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Chromosomal mosaicism in human blastocysts: the ultimate diagnostic dilemma

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BACKGROUND: Trophectoderm (TE) biopsy and next generation sequencing (NGS) are currently the preferred techniques for preimplantation genetic testing for aneuploidies (PGT-A). Although this approach delivered important improvements over previous testing strategies, increased sensitivity has also prompted a rise in diagnoses of uncertain clinical significance. This includes reports of chromosomal mosaicism, suggesting the presence of karyotypically distinct cells within a single TE biopsy. Given that PGT-A relies on the chromosomal constitution of the biopsied cells being representative of the entire embryo, the prevalence and clinical implications of blastocyst mosaicism continue to generate considerable controversy.

OBJECTIVE AND RATIONALE: The objective of this review was to evaluate existing scientific evidence regarding the prevalence and impact of chromosomal mosaicism in human blastocysts. We discuss insights from a biological, technical and clinical perspective to examine the implications of this diagnostic dilemma for PGT-A.

SEARCH METHODS: The PubMed and Google Scholar databases were used to search peer-reviewed publications using the following terms: 'chromosomal mosaicism', 'human', 'embryo', 'blastocyst', 'implantation', 'next generation sequencing' and 'clinical management' in combination with other keywords related to the subject area. Relevant articles in the English language, published until October 2019 were critically discussed.

OUTCOMES: Chromosomal mosaicism predominately results from errors in mitosis following fertilization. Although it appears to be less pervasive at later developmental stages, establishing the true prevalence of mosaicism in human blastocysts remains exceedingly challenging. In a clinical context, blastocyst mosaicism can only be reported based on a single TE biopsy and has been ascribed to 2–13% of embryos tested using NGS. Conversely, data from NGS studies disaggregating whole embryos suggests that mosaicism may be present in up to ~50% of blastocysts. However, differences in testing and reporting strategies, analysis platforms and the number of cells sampled inherently overshadow current data, while added uncertainties emanate from technical artefacts. Moreover, laboratory factors and aspects of *in vitro* culture generate further variability. Outcome data following the transfer of blastocysts diagnosed as mosaic remain limited. Current studies suggest that the transfer of putative mosaic embryos may lead to healthy live births, but also results in significantly reduced ongoing pregnancy rates compared to the transfer of euploid blastocysts. Observations that a subset of mosaic blastocysts has the capacity to develop normally have sparked discussions regarding the ability of embryos to self-correct. However, there is currently no direct evidence to support this assumption. Nevertheless, the exclusion of mosaic blastocysts results in fewer embryos available for transfer, which may inevitably compromise treatment outcomes.

WIDER IMPLICATIONS: Chromosomal mosaicism in human blastocysts remains a perpetual diagnostic and clinical dilemma in the context of PGT-A. This review offers an important scientific resource, informing about the challenges, risks and value of diagnosing mosaicism. Elucidating these uncertainties will ultimately pave the way towards improved clinical and patient management.

Key words: chromosomal mosaicism / preimplantation genetic testing / aneuploidy / blastocyst / next generation sequencing / preimplantation genetic testing for aneuploidies / preimplantation genetic screening

Introduction

Human reproduction is an intrinsically complex phenomenon. Inherently inefficient (Macklon *et al.*, 2002) or perhaps remarkably selective (Semprini and Simoni, 2000), ultimately, even in the most optimal circumstances the highest chance of achieving a pregnancy is estimated to be around 30–40% per ovulatory cycle (van Noord-Zaadstra *et al.*, 1991; Zinaman *et al.*, 1996; Evers, 2002). This inefficiency is reflected in the high incidence of preclinical losses during the first week following conception and remains largely attributed to embryo aneuploidy (Jamieson *et al.*, 1994; Munné *et al.*, 1994; Macklon *et al.*, 2002; Munné, 2006; van den Berg *et al.*, 2012). Human embryos frequently harbour cytogenetic imbalances that show a strong association with clinical phenotypes, including infertility and spontaneous miscarriage (Angell *et al.*, 1983; Fragouli *et al.*, 2013; Rodriguez-Purata *et al.*, 2015). Accordingly, the high prevalence of aneuploidy during preimplantation development also constitutes a prominent factor contributing to failed ART treatment (Munné, 2003; Baltaci et al., 2006). Preimplantation genetic testing for aneuploidies (PGT-A) was thus introduced into clinical ART practices to screen a cohort of embryos for those that are chromosomally normal, with an aim to improve pregnancy outcomes. At present, blastocyst culture followed by trophectoderm (TE) biopsy of 5–10 cells and next generation sequencing (NGS) have become the preferred techniques for embryo testing (Fiorentino *et al.*, 2014; Coll *et al.*, 2018).

While the technology has undoubtedly evolved, the value of PGT-A remains controversial, with many opposing to its routine application without solid data attesting to its clinical utility (Sermon *et al.*, 2016; Gleicher and Orvieto, 2017; Braude, 2018; Macklon *et al.*, 2019). Diagnostic accuracy remains at the forefront of this enduring debate. Due to its greater sensitivity, the implementation of NGS has prompted a rise in diagnoses of uncertain clinical significance. This includes reports of chromosomal mosaicism, suggesting the presence of heterogenous chromosomal content among the sampled embryonic cells (Delhanty *et al.*, 1993, 1997). Although the occurrence of cytogenetically distinct cell lines in human embryos is well recognised (Delhanty *et al.*, 1993, 1997; Munné *et al.*, 1993, 1994; Veiga *et al.*, 1999; Bielanska *et al.*, 2002a; Coonen *et al.*, 2004), the higher frequency of mosaicism reported in TE biopsies raised an unsettling uncertainty surrounding the prevalence of mosaicism in human blastocysts, and ultimately, its biological significance. Given that PGT-A relies on the chromosomal constitution of the biopsied cells being representative of the entire embryo, the presence of mosaicism may potentially lead to incorrect classification, undermining the principal strategy of improved embryo selection. While the prevalence and degree of chromosomal heterogeneity in human blastocysts remains elusive, the lack of standardisation in reporting and interpreting mosaic diagnoses among ART centres confounds clinical management. These challenges continue to substantiate reservations regarding the clinical predictive value of diagnosing chromosomal mosaicism by PGT-A.

Here, we examine existing scientific evidence regarding the incidence and impact of chromosomal mosaicism in human blastocysts. To comprehensively assess the implications of this phenomenon for PGT-A, we evaluate insights from a conceptual, technical, biological and clinical perspective. Examining the limitations of diagnosing mosaicism will be critical for reliably defining more specific criteria for a clinical diagnosis. Likewise, increasing knowledge surrounding clinical outcomes following the transfer of embryos classified as mosaic will ultimately contribute to a clearer consensus regarding the clinical management of mosaicism. Overall, we aim to facilitate improved patient counselling by elucidating the risks, challenges and value of diagnosing mosaicism. Uncovering these uncertainties may pave the way towards improved therapeutic strategies in the future.

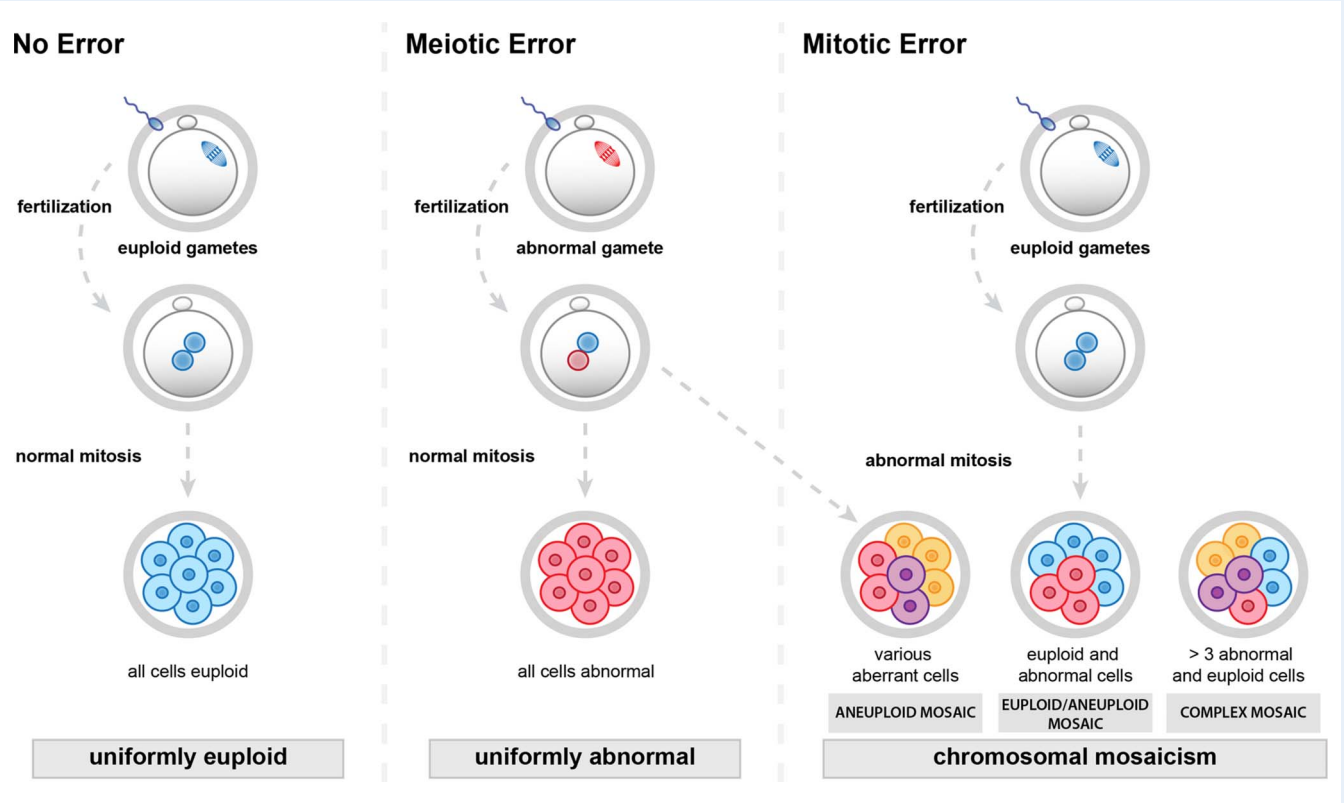


Figure 1 Classification of human preimplantation embryos based on their chromosomal status. In the left panel, the embryo is uniformly euploid, as no errors have occurred during meiosis or mitosis. The middle panel depicts a uniformly aneuploid embryo. Here, all blastomeres contain an identical abnormality, resulting from a meiotic error. In the right panel, a mitotic error leads to chromosomal mosaicism. The embryo may harbour both euploid and abnormal cells (euploid/aneuploid mosaic) or may comprise several clones with varying aberrant chromosomal constitutions (aneuploid mosaic). Complex mosaics consist of three or more abnormal cell lines in combination with euploid cells.

Defining chromosomal mosaicism in the context of early human development

The aetiology of chromosomal abnormalities in human preimplantation embryos is multifaceted. Errors may be derived from the oocyte, sperm or during the mitotic divisions underlying embryogenesis (Delhanty *et al.*, 1993; Munné *et al.*, 1994; Hassold *et al.*, 1996; Bielanska *et al.*, 2005). While meiotically derived abnormalities generally affect all embryonic cells uniformly, post-zygotic errors lead to mosaicism, the presence of chromosomally distinct cells within a single embryo (Delhanty *et al.*, 1993, 1997) (Fig. 1). Mosaic embryos may harbour both euploid and aneuploid cells (euploid/aneuploid mosaics) or be entirely composed of abnormal cell populations (aneuploid mosaics) (Fig. 1). Ploidy mosaics, comprising any combination of haploid, diploid and polyploid cells, have also been observed at both the cleavage and blastocyst stages of development (Munné *et al.*, 1994; Bielanska *et al.*, 2002b; van Echten-Arends *et al.*, 2011). Notably, some polyploid variations constitute a normal feature of trophoblast differentiation during implantation (Evsikov and Verlinsky, 1998; de Boer *et al.*, 2004).

Although frequently described during human preimplantation development (Delhanty *et al.*, 1993, 1997; Voullaire *et al.*, 2000; Wells and Delhanty, 2000; Munné *et al.*, 2002; Coonen *et al.*, 2004; Fragouli *et al.*, 2008, 2011a, 2011b; Vanneste *et al.*, 2009; Voet *et al.*, 2011; van Echten-Arends *et al.*, 2011; Mertzanidou *et al.*, 2013b), mosaicism is thought to be less pervasive as development progresses. It has been diagnosed in <2% of prenatal samples, with true fetal mosaicism identified in only ~0.4% of cases and has been estimated in <0.2% of live births (Hansteen *et al.*, 1982; MRC Working Party on the evaluation of chorion villus sampling, 1991; Smidt-Jensen *et al.*, 1993; Huang *et al.*, 2009; Malvestiti *et al.*, 2015). Yet it is possible that the true prevalence of low-grade chromosomal heterogeneity in clinical pregnancies and the general population remains underestimated (Woods *et al.*, 1994; Rohlin *et al.*, 2009; Robberecht *et al.*, 2010; Cai *et al.*, 2014; Campbell *et al.*, 2014; Jamuar *et al.*, 2014; Gajecka, 2015).

Cellular events leading to embryo mosaicism

To date, several studies have provided critical insights into the series of events that lead to embryo mosaicism (Coonen *et al.*, 2004; Daphnis *et al.*, 2005; Vanneste *et al.*, 2009; Fragouli *et al.*, 2013; Mertzanidou *et al.*, 2013a; further reviewed in Voet *et al.*, 2011; Taylor *et al.*, 2014; McCoy, 2017). During mitosis, nondisjunction leads to complementary chromosomal abnormalities (reciprocal gains and losses) in independent blastomeres of the same embryo. Conversely, the detection of monosomies without reciprocal trisomies points to anaphase lagging. These findings have been confirmed on single cells obtained from embryos at varying developmental stages using both fluorescent *in*

situ hybridisation (FISH) and comprehensive chromosomal screening (CCS) (Voullaire *et al.*, 2000; Wells and Delhanty, 2000; Coonen *et al.*, 2004; Vanneste *et al.*, 2009; Ioannou *et al.*, 2012; Mertzanidou *et al.*, 2013a,b). Coonen *et al.* (2004) analysed cytogenetic patterns in single cells obtained from 299 blastocysts using three-chromosome FISH. The higher prevalence of monosomic over trisomic cells in mosaic blastocysts suggested anaphase lagging as the prevailing mechanism leading to mosaicism. While the entire chromosome complement was not accounted for, similar findings were confirmed using 24-chromosome FISH in a later study (Ioannou *et al.*, 2012). However, the possibility of technical artefacts resulting from hybridisation failure and overlapping signals cannot be ruled out. Mitotic non-disjunction and anaphase lagging have also been described as a common occurrence in human cleavage stage embryos and morulas (Vanneste *et al.*, 2009; Mertzanidou *et al.*, 2013a,b). Nevertheless, the risk of false-positives as a consequence of whole genome amplification (WGA) artefacts, particularly when analysing single cells, warrants careful interpretation (Capalbo *et al.*, 2015; Deleye *et al.*, 2015a).

Chromosomal breakages, leading to structural abnormalities have also been associated with mosaicism (Voullaire *et al.*, 2000; Wells and Delhanty, 2000; Daphnis *et al.*, 2005). Recently, Babariya *et al.* (2017) examined the prevalence of structural aberrations in 1327 blastocysts using array comparative genomic hybridisation (aCGH). Interestingly, segmental aneuploidies occurred more frequently in blastocysts (16%) than in oocytes (10%), with chromosomes harbouring fragile sites more susceptible to breakages (Babariya *et al.*, 2017). Nevertheless, the impact of structural abnormalities on ART outcomes is not entirely understood. Duplications and deletions have been detected in ∼6% of established pregnancies that miscarry (Martínez *et al.*, 2010), while those compatible with live birth result in a range of clinical phenotypes (Theisen and Shaffer, 2010).

Chromosomal mosaicism may also originate from trisomic or monosomic rescue, involving a mitotic error that rescues euploidy in an aneuploid cell population (resulting in a euploid/aneuploid karyotype). If both homologs are derived from a single parent, this event may lead to uniparental disomy (UPD), associated with rare imprinting disorders (Engel, 1980; Kotzot, 2004; Daughtry and Chavez, 2016; McCoy, 2017). Nevertheless, UPD has only been reported in a very small proportion of preimplantation embryos (∼1%) (Northrop *et al.*, 2010; McCoy *et al.*, 2015).

It has been long proposed that relaxed cell cycle control and the complete absence of the spindle assembly checkpoint (SAC) may lead to the high rate of mitotic errors observed in human embryos (Harper *et al.*, 1995; van Echten-Arends *et al.*, 2011; Mantikou *et al.*, 2012). Jacobs *et al.* (2017), however, recently demonstrated that the SAC may in fact be functional. Embryos treated with the mitotic inhibitor nocodazole showed transient mitotic arrest, however, did not undergo apoptosis within the first 5 days of development, leading to aneuploidy (Jacobs *et al.*, 2017). It has been suggested that SAC function may become increasingly robust as development progresses, as cells become smaller (Kyogoku and Kitajima, 2017). Indeed, a recent study in *Caenorhabditis elegans* embryos demonstrated a cell size dependant increase in SAC activity (Galli and Morgan, 2016). The permissiveness of cell cycle checkpoints may thus allow for aneuploid cells to proceed through mitosis.

Understanding the aetiology of mitotic errors during human development remains incredibly complex. Due to the limited availability of human embryos for research, current findings remain largely descriptive. Future gene expression analysis, interventional experiments and monitoring of preimplantation events in real time may shed light on the many events leading to chromosomal segregation errors during mitosis (Vázquez-Diez and FitzHarris, 2018).

The prevalence of chromosomal mosaicism in human blastocysts

Data harmonisation: current challenges and future considerations

Establishing the true prevalence of chromosomal mosaicism at the blastocyst stage of development remains exceptionally challenging. As current reports reveal little consensus in evaluation approaches, conceptual, biological and technical variables all overshadow current findings, leading to substantial discrepancies in the estimates reported. Therefore, careful interpretation remains imperative while prevalence rates of blastocyst mosaicism cannot be declared without caution.

Principally, uncertainties stem from the specification of two different frequencies (McCoy, 2017). Firstly, the frequency of mosaic blastocysts within a cohort of embryos, which we herein refer to as the prevalence of mosaicism; and secondly, the frequency of mosaicism within a single blastocyst, denoting the proportion of abnormal cells, which we identify as the degree of mosaicism. Inferring both frequencies is largely influenced by the technological approach used to evaluate mosaicism.

In a clinical context, blastocyst mosaicism can inherently only be reported based on a single TE biopsy. However, due to sampling errors, these clinical rates may ultimately be an underestimate. To date, several studies have indicated that the distribution of abnormal cells is not always uniform within the TE of a mosaic blastocyst (Chuang *et al.*, 2018; Popovic *et al.*, 2018; Victor *et al.*, 2019a). Accordingly, estimating the precise degree and prevalence of mosaicism based on a single biopsy remains conceptually unachievable. Ultimately, a blastocyst diagnosed with chromosomal mosaicism following PGT-A can only truly be considered at risk of being mosaic.

Furthermore, the lack of standardisation in reporting putative mosaicism creates the potential for imprecise quantitative and qualitative comparisons. Fundamentally, reporting the prevalence of mosaicism on a per embryo basis may, in itself, introduce a selection bias. In a clinical context, reports of mosaicism predominately denote embryos diagnosed with single mosaic abnormalities and are therefore limited to blastocysts presumed to be euploid/aneuploid mosaic. Conversely, if aneuploid mosaics are considered, the overall prevalence of mosaicism, as reported per embryo, will inevitably be confounded by the maternal age of the patient cohort, reflecting the incidence of meiotic aberrations within the testing data set. Ultimately, the incidence of euploid/aneuploid mosaics decreases with advancing maternal age, coinciding with the reduced prevalence of euploid embryos, while aneuploid mosaics are diagnosed more frequently (Munné *et al.*, 2016; Munné and Wells, 2017). To obtain a more representative biological evaluation based on clinical data, it may thus be more appropriate to report the prevalence of all events pertaining to mosaicism, independent from the presence of additional uniform aberrations. Importantly, all chromosomes should be considered. For a more comprehensive overview, putative mosaic abnormalities may

also be reported per individual chromosome. This approach will allow further correlations with prenatal samples based on age and specific chromosomes involved, ultimately providing greater insights into the biological mechanisms underlying mosaicism.

Based on NGS analysis of a single TE biopsy, recent prevalence estimates of mosaicism range from 2% to 13% per blastocyst (Ruttanajit *et al.*, 2016; Katz-Jaffe *et al.*, 2017; Stankewicz *et al.*, 2017). In a large multicentre study of 16 352 TE biopsies, Katz-Jaffe *et al.* (2017) reported diagnoses consistent with mosaicism in 3% of embryos. This was comparable to the prevalence of mosaic calls deemed clinically relevant (2%), including embryos diagnosed with mosaic abnormalities only, and excluding putative mosaic monosomies or trisomies affecting chromosomes 13, 16, 18, 21, X and/or Y (Stankewicz *et al.*, 2017). Taking into account all chromosomes, the prevalence of chromosomal mosaicism reported increased to 5% (Stankewicz *et al.*, 2017). Ruttanajit *et al.* (2016) reported euploid/aneuploid mosaicism across all chromosomes in 9% of blastocysts analysed following TE biopsy and NGS. Yet, when extended to embryos presenting with additional uniform aneuploidies the prevalence increased to 13% (Ruttanajit *et al.*, 2016). Similarly, a recent multicentre randomised control trial (RCT) evaluating NGS-based PGT-A reported mosaicism in 17% of the blastocysts analysed (Munné *et al.*, 2019a). This included embryos diagnosed with both mosaic and uniform aberrations, which constituted 11% of the total number of blastocysts reported as mosaic. Nevertheless, rates varied among laboratories, ranging from ∼10% to ∼26%. Notably, the criteria for identifying copy number deviations consistent with mosaicism differed during the trial (Munné *et al.*, 2019a). When considering individual chromosomes, putative mosaic abnormalities have been reported across all autosomes and sex chromosomes (Greco *et al.*, 2015; Fragouli *et al.*, 2017; Spinella *et al.*, 2018; Munné *et al.*, 2019a,b). Interestingly, preliminary NGS data suggest that specific chromosomes, including chromosomes 22, 4 and 19, may be more frequently associated with diagnoses of mosaicism (Osman *et al.*, 2019). Nevertheless, further studies and more comprehensive data sets will be necessary to confirm these findings.

Evaluating mosaicism in a research setting

At present, the most suitable approach to investigate the prevalence and degree of mosaicism involves the disaggregation of whole blastocysts. Earlier studies applied FISH to evaluate single embryonic cells, while more recently, blastocyst portions encompassing the inner cell mass (ICM) and TE have been compared using CCS. Although such reports provide more representative insights into blastocyst mosaicism, the lack of standardisation in analytical approaches inevitably confounds valid comparisons. Differences in testing strategies, analysis platforms and the number of cells sampled inherently impact current data, while added uncertainties emanate from technical artefacts. These factors inevitably affect both the prevalence and the degree of chromosomal mosaicism reported. In addition, abnormal PGT-A embryos are often over-represented, which may not truthfully reflect rates of mosaicism in all blastocysts and across all patient demographics.

In a research setting, a key consideration relates to the stringency of criteria used to categorise a blastocyst as chromosomally mosaic. While some studies considered embryos with very low numbers of abnormal cells mosaic, others employed higher thresholds. Yet, in some instances, specific cut-offs were not clearly defined. Ultimately, the most reliable level of evidence for genuine mosaicism involves a reciprocal aberration (Capalbo *et al.*, 2017c; Munné and Wells, 2017). The detection of a monosomy in one population of cells and a reverse trisomy in another strongly suggests a mitotic non-disjunction event, minimizing the risk of false-positive calls of mosaicism due to amplification artefacts. However, from a biological viewpoint, such events may be less common in blastocysts, as clonal expansion may lead to one abnormal cell line being more prominent at later stages of development (Munné and Wells, 2017).

Blastocysts containing multiple cell populations with identical abnormalities may also be more credibly classified as mosaic. However, the number of cells analysed will inevitably influence such findings, as detecting low levels of aneuploid cells within a sample containing a considerable number of euploid cells may not be possible. Moreover, depending on the sensitivity of the analysis platform, the presence of reciprocal aneuploidies at equal ratios may mask the presence of mosaicism (Capalbo and Rienzi, 2017; Treff and Franasiak, 2017). Finally, technical challenges continue to confound both FISH and CCS studies and may potentially contribute to an overestimation of the prevalence and degree of blastocyst mosaicism. As such, the size of the putative mosaic aberration (Popovic *et al.*, 2018) and the chromosome(s) affected should also be taken into consideration. Certain abnormalities are more likely to compromise viability prior to the blastocyst stage of development if they were to be present in all embryonic cells (Rodriguez-Purata *et al.*, 2015). Moreover, amplification bias involving smaller chromosomal regions is more probable than under- or over-representation of whole chromosomes (Deleye *et al.*, 2015a).

FISH studies

The first studies to directly evaluate mosaicism in blastocysts were based on FISH (Evsikov and Verlinsky, 1998; Magli *et al.*, 2000; Ruangvutilert *et al.*, 2000a; Sandalinas *et al.*, 2001; Bielanska *et al.*, 2002a, 2005; Derhaag *et al.*, 2003; Hardarson *et al.*, 2003; Coonen *et al.*, 2004; Daphnis *et al.*, 2005). Although limited to a subset of chromosomes, these evaluations are particularly valuable as they involve the analysis of single embryonic cells from a substantial number of whole blastocysts. In some cases, the ICM (Evsikov and Verlinsky, 1998; Magli *et al.*, 2000) or both ICM and TE lineages (Derhaag *et al.*, 2003) were specifically evaluated. In these instances emphasis was placed on the degree of mosaicism within diploid/aneuploid mosaics, which was shown to be considerably reduced (∼10% to 30%) compared to cleavage stage embryos (Evsikov and Verlinsky, 1998; Magli *et al.*, 2000; Derhaag *et al.*, 2003; van Echten-Arends *et al.*, 2011). Importantly, these studies demonstrated the lack of preferential allocation of euploid cells to the ICM.

FISH analysis of whole blastocysts provided more direct data (Ruangvutilert *et al.*, 2000a; Sandalinas *et al.*, 2001; Bielanska *et al.*, 2002a, 2005; Hardarson *et al.*, 2003; Coonen *et al.*, 2004; Daphnis *et al.*, 2005). Nevertheless, the rates reported in close to 600 blastocysts varied, ranging from ∼50% to over 90%. Discrepancies largely stem from variations in the chromosomal classification of embryos (van Echten-Arends *et al.*, 2011), particularly in regard to the type and degree of mosaicism. For instance, in the studies of

Ruangvutilert et al. (2000a), Bielanska et al. (2002a), Hardarson et al. (2003) and Daphnis et al. (2005) blastocysts containing haploid and tetraploid cells were classified as mosaic and comprised over half of all mosaic embryos. Conversely, Sandalinas et al. (2001) classified diploid/tetraploid blastocysts as chromosomally normal, given that they contained <38% tetraploid cells. Similarly, Coonen et al. (2004) considered tetraploid chromosomal constitutions a normal feature of embryo development. In turn, the prevalence of diploid/aneuploid mosaics was more comparable among the studies, ranging from ~15% to 30%. Nevertheless, the threshold of abnormal cells used to categorise a blastocyst as mosaic was not always clearly defined. Ruangvutilert et al. (2000a) and Hardarson et al. (2003) applied a threshold percentage of >10% abnormal cells, while Coonen et al. (2004) classified blastocysts as mosaic if they contained two or more abnormal cells.

Variations in experimental design and the inherent technical limitations of FISH may underlie further discrepancies. While Sandalinas et al. (2001) examined blastocysts that developed from chromosomally abnormal cleavage stage embryos, the remaining studies utilised untested embryos not suitable for transfer or cryopreservation. Moreover, errors arising from probe inefficiency or split signals may account for an overestimation in the reported prevalence of mosaicism (Ruangvutilert et al., 2000b; Velilla et al., 2002; Munné and Wells, 2017). In addition, compared to a TE biopsy, more cells were analysed using FISH on whole blastocysts. Statistically, this will lead to a higher proportion of embryos classified as mosaic.

In a further investigation, Capalbo et al. (2013) used FISH to re-analyse 70 blastocysts classified as clinically unsuitable following aCGH. Notably, the ICM and TE were assessed separately. While concordance among the embryo portions was high, mosaicism was detected in 20% of the re-analysed blastocysts (Table I). Of these, the majority either contained a combination of uniform and mosaic aberrations or varying aneuploid cells. In contrast to the previous studies, diploid/aneuploid mosaicism was determined in only 3% of the embryos analysed (Table II). Notably, Capalbo et al. (2013) classified an embryo as mosaic if >10% of nuclei presented with the same abnormality and if the same aneuploid signal was detected in at least two cells within one blastocyst section. The blastocysts examined, however, were all previously diagnosed as clinically unsuitable, presenting with either unbalanced translocations, or single or double aneuploidies, which may skew data.

Microarray studies

The shift towards CCS failed to provide greater clarity. Fragouli et al. evaluated 64 blastocysts across two reports, using a combination of CGH, aCGH and FISH (Fragouli et al., 2008, 2011a). Here, the prevalence of mosaicism was 33% and was comparable in the two studies. Specifically, the prevalence of euploid/aneuploid mosaics was also similar, close to 17%, while the remaining mosaic blastocysts harboured no euploid cells. In the study of Fragouli et al. (2008), the ICM and TE of an additional 10 blastocysts were evaluated independently using CGH. Here, samples from the same blastocyst were determined to be concordant in all instances (Tables I and II).

Further microarray studies examining different embryo portions generally show a high concordance between the ICM and TE and a low prevalence of mosaicism (Johnson et al., 2010; Northrop et al., 2010) (Table I). Johnson et al. (2010) evaluated a total of 51 ICM samples and 80 matching TE portions, obtained from 51 blastocysts. Overall, only two embryos (4%) were non-concordant between ICM and TE; however, they contained no euploid cells, while all matching TE portions showed consistent results (Tables I and II). Similarly, Northrop et al. (2010) evaluated 50 blastocysts using single-nucleotide polymorphism (SNP) array. These were previously classified as abnormal on Day 3 using FISH. Here, embryos were separated into four portions, including the ICM and three TE samples, and the overall prevalence of mosaicism, including aneuploid mosaics, was determined to be 24% ($n = 12$). Of these, four embryos presented with a normal ICM. One blastocyst contained an abnormal ICM for which all three TE samples were determined to be euploid. Interestingly, the aberration detected in the ICM was also diagnosed at the cleavage stage. Overall, 16% of the blastocysts were euploid/aneuploid mosaics (Table I).

Here, an important consideration is the genetic testing methodology used. Microarray platforms utilise reference sequences that cover larger chromosomal regions and thus provide less sensitivity. Mamas et al. (2012) investigated the detection rate of aCGH on mixtures of euploid and aneuploid cells and determined that it was only possible to detect mosaicism when >50% of the cells were abnormal. Compared to aCGH, NGS has a higher resolution and broader dynamic range for interpretation of copy number values (Fiorentino et al., 2014; Wells et al., 2014; Deleye et al., 2015b; Harton et al., 2017; Lai et al., 2017). Accordingly, several studies have demonstrated the capacity of NGS to detect as low as 20% abnormal cells within a mosaic sample (Maxwell et al., 2016; Fragouli et al., 2017; Munné et al., 2017b; Popovic et al., 2018; Spinella et al., 2018; Tšuiko et al., 2018). As NGS is a more sensitive technique, it will inevitably lead to an increase in reports consistent with mosaicism. In a recent comparative analysis of 49 blastocyst biopsies using aCGH and NGS, Ruttanajit et al. (2016) demonstrated a 12% discordant diagnosis between the two platforms. This was attributed to low and medium levels of putative mosaicism not detected by aCGH.

NGS studies

In a recent effort to evaluate the diagnostic accuracy of a TE biopsy for PGT-A, several studies have assessed mosaicism by comparing the chromosomal constitutions of multiple blastocysts portions using NGS (Orvieto et al., 2016; Huang et al., 2017; Chuang et al., 2018; Popovic et al., 2018; Tšuiko et al., 2018; Fragouli et al., 2019; Lawrenz et al., 2019; Victor et al., 2019a).

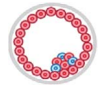
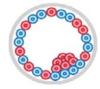
Orvieto et al. (2016) reported on a small series of eight blastocysts, with a previously unknown chromosomal status. Three TE biopsies were examined from each of the embryos, while for four of the blastocysts the ICM was also collected. Of the 28 samples analysed, five presented with considerable background noise, while a further five samples revealed inconclusive results. As two of these involved the embryo portion containing the ICM, comparisons remain limited. If the aforementioned samples are excluded, one embryo showed discordance between the ICM and TE (Tables I and II). In addition, two out of the eight embryos presented with euploid/aneuploid mosaicism within the TE. However, the lack of thorough validation data of the NGS platform used and the small number of embryos analysed warrants careful interpretation.

Table I Comparison of mosaicism rates across human blastocyst disaggregation studies.

Study	Average maternal age (years)	Blastocysts analysed	Ploidy concordance ICM and TE	Concordance ICM and TE*	All mosaic blastocysts (%)	Euploid/aneuploid blastocysts (%)	Genetic testing platform	Blastocyst portions analysed	Original embryo diagnosis
Fragouli *et al.*, 2008	34.6 (age range 24–41)	10	100%	100%	0 (0.0)	0 (0.0)	CGH	2	abnormal cleavage biopsy (FISH)
Capalbo *et al.*, 2013	36.4 (age range 31–42)	70	100%	100%	14 (20.0)	2 (2.9)	FISH	4	abnormal blastocyst biopsy (aCGH)
Johnson *et al.*, 2010	31.0 (age range 22–37)	51	100%	96%	2 (3.9)	0 (0.0)	SNP array	2	unknown chromosomal status
Northrop *et al.*, 2010	35.1 (age range not specified)	50	90%	76%	12 (24.0)	8 (16.0)	SNP array	4	abnormal cleavage biopsy (FISH)
Orvieto *et al.*, 2016	33.1 (age range 26–41)	2	50%	50%	1 (50.0)	1 (50.0)	NGS	2	unknown chromosomal status
Huang *et al.*, 2017	not specified (age range 24 to 44)	51	98%	84%	8 (15.7)	1 (2.0)	NGS	4	abnormal blastocyst biopsy (aCGH)
Popovic *et al.*, 2018	32.2 (age range 23–39)	34	65%	56%	15 (44.1)	11 (32.4)	NGS	4	unknown chromosomal status
	32.6 (age range 23–40)	24	67%	33%	16 (66.6)	7 (29.2)	NGS	4	abnormal or mosaic blastocyst biopsy (NGS)
Chuang *et al.*, 2018	34.4 (age range 26–43)	29	79%	55%	13 (44.8)	6 (20.7)	NGS	4	unknown chromosomal status
Tšuiko *et al.*, 2018	33.8 (age range 23–42)	14	93%	86%	4 (28.6)	3 (21.4)	NGS	2	unknown chromosomal status
Lawrenz *et al.*, 2019	33.9 (age range 24–46)	84	93%	86%	12 (14.3)	6 (7.1)	NGS	2	unknown chromosomal status
Victor *et al.*, 2019a	36.5 (age range not specified)	100	93%	79%	21 (21.0)	7 (7.0)	NGS	2	abnormal blastocyst biopsy (NGS)

*As multiple TE samples were analysed, concordance refers to the complete concordance between the ICM and all TE samples analysed.
Percentages (%) are of the total number of blastocysts analysed.
FISH: fluorescent *in situ* hybridisation, aCGH: array comparative genomic hybridisation, NGS: next generation sequencing, SNP: single-nucleotide polymorphism.

Table II Concordance between ICM and TE as reported across human blastocyst disaggregation studies.

	 ICM: normal TE: normal	 ICM: abnormal TE: abnormal	False Positive  ICM: normal TE: abnormal	 ICM: normal TE: mosaic	False Negative  ICM: abnormal TE: normal	 ICM: mosaic TE: normal	 ICM: mosaic TE: abnormal	 ICM: abnormal TE: mosaic	 ICM: mosaic TE: mosaic	Total
Fragouli *et al.*, 2008	4	6								**10**
Capalbo *et al.*, 2013	0	68							2	**70**
Johnson *et al.*, 2010	41	10								**51**
Northrop *et al.*, 2010	29	13		4	1			3		**50**
Orvieto *et al.*, 2016	0	1			1					**2**
Huang *et al.*, 2017	0	50			1					**51**
Popovic *et al.*, 2018	14	9		7		1		1	2	**34**
	0	17		6					1	**24**
Chuang *et al.*, 2018	8	15		1		1		2	2	**29**
Tšuiko *et al.*, 2018	8	3		1					2	**14**
Lawrenz *et al.*, 2019	41	37	3		3					**84**
Victor *et al.*, 2019a	0	93		5			2			**100**

Huang *et al.* (2017) showed a high overall concordance between the ICM and TE (98%). Here, 51 blastocysts previously diagnosed as abnormal by aCGH were re-biopsied in four regions. Samples included the ICM and three TE biopsies, which were then sequenced. Findings revealed that 50 out of 51 ICM samples matched at least one of the TE biopsies from the same blastocyst. Yet, consistent results across all four biopsies were observed in 43 embryos (84%), with an overall mosaicism prevalence rate of 16% (Table I). One blastocyst presented with an aneuploid ICM, while all three TE samples were diagnosed as euploid (Table II). The remaining embryos were all aneuploid mosaics. Notably, Huang *et al.* (2017) utilised multiple annealing and looping-based amplification cycles (MALBAC) WGA, as part of their NGS protocol. While their approach was validated, it differs to that of standard PGT sequencing platforms (Deleye *et al.*, 2015a). Lawrenz *et al.* (2019) also reported a high overall concordance between the ICM and TE in their analysis of 84 blastocysts (Table I). Here, three blastocysts had a euploid ICM and abnormal TE, while a further three blastocysts had an abnormal ICM and normal TE (Table II). Discordance between the ICM and TE largely stemmed from the detection of structural abnormalities in either of the lineages. For one embryo, a monosomy was detected in the ICM but not in the TE, while a second blastocysts presented with several trisomies in the TE, while the ICM was euploid.

We analysed corresponding chromosomal profiles of the ICM and three TE portions obtained from 58 blastocysts (Popovic *et al.*, 2018). Of these, 34 were previously untested, while the remaining embryos were classified as either abnormal or mosaic following PGT-A ($n = 24$). Mosaicism was reported in up to 38% of blastocysts analysed, including both whole chromosome and segmental aberrations. Within the previously untested group, 11 blastocysts were classified as euploid/aneuploid mosaic, seven of which contained a euploid ICM (Tables I and II). Moreover, one embryo presented with several mosaic whole chromosome aberrations within the ICM, while all three TE samples were euploid (Table II). Finally, six blastocysts originally diagnosed as mosaic presented with a euploid ICM (Tables I and II).

In a further study, Chuang *et al.* (2018) examined serial biopsies obtained from 29 blastocysts. Samples included two biopsies of the TE and one of the ICM. Complete concordance among all three samples was observed in 16 embryos (55%), while the chromosomal status of the ICM matched that of the TE, in 79% of the cases. Over half of the discrepant results were attributed to aneuploid mosaics, and six blastocysts (21%) were reported as euploid/aneuploid (Table I). Moreover, one embryo had a euploid ICM and a mosaic TE, with a reciprocal monosomy and trisomy detected in the two portions (Table II). Similarly, Tšuiko *et al.* (2018) analysed the ICM and TE of 14 blastocysts and determined concordance in 86% of cases. Mosaicism was diagnosed in four blastocysts (29%), of which one presented with a euploid ICM, while several mosaic numerical aberrations were diagnosed in the TE. Two further embryos presented with concordant mosaic aberrations within the TE and ICM and one embryo was mosaic aneuploid.

Contrary to Huang *et al.* (2017) and Lawrenz *et al.* (2019) who only report on uniform aberrations, Chuang *et al.* (2018), Popovic *et al.* (2018) and Tšuiko *et al.* (2018) also diagnosed mosaicism within a single blastocyst sample. This approach inevitably leads to a higher chance of discordance between the ICM and multiple TE portions, and thus a higher rate of mosaicism reported. While the risk of overestimating mosaicism due to technical artefacts cannot be ruled out, the overall prevalence of mosaicism may be more representative, as differences in cell populations within the embryo samples are also considered. Nevertheless, the number of cells biopsied will also affect the rates reported. Lawrenz *et al.* (2019) and Tšuiko *et al.* (2018) analysed two embryo portions and as such the overall rate of false-positive diagnoses will inevitably be lower (Tables II and III). Huang *et al.* (2017) also reported a low rate of false-positives (Tables II and III). Although multiple portions of the embryos were compared in this study, only aneuploid blastocysts were evaluated. Victor *et al.* (2019a) also showed a high concordance among samples obtained from uniformly abnormal embryos (Table I). In this study, five blastocysts presented with a euploid ICM, while a further two embryos appeared to harbour some aneuploid cells within their ICM (Table II). The false-positive rate in Popovic *et al.* (2018) is comparable to that of Fragouli *et al.* (2019), who recently reported on results obtained following the re-analysis of several embryo portions obtained from 65 blastocysts (Table III). These embryos were previously diagnosed as uniformly abnormal or mosaic. While a high concordance was observed for uniform abnormalities, over half of the blastocysts originally diagnosed as mosaic harboured a euploid ICM (also reported in Garrisi *et al.*, 2016; Munné and Wells, 2017) (Table III). This suggests that misidentification of embryos due to false-positive errors is largely associated with the diagnosis of mosaicism.

Evaluating mosaicism: future considerations

As suggested by Capalbo *et al.* (2017b), the optimal approach to investigate mosaicism in human blastocysts should involve the analysis of all individual embryonic cells using a CCS platform with a well-defined error rate. This method would also allow for a more credible assessment of the mechanisms underlying mosaicism. At present, micromanipulation approaches facilitate reliable isolation of the ICM from human blastocysts (Capalbo *et al.*, 2013; Warrier *et al.*, 2018). However, chromosomal studies that further segregate the ICM and TE into individual cells remain remarkably scarce. In a small proof of principle investigation, Taylor *et al.* (2016) used a holding pipette to dissociate sections of the blastocyst into individual cells, which were analysed by aCGH. However, total cell recovery was limited, with only 18 cells obtained from one blastocyst. Single-cell studies profiling the transcriptome and epigenome of human blastocysts have revealed similar challenges in isolating viable cells (Yan *et al.*, 2013; Blakeley *et al.*, 2015; Petropoulos *et al.*, 2016; Zhu *et al.*, 2018). Therefore, the requirement for manual collection and difficulties in cell separation continue to limit efficiency and throughput. Moreover, the cost associated with downstream high-resolution single-cell sequencing considerably limits well-designed, high-powered studies, while the technical and analytical hurdles associated with single-cell genomics persist (Macaulay and Voet, 2014; Capalbo *et al.*, 2017b). Nevertheless, technologies allowing automated cell capture and nanofluidic approaches have already provided substantial advancements in acquiring higher quality single-cell data (Macosko *et al.*, 2015; Zheng *et al.*, 2017; Romagnoli *et al.*, 2018). Further improvements will certainly broaden research strategies and ultimately hold great promise for more precise analysis of chromosomal mosaicism in human embryos.

Technical and bioinformatic advancements are now also allowing genome-wide haplotyping combined with copy number analysis, using platforms such as karyomapping (Handyside *et al.*, 2010), haplarithmisis (Zamani Esteki *et al.*, 2015) and OnePGT Solution

Table III Overview of false-positive and false-negative rates reported in human NGS studies to date.

Study	False-positive rate, %	False-negative rate, %	Blastocyst portions analysed	Original embryo diagnosis
Orvieto et al., 2016	0.0	50.0	2	unknown chromosomal status
Huang et al., 2017	0.0	2.0	4	abnormal blastocyst biopsy (aCGH)
Popovic et al., 2018	20.6	2.9	4	unknown chromosomal status
	25.0	0.0	4–5	abnormal or mosaic blastocyst biopsy (NGS)
Chuang et al., 2018	3.4	3.4	4	unknown chromosomal status
Tšuiko et al., 2018	7.1	0.0	2	unknown chromosomal status
Lawrenz et al., 2019	3.6	3.6	2	unknown chromosomal status
Victor et al., 2019a	5.0	0.0	2	abnormal blastocyst biopsy (NGS)
Fragouli et al., 2019	27.7	not specified	4–5	abnormal or mosaic blastocyst biopsy (NGS)

Rates are based on concordance between TE and ICM.

Technology (Masset et al., 2018). These approaches allow for the mechanistic origin of trisomies to be deduced based on chromosome recombination patterns (Vermeesch et al., 2016). This may provide an important advantage over current NGS approaches for the diagnosis of chromosomal mosaicism and its clinical interpretation. Moreover, unlike NGS that relies solely on copy number to evaluate chromosomal content, genome-wide haplotyping enables the detection of triploidy, parthenogenetic activation and uniparental heterodisomy (Vermeesch et al., 2016). Similarly, combined ploidy and copy number analysis using targeted NGS approaches (Capalbo et al., 2017a; Marin et al., 2018) may provide greater insights into the prevalence of ploidy mosaics at the blastocyst stage of development. Nevertheless, a clear distinction between ploidy types should be made to avoid the risk of over-reporting mosaic variations that represent a normal feature of development.

The value of diagnosing chromosomal mosaicism by PGT-A

In the context of PGT-A, uniform whole chromosome abnormalities can be detected with sufficient accuracy, as the TE biopsy is likely to reflect the chromosomal constitution of the embryo (Capalbo and Rienzi, 2017; Chuang et al., 2018; Popovic et al., 2018; Fragouli et al., 2019). Moreover, such diagnoses are largely predictive of clinical outcomes (Schoolcraft et al., 2010, 2011; Scott et al., 2012; Dahdouh et al., 2015). The most compelling evidence was provided by the blinded, non-selection study performed by Scott et al. (2012), in which blastocysts were biopsied and transferred in the absence of genetic testing. Once clinical outcomes were known, SNP array was used to evaluate the clinical predictive value of a TE biopsy. Overall, 96% of embryos determined to be aneuploid did not implant, while 41% of the euploid embryos led to an ongoing pregnancy or live birth (Scott et al., 2012). Similarly, no viable pregnancies were obtained following the transfer of blastocysts ($n = 10$) diagnosed with uniform monosomies or a combination of uniform monosomies and trisomies using aCGH (Munné et al., 2019b). Furthermore, uniform numerical aneuploidies diagnosed in the original blastocyst biopsy were concordant at both Days 8 and 12 of development, leading to poorer developmental outcomes compared to euploid embryos during extended *in vitro* culture (Popovic et al., 2019). Collectively, these data suggest that if a meiotic error is present, PGT-A is very likely to provide an accurate diagnosis.

In contrast, diagnosing mosaicism remains remarkably complex, particularly when considering euploid/aneuploid mosaics. In such instances, distinguishing genuine mosaicism may not always be possible, while the developmental potential of euploid/aneuploid blastocysts remains unclear. While a conservative approach would be to classify all mosaic embryos as clinically unsuitable, a very realistic consideration is that no euploid blastocysts are available for transfer. This risk increases substantially with maternal age (Franasiak et al., 2014; Ubaldi et al., 2017). Both Popovic et al. (2018) and Fragouli et al. (2019) suggest that misidentification due to false-positive errors is largely associated with the diagnosis of mosaicism. Therefore, classifying mosaic blastocysts as clinically unsuitable may ultimately reduce the probability of pregnancy for the patient. This presents a legitimate concern particularly if mosaicism is not reported, that is, if threshold values for classifying an embryo as abnormal are set too low (e.g. 30% abnormal cells). Friedenthal et al. (2018) recently demonstrated improved ongoing pregnancy rates following NGS-based PGT-A compared to aCGH. However, patients undergoing PGT-A with NGS had significantly fewer embryos available for transfer, due to the exclusion of putative mosaic embryos.

To preclude the inadvertent classification of viable embryos as clinically unsuitable, embryos classified as mosaic are currently emerging as a third diagnostic category in PGT-A (Munné and Wells, 2017). Mosaic blastocysts are inevitably being transferred during routine IVF cycles. Importantly, there is currently no evidence of an increased risk of chromosomal mosaicism in children born following ART. Therefore, if a proportion of embryos diagnosed as mosaic can achieve viable pregnancies, treatment outcomes will be compromised by their exclusion. Yet the transfer of mosaic blastocysts requires careful consideration, and currently a clear, standardised framework for clinical care is lacking. While diagnosing chromosomal mosaicism may lead to fewer normal embryos being discarded,

abnormal blastocysts may also be inadvertently transferred under its premise. These will invariably lead to implantation failure or miscarriage.

In contrast, misidentification of embryos due to false-negative errors remains very low (<4%) and consistent across investigations comparing ICM and TE portions (Table III). This confirms previous findings suggesting that a TE biopsy is sufficient for accurately classifying embryos as euploid (Schoolcraft *et al.*, 2010, 2011; Scott *et al.*, 2012; Dahdouh *et al.*, 2015). When considering clinical PGT-A outcomes to date, false-negative diagnoses are indeed exceptionally rare. Embryos with an abnormal or mosaic ICM and euploid TE may potentially have an impaired developmental capacity compared to those with a euploid ICM.

The accuracy of diagnosing mosaicism

A major challenge in performing PGT-A using CCS is the low DNA input available for analysis. A single cell contains ~7 picograms of genomic DNA, which is insufficient for most genetic tests presently available. Consequently, CCS-based PGT-A has fundamentally relied on WGA, allowing vast amounts of DNA (>2 µg) to be generated from single cells (Zhang *et al.*, 1992; Handyside *et al.*, 2004). However, WGA methods inherently lead to amplification bias, namely under- or over-representation of the genome at specific loci (Capalbo *et al.*, 2017c). As such, the representation of the original genome may in some instances be incorrect. The type and extent of the bias vary with each WGA method and also depend on aspects of the DNA sample itself (Sabina and Leamon, 2015). This becomes particularly relevant when diagnosing mosaic structural aberrations. Remarkably, Victor *et al.* (2019a) demonstrated that 43% of embryos originally diagnosed with a structural abnormality presented with a normal ICM. Accordingly, our intra-embryo comparison revealed that a high proportion of mosaic structural variants diagnosed in the original TE biopsy could not be confirmed in the re-biopsied portions of the blastocyst (Popovic *et al.*, 2018). Distinguishing true mosaicism from possible technical artefacts in such instances remains particularly challenging. Furthermore, biological variability including DNA degradation, S-phase artefacts, as well as the loss or gain of chromosomes in triploid embryos, further confounds the accurate diagnosis of mosaicism (Capalbo *et al.*, 2017c). Certainly, methodological drawbacks of sampling mosaicism must be considered when defining more specific diagnostic criteria for PGT-A (Gleicher *et al.*, 2017).

To date, a number of studies have performed extensive validation of both aCGH and NGS for the detection of mosaicism (Greco *et al.*, 2015; Maxwell *et al.*, 2016; Fragouli *et al.*, 2017; Popovic *et al.*, 2018; Spinella *et al.*, 2018). Modelling mosaicism by combining euploid and aneuploid cell lines at different ratios determined that NGS is capable of accurately identifying abnormal cells when present in as low as 20% of a mixed cell sample. Nevertheless, all platform validations to date have been performed on cell line models, which are inherently more stable compared to clinical TE biopsy samples. Extrapolation of cell line derived cut-offs for the diagnosis of mosaicism by PGT-A should thus be considered with caution. Furthermore, differences in WGA protocols, sequencing methods and bioinformatic approaches may all influence thresholds for detection, leading to false-positives. Nevertheless, it is critical to validate each NGS platform individually. Goodrich *et al.* (2017) demonstrated that applying custom analysis criteria significantly increased sensitivity, however at the cost of specificity, leading to a high rate of false-positive calls, of up to 33%. Therefore, the use of insensitive or unvalidated methods may have negative clinical consequences, due to the inappropriate categorisation of embryos.

Predisposition to chromosomal mosaicism in the context of ART

As chromosomal mosaicism originates during the first embryonic cleavages, it is conceivable that in the context of ART, both patient characteristics and various treatment-related factors ultimately predispose embryos to mitotic errors (Munné and Wells, 2017). At present, however, the association between specific parameters and chromosomal mosaicism has not been thoroughly established. A vast challenge in evaluating such data remains the inherent complexity and variability of all factors involved.

Maternal age remains the most powerful contributor to the incidence of meiotic abnormalities (Hassold and Hunt, 2001; Handyside *et al.*, 2012; Nagaoka *et al.*, 2012; Fragouli *et al.*, 2013). However, age-related effects do not appear to impact the occurrence of mitotic errors (Munné *et al.*, 2007). When considering clinical outcomes, however, Victor *et al.* (2019b) revealed that putative mosaic blastocysts originating from younger patients (≤34 years) showed a significantly higher ongoing implantation rate compared to mosaic embryos obtained from older women (>34 years). This is in contrast to the transfer of euploid embryos, which eliminates the impact of advancing female age on implantation rate (Harton *et al.*, 2013). However, the findings of Victor *et al.* (2019b) may potentially be confounded by a technical bias. When considering blastocysts from older patients, TE profiles suggestive of mosaicism may in fact represent false-negative diagnoses of uniform abnormalities. Meiotic aberrations are inherently more prevalent within a cohort of older patients, inevitably leading to reduced ongoing implantation rates. Accordingly, false-positive diagnoses of mosaicism may account for the observed improvement in clinical outcomes within the young patient group. Further clinical outcome data across specific age groups will be necessary to definitively attribute such differences to genuine biological mechanisms.

While the effects of patient parameters remain difficult to delineate, it has been well established that treatment-related factors have a significant impact on the occurrence of chromosomal abnormalities in embryos. Historically, various aspects have been reported, ranging from temperature, levels of oxygen tension and types of stimulation protocol (Almeida and Bolton, 1995; Dumoulin *et al.*, 1995; Baart *et al.*, 2007; Weghofer *et al.*, 2008, 2009; Rubio *et al.*, 2010). Interestingly, an *in vivo* culture system of human embryos based on encapsulation technology revealed a higher proportion of euploid embryos in the *in vivo* cultured cohort compared to *in vitro* controls (Blockeel *et al.*, 2009). Moreover, following FISH-based PGT-A, Munne *et al.* (1997) demonstrated that the prevalence of putative mosaic embryos varied significantly among fertility clinics. While these findings may be difficult to extrapolate to laboratories today, it is reasonable to assume that *in vitro* conditions may introduce differing effects on

the occurrence of chromosomal aberrations in human embryos. Indeed, a large retrospective analysis, including data from 13 282 blastocyst biopsies obtained from 1645 donor cycles, performed across 42 different ART centres established that the rates of euploidy ranged from 40% to 83% between clinics (Munné *et al.*, 2017a). Nevertheless, significant differences were only identified among centres reporting on a limited number of cycles, which may reflect a sampling bias. Furthermore, the logistic regression analysis did not account for biopsy operator, which may confound the reported findings.

Echoing earlier reports, various laboratory factors have been recently suggested to impact the prevalence of mosaicism in human *in vitro* cultured embryos, ranging from culture medium, pH fluctuations and even insemination method (Palmerola *et al.*, 2019; Swain, 2019). A further potential source of error involves the biopsy itself. Excessive use of the laser, mechanical damage to the cells or DNA degradation may cause an artefactual loss or gain of chromosomal regions (Munné and Wells, 2017). However, at present, there is no direct evidence to support this assumption. Preliminary NGS data based on 1492 blastocyst biopsies obtained from donor cycles across 91 different ART centres revealed varying prevalence rates of mosaicism across clinics (Sachdev *et al.*, 2016). Yet other studies show no differences in the reported rate of mosaicism among centres (Katz-Jaffe *et al.*, 2017). However, careful interpretation of the data remains imperative. While differences in prevalence rates of mosaic embryos across ART centres do implicate procedural effects, identifying single causative factors among the extensive number of interdependent variables affecting *in vitro* embryo culture remains an immense challenge. As discussed, differing criteria used to diagnose mosaicism may also affect the prevalence rates reported. Nevertheless, laboratory practices should not be overlooked. Further studies will be important for unravelling and optimising procedural conditions associated with chromosomal instability. As recommended by the Preimplantation Genetic Diagnosis International Society (PGDIS), centres may consider reviewing clinical and laboratory practices if a consistently high prevalence of mosaicism is reported (PGDIS, 2019).

The developmental fate of mosaic embryos

Clinical considerations

Knowledge regarding the developmental capacity of blastocysts diagnosed as mosaic predominately stems from clinical outcome data following PGT-A (Greco *et al.*, 2015; Maxwell *et al.*, 2016; Fragouli *et al.*, 2017; Lledó *et al.*, 2017; Munné *et al.*, 2017b; Spinella *et al.*, 2018; Victor *et al.*, 2019b; Zhang *et al.*, 2019). Although limited, current findings consistently suggest that blastocysts classified as mosaic have the capacity to result in viable pregnancies. Crucially, however, compared to euploid embryos, putative mosaic blastocysts also entail higher rates of miscarriage.

Clinical outcomes were primarily reported following the transfer of 18 embryos classified as mosaic in cases in which no euploid embryos were available (Greco *et al.*, 2015). Ultimately, eight biochemical pregnancies (44% implantation rate) and six live births were achieved (33% live birth rate), demonstrating, for the first time, that putative mosaic embryos may result in healthy live births (Greco *et al.*, 2015). The impact of a mosaic diagnosis on clinical outcomes was further examined by Maxwell *et al.* (2016) who used NGS to reanalyse TE biopsies following miscarriage ($n = 38$) or live birth ($n = 38$). All blastocysts were originally classified as euploid by aCGH. Remarkably, of the embryos that miscarried, 12 blastocysts (32%) were diagnosed as mosaic by NGS. Interestingly, putative mosaicism was also detected in the group of embryos that led to live births, albeit at a significantly lower frequency of 16%. This study, in fact, provided the first evidence that the diagnosis of mosaicism may be associated with early pregnancy loss.

To establish a more definitive assessment of the effects of mosaicism on peri-implantation development, several studies further compared clinical outcomes of embryos categorised as mosaic to those of euploid counterparts across matched patient cohorts (Table IV). In a retrospective analysis, Fragouli *et al.* (2017) reanalysed 150 TE biopsies using NGS. These were originally classified as euploid and were transferred. Overall, their data revealed that blastocysts reported as mosaic following NGS were significantly more likely to result in miscarriage compared to euploid embryos (Table IV). Interestingly, however, the implantation rate of blastocysts diagnosed with mosaic structural aberrations ($n = 14$) was comparable to that of euploid embryos, with no losses observed (8 out of 14, 57% per embryo transferred). The remaining blastocysts, in which mosaicism appeared to affect one or more whole chromosomes ($n = 18$) or that presented with a combination of mosaic whole and segmental aneuploidies ($n = 12$) revealed a lower implantation rate (30%) and a significantly reduced live birth rate (13%), which was even lower (6%) when considering embryos classified as complex mosaic. Munné *et al.* (2017b) revealed comparable clinical outcomes (Table IV). This study in fact included the embryos reported in Fragouli *et al.* (2017), while data from the remaining 99 blastocysts originated from three different centres. Embryos presenting with single and double mosaic aneuploidies as well as mosaic structural aberrations showed comparable ongoing pregnancy rates (50%, 45% and 41%, respectively). Interestingly, no difference was observed in pregnancy rates between embryos diagnosed with a mosaic monosomy or mosaic trisomy, nor did the mosaic chromosome involved have an effect.

Lledó *et al.* (2017) also retrospectively re-analysed aCGH results with known clinical outcomes (Table IV). However, unlike Munné *et al.* (2017b) who re-evaluated samples using NGS, PGT-A data were re-assessed using a more sensitive bioinformatics approach. This allowed for the identification of mosaic embryos according to log2 ratio calls. While biochemical miscarriage rates appeared higher following the transfer of putative mosaic embryos compared to euploid counterparts, no significant differences were observed in implantation or live birth rates between the two transfer groups. These findings were comparable to those of Zhang *et al.* (2019) who employed the same analysis approach (Table IV). However, in the study of Zhang *et al.* (2019), subgroup analysis revealed a significantly lower live birth rate following the transfer of embryos diagnosed with mosaic numerical aneuploidies compared to euploid controls (43.5% compared to 59.1%, respectively). Collectively, however, these results denote the poorer sensitivity of aCGH for evaluating potential mosaicism, which may ultimately affect accurate classification. Mosaic embryos may be incorrectly identified as euploid, particularly

Table IV Overview of studies reporting on clinical outcomes of human mosaic embryos and matched euploid controls

	Fragouli *et al.*, 2017[a]		Munné *et al.*, 2017b[a]		Lledó *et al.*, 2017[b]		Spinella *et al.*, 2018[c]		Victor *et al.*, 2019b[c]		Zhang *et al.*, 2019[b]		Zore *et al.* 2019[a]		Munné *et al.* 2019b[a,c]	
Embryo group	**Mosaic**	**Euploid**	**Mosaic**	**Euploid**	**Mosaic**	**Euploid**	**Mosaic**	**Euploid**	**Mosaic**	**Euploid**	**Mosaic**	**Euploid**	**Mosaic**	**Euploid**	**Mosaic**	**Euploid**
Average maternal age (years)	37.2	37.9	35.8	37.3	31.0	30.6	37.6	37.0	36.4	37.4	31.4	31.3	39.1	38.3	36.6	34.1
Embryos transferred	44	51	143	1045	52	382	78	251	100	478	102	268	20	357	253	1338
Embryos implanted	17	29	76	736	14	142	30	137	37	237	59	181	8	215	125	1107
Implanation rate	38.6%	56.9%	53.1%	70.4%	26.9%	37.2%	38.5%	54.6%	38.0%	49.6%	57.8%	67.5%	40.0%[§]	60.2%[§]	49.4%	82.7%
Live births	12	24	57	661	10	54	24	117	30	225	48	159	6	192	94	1022
Live birth rate	27.3%	47.1%	39.9%[*]	63.3%[*]	19.2%	14.1%	30.8%	46.6%	30.0%[†]	47.1%[*]	46.6%[‡]	59.1%[‡]	30.0%	53.8%	37.2%[*]	76.4%[*]

Implantation rate was defined as the presence of a gestational sac at 6–10-week ultrasound per embryo transferred.
[a]Re-analysis of biopsy samples originally diagnosed as euploid by aCGH using NGS.
[b]Re-analysis of aCGH results according to $\log_2$ copy number values.
[c]Prospective study.
*Extrapolated based on ongoing implantations, as live births not reported.
†Extrapolated based on 19 live births and 11 ongoing pregnancies reported.
‡Includes one monozygotic twin live birth.
Implantation rate defined as presence of fetal heart at ultrasound, as only clinical pregnancies reported.

in cases in which the degree of mosaicism within the TE biopsy is low. This will inevitably obscure differences between the two groups.

Spinella et al. (2018) and Victor et al. (2019b) adopted a prospective study design, reporting on clinical outcomes following the transfer of 78 and 100 putative mosaic blastocysts, respectively (Table IV). While Spinella et al. (2018) employed both aCGH and NGS for diagnosis, Victor et al. (2019b) were the first to report on transfers of blastocysts diagnosed as mosaic following NGS-based PGT-A. Compared to euploid blastocysts, both studies revealed significantly lower implantation and live birth rates following the transfer of embryos classified as mosaic, comparable to those reported by Munné et al. (2017b) (Table IV). Yet, unlike Munné et al. (2017b), Victor et al. (2019b) demonstrated that embryos diagnosed with single mosaic structural aberrations led to improved clinical outcomes compared to all other types of putative mosaicism. In a further retrospective study, Zore et al. (2019) correlated clinical outcomes to the diagnosis of mosaic structural aberrations following NGS re-analysis. Unlike Victor et al. (2019b), the authors demonstrated that the transfer of blastocysts diagnosed with mosaic structural abnormalities resulted in significantly lower live birth and higher miscarriage rates compared to euploid embryos (Zore et al., 2019). Nevertheless, Zore et al. (2019) set their detection limit for diagnosing mosaicism at 40%, which is higher than previous studies. Furthermore, they did not apply clearly defined criteria for designating a structural mosaic embryo as euploid following re-analysis, which may account for the observed differences between the reports.

In a recent study, Munné et al. (2019b) reported on a further 253 PGT-A cycles across 14 centres, following the transfer of embryos characterised as mosaic (Table IV). This series included data published in Munné et al. (2017b) and Spinella et al. (2018) and confirmed previously reported findings (Table IV). No differences in implantation potential were found between blastocysts diagnosed with whole chromosome mosaicism (42%) and structural mosaicism (40%), nor blastocysts diagnosed with mosaic monosomies (42%), mosaic trisomies (42%) and two mosaic aberrations (43%). Interestingly, Spinella et al. (2018) and Munné et al. (2019b) demonstrated that embryos diagnosed with lower levels of mosaicism (<50%) were associated with more favourable clinical outcomes compared to those presenting with a higher degree of heterogeneity. Yet Victor et al. (2019b) did not establish any correlation between the degree of mosaicism reported and clinical outcome. In a mouse model of embryo mosaicism, Bolton et al. (2016) demonstrated that blastocysts containing a sufficient number of euploid cells retained a normal developmental potential. Nevertheless, Spinella et al. (2018) employed both aCGH and NGS for genetic analysis and do not correlate the mosaic diagnoses to the respective platforms (Spinella et al., 2018; Munné et al., 2019b). Although the authors provide extensive validation of both techniques (Greco et al., 2015; Spinella et al., 2018), this could potentially impact the interpretation of their findings. The study of Bolton et al. (2016) may also encompass a potential bias, as the mouse model relied on artificially induced chaotic abnormalities by impairing the SAC. Ultimately, the effect of the degree of mosaicism in TE biopsies on clinical outcomes remains to be thoroughly investigated.

The impact of chromosomal mosaicism on implantation and post-implantation development has also been evaluated in a more fundamental context. Nevertheless, experimental data remain exceptionally scarce, as studying the implantation period of early human embryos *in vitro* continues to present significant challenges. To assess the fate of euploid, chromosomally abnormal and putative mosaic blastocysts during peri-implantation development ($n = 80$), we developed an optimised system for the extended *in vitro* culture of human embryos up to 12 days post fertilization (dpf) (Popovic et al., 2019). We compared original TE biopsy profiles with both culture outcomes and the chromosomal status of the embryos during later development. Viable Day 12 outgrowths were predominantly generated from euploid blastocysts and those diagnosed with trisomies, structural duplications or mosaic aberrations. Conversely, monosomies, deletions and more complex chromosomal constitutions significantly impaired *in vitro* development to 12 dpf. Echoing clinical data, in our study 58% of embryos originally diagnosed with chromosomal mosaicism remained viable at 12 dpf, of which 71% presented with euploid profiles. The remaining embryos were either found to be abnormal or failed to stay attached during the *in vitro* culture.

To date, karyotyping information from miscarriages, ongoing pregnancies and live-births following the transfer of mosaic embryos remains exceptionally limited. Thus far, live births following the transfer of putative mosaic embryos have all revealed normal karyotypes (Greco et al., 2015; Munné et al., 2019b; Victor et al., 2019b). Notably, both cytogenetic analysis of miscarriage tissues and prenatal testing data are yet to reveal a true positive clinical diagnosis of mosaicism, thereby confirming the original mosaic diagnoses following PGT-A. Interestingly, however, it has been hypothesised that the types of mosaicism reported during preimplantation development may represent a different phenomenon to those affecting the fetus during pregnancy and at birth (Munné and Wells, 2017). Yet to truly assess the positive and negative predictive values of diagnosing mosaicism on clinical outcomes, a blinded, non-selection NGS study adopting the approach of Scott et al. (2012) is eagerly required. This analysis will be crucial for defining both the clinical significance as well as the risks of reporting and transferring putative mosaic embryos. In the interim, it is important to acknowledge that current studies examining clinical outcomes following the transfer of blastocysts diagnosed as mosaic are retrospective by design, thus subjected to patient and embryo selection bias. Putative mosaic embryos are only considered for transfer if no euploid embryos are available and thus largely involve patients that have undergone multiple cycles resulting in few or no euploid embryos. Although clinical outcomes are compared to those of euploid controls across matched patient cohorts, there may be a selection bias towards poor prognosis patients. This may inherently impact the currently reported ongoing pregnancy rates following the transfer of blastocysts diagnosed with chromosomal mosaicism.

Biological considerations

Observations that a subset of mosaic blastocysts has the capacity to develop normally have sparked discussions regarding the ability of embryos to 'self-correct'. While the initial mosaicism within the embryo may be self-limiting, the underlying events allowing the normalisation of euploid cells throughout development remain theoretical. The depletion of abnormal cells and cellular self-

correction have both been proposed as potential mechanisms for self-limiting mosaicism. Nevertheless, providing direct evidence for corrective mechanisms during early human development is exceptionally difficult. Existing tools for visualising single abnormal and euploid cells within a mosaic embryo in real time certainly remain limited.

It has been hypothesised that TE mosaicism may be a normal feature of early embryonic development, facilitating implantation, as in tumour invasion (Ogden *et al.*, 2013; Gleicher *et al.*, 2016). Although an interesting proposition, several studies have indicated no preferential allocation of aneuploid cells to either embryonic lineage (Evsikov and Verlinsky, 1998; Magli *et al.*, 2000; Fragouli *et al.*, 2008; Capalbo *et al.*, 2013; Popovic *et al.*, 2018; Victor *et al.*, 2019a). Experimental evidence in mouse, however, does suggest that lineage-specific mechanisms may take place within the blastocyst (Bolton *et al.*, 2016). Accordingly, abnormal cells were shown to be depleted from the ICM by apoptosis, while proliferative defects led to the reduction of aneuploid cells in the TE. While these findings may not be directly translatable to human (Fragouli *et al.*, 2019), indirect evidence for the depletion of abnormal cells stems from studies analysing human embryos at different stages of development. Santos *et al.* (2010) revealed a significant decrease in the number of abnormal cells over time in embryos at 4, 5 and 8 dpf. Similarly, we observed euploid profiles in both ICM and TE-derived lineages of viable Day 12 embryo outgrowths generated from blastocysts classified as mosaic (Popovic *et al.*, 2019). Coinciding with these findings, several studies have demonstrated the derivation of euploid human embryonic stem cells from abnormal embryos, suggesting that aneuploid cells may be depleted under conditions of euploid cell competition (Munné *et al.*, 2005; Lavon *et al.*, 2008; Peura *et al.*, 2008). Interestingly, Victor *et al.* (2019b) evaluated markers of mitosis and apoptotic activity in euploid, abnormal and mosaic human embryos. They revealed considerably higher levels of cell proliferation and death in putative mosaic and aneuploid blastocysts compared to euploid counterparts.

The hypothesis of cellular self-correction assumes that abnormal cells can revert to a euploid state by losing a chromosome (trisomic rescue) or by duplicating one (monosomic rescue). As aforementioned, some cases of cellular self-correction may result in UPD. While UPD is exceptionally rare (Northrop *et al.*, 2010; McCoy *et al.*, 2015), correction events that do not result in uniparental homologs are impossible to detect and as such may be underestimated. Using time-lapse imaging and single-cell DNA sequencing on rhesus macaque embryos, Daughtry *et al.* (2019) proposed an alternative mechanism of cellular self-correction. They reveal that whole or partial chromosomes may be lost via cellular fragmentation, while abnormal blastomeres exhibit extensive DNA damage and are not further incorporated into blastocysts. Although challenging, future studies in human embryos may provide fascinating insights into potential mechanisms of self-correction and the capacity of blastocysts to cope with populations of abnormal cells.

Clinical management and patient counselling

Clinical data following the transfer of mosaic blastocysts will certainly continue to shed light on the significance of diagnosing chromosomal mosaicism by PGT-A. Yet, as our understanding of blastocyst mosaicism continues to advance, clinical management and counselling strategies must also concomitantly improve. Accordingly, PGDIS recently updated their recommendations regarding the transfer of blastocysts diagnosed as mosaic (PGDIS, 2019).

Principally, PGDIS recommendations stipulate that laboratory reports should include embryos at risk of mosacism, incorporating the cut-off scores used for diagnosing mosaicism, as well as the nature of the chromosomal abnormality involved. Importantly, this information should be made available by commercial testing laboratories to ensure appropriate patient counselling (PGDIS, 2019). From a technical standpoint, platform validation is critical when reporting mosaicism. Nonetheless, patients must be made aware that PGT-A remains an embryo screening strategy and, like any diagnostic test, cannot be 100% accurate. To facilitate informed decision-making, patients should be further informed of the technical and biological challenges in interpreting PGT-A results (Besser and Mounts, 2017). While deviations from threshold values may suggest chromosomal mosaicism, false-positive diagnoses are also possible and are highly influenced by the genetic testing platform used (Deleye *et al.*, 2015a; Maxwell *et al.*, 2016; Ruttanajit *et al.*, 2016). Ultimately, both technical and biological factors confound the diagnosis of mosaicism from a single TE biopsy.

Prioritising embryos for transfer following a mosaic diagnosis constitutes a further clinical challenge. As the clinical consequences of blastocyst mosaicism are determined by a multitude of factors, each diagnosis must be considered as a unique circumstance. PGDIS suggests that the degree of mosaicism should be considered as a continuous risk gradient, ranging from lower risk at 20%, to higher risk towards 80% (PGDIS, 2019). However, as the degree of chromosomal heterogeneity reported may not always be indicative of the rate of mosaicism for the entire blastocyst, specifying a single mosaic value can only serve as a reference. Original PGDIS recommendations (PGDIS, 2016) further proposed categorising putative mosaic embryos in regard to the chromosomes involved. However, current findings suggest that these guidelines may be overly cautious (Munné and Wells, 2017). Nevertheless, Grati *et al.* (2018) offer comprehensive recommendations by determining specific risk scores across individual chromosomes, based on chorionic villus sampling and the analysis of miscarriage samples. Accordingly, chromosomes associated with UPD, severe intrauterine growth restriction or congenital disorders may be assigned lower priority (PGDIS, 2019). As PGT-A ultimately endeavours to identify embryos with the highest developmental potential per transfer, uniformly euploid embryos are prioritised; however, there are currently no prospective case-controlled studies or RCTs that definitively support this notion. It is plausible that good quality blastocysts diagnosed as mosaic may perform better compared to uniformly euploid embryos of poor morphology.

Certainly, the transfer of mosaic blastocysts requires careful consideration. Besser *et al.* (2019) recently reported that patients who opted for additional cycles were twice as likely to have an ongoing pregnancy compared to those who pursued the transfer of an embryo classified as mosaic. As such, blastocysts diagnosed with mosaicism should only be considered for transfer in cases in which patients are unable or unwilling to attempt further treatment (Sachdev *et al.*, 2017). Accordingly, older patients and patients who previously underwent a number of oocyte retrievals were more likely to undergo the transfer of a putative mosaic embryo (Besser *et al.*, 2019). Nevertheless, it is

important to appreciate that clinical management remains inherently challenging when considering these patient populations. Therefore, decisions regarding transfer or storage of mosaic embryos may confer an added emotional burden and be even more difficult in these cases (Besser and Mounts, 2017). As clinical outcome data remains preliminary, patients should be made aware of the potential risks of transferring blastocysts with a mosaic diagnosis. Above all, the emotional and financial repercussions of a failed embryo transfer or pregnancy loss should remain at the forefront of these discussions (Sachdev *et al.*, 2017). If the transfer of a putative mosaic embryo is considered, extensive counselling regarding the limited predictive data available, the potential risks, as well as prenatal testing options should all be discussed (Besser and Mounts, 2017; Sachdev *et al.*, 2017). Prenatal diagnosis of the fetus and the placenta are highly recommended, with amniocentesis currently considered most representative of fetal tissues (PGDIS, 2019).

While further outcome data may contribute to individualised treatment strategies, at present the potential risks must be carefully weighed against the possibility of discarding potentially viable embryos. At present, ART centres routinely classify mosaic embryos as clinically unsuitable. Nevertheless, the exclusion of mosaic blastocysts results in fewer embryos available for transfer, which may inevitably compromise treatment outcomes.

Conclusion

While NGS delivered important improvements in the diagnostic accuracy of PGT-A, its improved sensitivity has inherently introduced new challenges for result interpretation, particularly regarding chromosomal mosaicism (Besser and Mounts, 2017). Paradoxically, less sensitive platforms facilitated more straightforward clinical decision-making, as embryos could be more readily categorised as either euploid or aneuploid. Nevertheless, in the age of constant technological progress in both diagnostic and research approaches, uncertainties challenging clinical management in PGT-A are perhaps inevitable. Ultimately, the finer the focus, the higher the incidence of diagnoses of unknown clinical significance. Therefore, greater standardisation of clinical and laboratory practices will be paramount for achieving consistency and substantiating relevant findings. In the context of chromosomal mosaicism, improved clinical decision-making will inevitably rely on evidence-based standards. Accordingly, analyses of larger patient cohorts and long-term follow-up studies present a demanding yet essential task ahead (Forman, 2019). Moreover, future efforts to define embryo characteristics that are important for maintaining chromosomal stability may provide a more definitive assessment of the impact of chromosomal mosaicism on early human development. Elucidating these uncertainties will ultimately pave the way towards improved embryo selection and clinical management.

Authors' roles

M.P.: manuscript conception and design, manuscript writing, literature search and critical discussions. L.D.: critical discussions, manuscript editing and critical revisions. A.B.: manuscript editing and revisions. B.M.: critical discussions, manuscript editing and critical revisions. B.H: manuscript conception and design, critical discussions, manuscript editing and critical revisions. All authors revised and approved the final version of the manuscript.

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Conflict of interest

The authors declare no conflict of interest.

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