *et al.* 2010, Rodrigo *et al.* 2019). Regarding sperm morphology, controversial results have been found. Some authors did not find a relationship between sperm aneuploidy and morphology (Celik-Ozenci *et al.* 2004, Sarrate *et al.* 2010, Rodrigo *et al.* 2019). Whereas others reported an increase in sperm aneuploidy in teratozoospermic males (Gole *et al.* 2001, Burrello *et al.* 2004). However, a general consensus seems to exist regarding severe teratozoospermia with large-headed and multiple-tailed spermatozoa or abnormal flagella for a higher risk of sperm aneuploidy, diploidy, and polyploidy, as reported in several studies with small number of patients included, ranging from a case report to 30 infertile men (Hristova *et al.* 2002, Tempest *et al.* 2004, Mateu *et al.* 2006, Brahem *et al.* 2012).

Compromised semen quality, mainly related to severe low sperm concentration, is purportedly associated with increased rates of mosaic blastocysts analysed in PGT-A cycles (Tarozzi *et al.* 2019). Moreover, higher rates of abnormal embryos may occur in couples with altered sperm FISH, with a high incidence of aneuploidy for sex chromosomes and mosaic embryos (Gianaroli *et al.* 2005, Rubio *et al.* 2005, Rodrigo *et al.*

2010, Sánchez-Castro *et al.* 2009). The percentage of abnormal embryos ranges 43–78% in patients with oligozoospermia and azoospermia in which abnormal sperm FISH results or impaired meiosis are reported (Silber *et al.* 2003, Aran *et al.* 2004, Gianaroli *et al.* 2005, Rubio *et al.* 2005, Magli *et al.* 2009, Sánchez-Castro *et al.* 2009, Rodrigo *et al.* 2010, 2014, Al-Asmar *et al.* 2012). Further, patients with severe oligozoospermia or cryptozoospermia who are carriers of Y-chromosome microdeletions show an increase in aneuploid embryos, highlighting monosomy X (Mateu *et al.* 2010).

Therefore, an increase in the percentage of spermatozoa with sex chromosome disomies has been associated with an increase in embryo aneuploidies compatible with life (Patau, Edwards, Down, Klinefelter, and Turner syndromes; and trisomies XXX and XYY). In contrast, an increase in diploid spermatozoa has been related to an increase in triploid embryos, which mostly miscarry before delivery (Rodrigo *et al.* 2010).

Embryo mosaicism and mitotic errors

Although the vast majority of aneuploidies in preimplantation embryos are due to meiotic errors, especially of maternal origin, defective mitosis may occur at all stages of early embryo development that result in chromosomally distinct cell populations – these are termed mosaic embryos, defined as embryos having cells with different chromosome constitution. The origin of mosaicism can be related to the failure of homologous chromosomes to separate due to anaphase lag and

* Most frequent types of mosaicism

Capalbo *et al.*, HR 2016; Vera-Rodriguez *et al.*, F&S, 2017; Popovic *et al.*, HR 2018

Figure 3 Mosaicism types according to the location of aneuploid cells, only in the inner cell mass (ICM), only in the trophectoderm (TE), or distributed in both blastocyst compartments.

chromosome nondisjunction during mitosis (Conlin *et al.* 2010, Daughtry *et al.* 2016). Other suggested causes are DNA endoreduplication, cell fusion, multipolar divisions, or defective cell cycle checkpoint machinery (Mantikou *et al.* 2012). Blastomere fragmentation and micronuclei formation have also been associated with the presence of mosaicism in human embryos (Kort *et al.* 2016).

A genetic condition may increase the risk of mosaicism in some couples, identifying an association between aneuploidy of putative mitotic origin and linked genetic variants on chromosome 4 of maternal genomes. This associated region contains a candidate gene, *Polo-like kinase 4 (PLK4)*, that plays a well-characterized role in centriole duplication and has the ability to alter mitotic fidelity upon minor dysregulation (McCoy *et al.* 2015).

Mosaicism can occur as early as the two-cell stage, although detection at the blastocyst stage is more common because more TE cells can be simultaneously analysed. At the blastocyst stage, three main types of mosaic embryos have been defined: diploid-aneuploid mosaicism, a combination of normal diploid cells and aneuploid cells; aneuploid mosaicism, with a combination of cell with different aneuploidies; and chaotic mosaicism, when the aneuploid cells have multiple aneuploid chromosomes (McCoy *et al.* 2017). According to the affected cell lineage a 'total mosaic' embryo is observed when aneuploid and euploid cells are found indistinctly in the inner cell mass (ICM) and TE. Alternatively, the mosaic population may be confined exclusively to one of these cell populations, thus generating 'ICM mosaicism' or 'TE mosaicism'. Finally, all cells in the ICM being aneuploid and those of the TE being euploid (or vice versa) confers 'ICM/TE mosaicism' (Fig. 3).

Accuracy in diagnosing mosaicism depends on the distribution and percentage of aneuploid cells. Therefore, biopsy information is only relevant to the biopsy itself. Nevertheless, the most common types of mosaicism detected in human blastocysts are TE mosaicism and total mosaicism, in which both TE and ICM are aneuploid. These two types of mosaicism would be detected in a TE biopsy if enough aneuploid cells are present (Capalbo *et al.* 2017, Vera-Rodriguez *et al.* 2018, Popovic *et al.* 2019).

Mosaicism was initially predicted more than 25 years ago from FISH analysis of two blastomeres that were assessed for PGT-A (Papadopoulos *et al.* 1989). Subsequently, multiple FISH studies analysing single cells of whole blastocysts have confirmed mosaicism in day 3 biopsies with non-concordance rates (when genotypes of analysed cells do not match one another) ranging 18–46% (Li *et al.* 2005, Baart *et al.* 2006, Barbash-Hazan *et al.* 2009, Vanneste *et al.* 2009). These high non-concordance rates on day 3 suggest very high mosaicism in the cleavage-stage embryo, suggesting TE

biopsy is a more reliable option for PGT-A than embryo biopsy (Harton *et al.* 2011).

Development of aCGH and NGS on TE biopsies has allowed new assessments of mosaicism. Several studies have used cell line mixture models to estimate the sensitivity and specificity of these methods for detecting mosaicism, estimating 40–50% levels of detection for aneuploid cells with the use of aCGH and 20% with the use of NGS (Mamas *et al.* 2012, Goodrich *et al.* 2016). Analysis of two to three biopsies in the same embryo shows concordance rates as high as 95–100%. In addition, these studies also analysed ICM of the same embryos to estimate discordance frequencies between cell lineages. TE and ICM show discordant mosaicism rates of 3–4% (Capalbo *et al.* 2013, Ruttanajit *et al.* 2016). Mosaicism detection in TE biopsies is challenging, and proper validation to define mosaicism thresholds is required for each platform to avoid overdiagnosis of mosaicism due to technical artefacts.

Mosaic embryos have been proposed for transfer if no euploid embryo are present in the PGT-A cycle (Greco *et al.* 2015, Fragouli *et al.* 2017, Victor *et al.* 2019). Clinical outcome has been related to the level of mosaicism (Spinella *et al.* 2018), chromosomes implicated (Grati *et al.* 2018), and number of affected chromosomes (Maxwell *et al.* 2016). One key aspect when reporting mosaicism in TE biopsies is to offer proper pre-cycle and post-cycle genetic counselling, informing of the limited information of risks of transferring mosaic embryos, including miscarriage, offspring with aneuploidies, intrauterine growth retardation, or uniparental disomy (Grati *et al.* 2018). Patients who do not have euploid embryos can opt for transfer of a mosaic embryo as an alternative if they opt for additional treatment cycles, which increases the chance of achieving an ongoing pregnancy than with mosaic transfer. However, future studies are needed to compare cost-effectiveness for both options (Besser *et al.* 2019).

Conclusions and future perspectives

We can conclude that PGT-A with NGS coupled with SET and deferred blastocyst transfer is the most extended approach. The published studies have shown similar cumulative pregnancy rates, but increased ongoing pregnancy rates per transfer, allowing to decrease the number of transfers required to achieve a healthy pregnancy, and therefore the time to pregnancy and the final cost in some cases. Several studies have confirmed the safety of embryo biopsy and that PGT does not impact neonatal and postnatal outcomes, indicating the safety of approaches used up to date.

A non-invasive approach to study the chromosomal status of embryos could have several important advantages over current invasive PGT-A with TE biopsy. A

major advantage is avoiding invasiveness with potential embryo harm, as well as extending the feasibility of PGT-A in a larger number of clinics and accessibility to a wider population of patients by minimising laboratory and personnel expenses. Therefore, this approach would be accessible worldwide. However, clinical studies and more basic research are needed to fully address remaining questions related to the origin of cfDNA, its representativeness of the full embryo chromosome content, and its potential clinical applications to improve IVF outcomes.

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