EDITORIAL
PGDIS Position Statement on the Transfer of Mosaic Embryos 2019

Post release of previous Preimplantation Genetic Diagnosis International Society (PGDIS) Position Statement 2016 (www.pgdis.org), there is still uncertainty in the classification of mosaicism and transfer options (Kim et al., 2018). The purpose of this document is to review the current information and update recommendations regarding the transfer of mosaic embryos.

BACKGROUND
The primary purpose of preimplantation genetic testing for aneuploidy (PGT-A) is to improve IVF transfer outcomes by reducing the impact of aneuploidy in an embryo cohort. Identification of aneuploidy and transfer of euploid embryos has demonstrated improved rates for implantation, pregnancy and live birth per transfer and reduced implantation failures.

Testing blastocysts for abnormal copy number (aneuploidy) using array CGH, SNP arrays and next generation sequencing (NGS) based PGT-A methods have been introduced into clinical practice in recent years. The majority of IVF laboratories now culture embryos to the blastocyst stage to identify the developmentally competent embryos and then biopsy small numbers of trophectoderm cells for aneuploidy testing. Analysis of multiple-cell biopsies introduces the possibility of aneuploidy results that lie somewhere between full aneuploid and normal euploid. Chromosome mosaicism is typically defined as the presence, in a single sample, of two or more cell lines with different chromosome sets, which has been observed commonly in a minority of embryos at all stages of preimplantation development. Sensitive technologies such as array CGH and NGS based copy number methods can variably distinguish simple, uniform aneuploidies (affecting all cells in the biopsy) from partial (mosaic) aneuploidies (affecting only some of the cells in the biopsy) and can quantify the extent of any copy number changes present (Ruttanajit et al., 2016). Using higher resolution NGS methods, segmental mosaicism can also be detected whereby small chromosome deletions or duplications (typically >10 Mb) are identifiable.

OVERVIEW OF NEW KNOWLEDGE
Incidence of mosaic embryos
At the blastocyst stage, the incidence of reported mosaicism using NGS methods is highly variable between clinics, ranging from as low as 2% to as high as 40%. However, the vast majority of clinics report that mosaic embryos represent between 5-10% of those tested (Ruttanajit et al., 2016, Munné et al., 2016, Fragoüli et al., 2019). A consistent high incidence of mosaic embryos in some clinics may be indicative of clinical treatment, embryology, analysis approach or in some cases be patient-related factors (Fragoüli et al., 2019) and in such cases further review of both clinical and laboratory practices may be warranted. All clinics referring PGT-A testing to outside service laboratories may request the laboratory to disclose their identified mosaic rates and cut off ranges.

Transfer outcomes from mosaic embryos
In the first published study using array CGH-based PGT-A (Greco et al., 2015), healthy live births were reported following transfer of apparent mosaic embryos. Since this initial report, several other studies involving the transfer of larger numbers of mosaic embryos have been conducted (Munné et al., 2017, Victor et al., 2019, Zhang et al., 2019, Zore et al., 2019). From the transfer outcomes, compared to euploid transfers, transfer of mosaic or mosaic segmental embryos do give rise to healthy pregnancies but may be associated with reduced implantation and higher miscarriage rates. In general, good success rates were achieved transferring mosaics with <40% mosaicism, whereas mosaics with 40-80% mosaicism were less likely to achieve a viable pregnancy. Poorer outcomes were achieved with the transfer of complex mosaics where more than 1 chromosome was involved. While the collective transfer data still only comprises less than 500 mosaic embryos, it is clear that a high proportion of mosaic embryos have some level of developmental competence. Equally important, prenatal diagnosis follow up of the established pregnancies by amniocentesis revealed normal euploid fetuses indicating that the trophectoderm mosaicism originally seen in the blastocyst was likely of limited nature. All live births reported to date were healthy with no evidence of chromosome based syndromes. In addition, these studies revealed that outcomes were generally independent of the original chromosome involved in the mosaicism.

Genetic analysis of mosaic blastocysts
Research studies re-analysing discarded blastocysts diagnosed aneuploid after NGS have consistently shown a high concordance (> 95%) of the original aneuploidy result with other sites in the embryo, including the ICM and other regions of the trophectoderm compartment (Victor et al., 2019, Victor et al., 2019, Huang et al., 2017). More recently, the analysis of mosaic embryos donated to research has begun to shed light on the chromosomal constitution of mosaic blastocysts (Viotti et al., 2018). In general, if the level of mosaicism was high (>40-80%) in the initial biopsy, subsequent trophectoderm biopsy and ICM analysis tended to similarly show some level of mosaicism. However, if the level of mosaicism was lower (<40%), subsequent trophectoderm and ICM biopsies often showed a lower degree of concordance for that mosaicism, with many embryos being found uniform euploid.

Technical considerations
From PGT-A practice, circumstantial evidence is emerging that suggests that...
the NGS and data analysis pipelines used to measure chromosome copy number may in some embryos, incorrectly indicate mosaicism, as a result of various technical effects (Fragouli et al., 2019). Such artifacts could result from situations such as:

(i) Method: Poor biopsy technique taking too few cells with cell damage or partial destruction and loss of cellular DNA affecting apparent chromosome profiles.

(ii) Analysis: Algorithms used for normalizing the chromosome mapping bins can also potentially alter profiles, especially if any bin counts used to normalize the profiles are variable or low. In addition, biases in library construction from poorer quality starting DNA (including compromised whole genome amplifications) could lead to under or over representation of chromosomes (whole chromosome mosaicism) or sub-chromosomal regions (segmental mosaicism). NIPT analyses of cell free DNA, which uses similar NGS methodology, suggest that biases can also occur in the library preparation step, leading to incorrect copy number calls, especially for chromosomal segments.

HOW DOES THIS AFFECT ANEUPLOIDY TESTING IN CLINICAL PRACTICE?

Most (>90%) trophoderm biopsy results are either uniform euploid for all chromosomes or full aneuploid involving one or more chromosomes. However, a small proportion of embryos may show intermediate copy number changes for one or more chromosomes, indicating possible cell mosaicism. Occasionally, these may be the only embryos which are available for potential transfer. Since mosaicism detected in trophoderm biopsies may theoretically have clinical implications for the pregnancy (including effects on placental function, and/or liveborn disease syndromes), transfer of these embryos should be considered only after appropriate counselling of the patient and alternatives have been discussed.

COMMENTS FOR THE LABORATORY

1. Clinics should understand the impact that poor biopsy technique may have on subsequent analyses. Ideally ≥ 5 cells should be biopsied to give subsequent robust and balanced amplification. Fewer than 5 cells may impact on amplification profiles (noise) and mosaic detection levels. It is recommended that no more than 10 cells be biopsied in order to minimize the impact of the process on the remaining embryo - care should be taken to ensure minimum effect on the embryo. Cell damage should be minimized to reduce amplification bias and yield a DNA product reflecting the original cells taken. If the biopsy is facilitated using a laser, the identified contact points should be minimal and preferably at cell junctions. If there is a consistently high incidence of mosaicism identified in embryo cohorts in your laboratory, consideration should be given to investigating both the embryology and PGT-A practice to identify the underlying causes.

2. For technical reasons only an analysis platform that can reproducibly measure copy number should be used for reporting of mosaic levels in the biopsy sample. Different platforms may have lesser (or greater) detection/ quantification abilities for mosaicism, as well as intrinsic baseline noise levels. Service laboratories can perform their own baseline control experiments for both euploid and aneuploid WGA products from a range of samples. Values that lie outside of these euploid/aneuploid ranges are deemed to be mosaic. Detection and quantification limits of mosaic level can be defined, if considered necessary, through cell mixing experiments. Low level DNA mixing experiments may not be as suitable a test specimen because of sampling theory issues at low copy number biasing relative chromosome ratios. Embryos in the selected lower range value may be reported as euploid while embryos above the upper value chosen may be reported as aneuploid. The typical lower cut off value from a number of published groups is 20% while the upper value is 80% (Maxwell et al., 2016, Vera-Rodriguez et al., 2016, Munne et al., 2017, Fragouli et al., 2017, Spinella et al., 2018). These limit values should be reported by the service group to the referring group. It is highly recommended that any clinic using commercial service laboratories for PGT-A ask for these ranges for confident reporting of mosaicism and counselling to patients.

3. Given the nature of the biology of the genesis and propagation of mosaicism, any biopsy piece analyzed as mosaic may not accurately reflect the surrounding trophoderm or the rest of the embryo. The inherent difficulty in assigning a single mosaic value to what is usually a broad data spread means the reported value should be considered only a reference point for counselling couples considering transfer (or discard) of an apparent mosaic embryo. We suggest that the mosaic spectrum be considered a continuous risk gradient ranging from relatively lower risk at 20% to higher risk as it approaches 80% (FIGURE 1). However, clinics should use

FIGURE 1 Relationship between level of mosaicism and increased risk of an adverse outcome following transfer of a mosaic embryo.
their own judgment in assigning risk and the impact this might have on reporting and counselling.

4. Laboratory report formats should be updated to include reporting of mosaics, the cut off values for mosaics and the nature of the chromosome abnormality identified.

**RECOMMENDATIONS FOR THE CLINICIAN**

1. Patients should continue to be advised that any genetic test based on sampling one or small number of cells biopsied from a preimplantation embryos cannot be 100% accurate for a combination of technical and biological factors, including chromosome mosaicism.

2. The patient information and consent forms for aneuploidy testing (if used) should be modified to include the possibility of mosaic results and any potential risks in the event of transfer and implantation. This needs to be explained to patients by the person recommending PGT-A.

3. Transfer of blastocysts with a normal euploid result should generally be prioritized over those with mosaic results.

4. In the event of considering the transfer of a mosaic blastocyst, the following options should be discussed with the patient:
   
   (i) Initiation of a further PGT-A cycle to increase the chance of identifying a euploid blastocyst for transfer
   
   (ii) Transfer of a blastocyst with lower level mosaicism, after appropriate counselling.

Prenatal diagnosis of the fetus and placenta of any established pregnancy after PGT is highly recommended - this especially applies after any mosaic embryo transfer. Amniocentesis analysis from week 14 onwards is currently considered to be the most representative of the fetus genetics. For earlier investigations (week 10 onwards) of the placenta, consideration can also be given to NIPT methodology that analyses placental copy number of all 24 chromosomes - simple 5 chromosome NIPT tests for chromosomes 21, 18, 13 X and Y may not be appropriate. Ultrasound may also be helpful in identifying fetal abnormalities while PAPP-A screening and Doppler Ultrasound may also be useful in identifying placental malfunction.

**SUGGESTED RECOMMENDATIONS TO ASSIST IN THE PRIORITIZATION OF MOSAIC EMBRYOS CONSIDERED FOR TRANSFER**

Based on our current knowledge of the reproductive outcomes of fetal and placental mosaicism from prenatal diagnosis and new knowledge gained from recent embryo analysis and transfer studies, the following is a guide only to assist the clinician (or a genetic counsellor if available) when a mosaic embryo is being considered for transfer:

1. Embryos with low-level mosaicism (low-risk) are preferable to embryos with higher level mosaicism, since those with a higher level may be associated with a higher risk for an adverse outcome. Relative percentage of mosaicism seems to be a better predictor of outcome rather than the specific chromosome(s) involved. Specific chromosomes are linked to specific syndromes and such should be discussed on a case by case basis with directed counselling. Higher risk mosaic embryos should be transferred with caution and only after appropriate genetic counselling.

2. If a decision is made to transfer embryos with similar levels of mosaicism and level identifiability in the biopsy piece. If there is a choice between the transfer of two mosaic embryos with similar levels of mosaicism, embryos mosaic for chromosomes that are associated with potential for uniparental disomy, severe intrauterine growth retardation or liveborn syndromes may be given lower priority. For further guidance, reference may be made to the review by (Grotti et al., 2018) for information on specific placental complications and fetal syndromes, in determining which chromosomes may be associated with various disorders. Recent ASRM guidelines (Practice Committee and Genetic Counselor Special Interest Group (GCSIG) of the American Society for Reproductive Medicine 2019) may be referred to for counselling issues related to transfer of mosaic embryos.

**OVERVIEW**

Developments in genomic technologies for PGT have revolutionized our ability to detect, at the level of the single cell or small numbers of cells, genetic abnormalities of various kinds. Perhaps inevitably, the increased sensitivity and resolution of these methods has allowed a more complete spectrum of chromosome abnormalities to also be identified, including chromosome and segmental mosaicism- areas where our knowledge of the biology and the outcomes is incomplete and still evolving. Prior IVF outcomes indicated no elevated risks of chromosome disorders compared to natural pregnancies and so from the available PGT-A data, transfer of mosaic embryos appears to be a relatively safe option for couples, with low or minimal risk of negative outcomes for the pregnancy. Nonetheless, transfer of blastocysts in which mosaic aneuploidies have been detected should only be considered following expert advice and appropriate genetic counselling of patients. The laboratory reporting recommendations should also be understood when advising patients of the reasoning behind any concerns regarding the transfer of a mosaic embryo and the appropriateness of pregnancy follow up by a non-invasive comprehensive NIPT that discloses all chromosomes or invasive tests such as amniocentesis where mosaicism can be identified.

To better understand the clinical consequences of transferring mosaic embryos and provide valuable information to improve genetic counselling for patients considering transfer of a mosaic embryo, where possible, long term follow up studies of all putative mosaic embryo transfers by clinicians should be encouraged. With the increasing uptake of NIPT for screening fetal aneuploidies, and more comprehensive NIPT screening technologies available, many pregnancies established after the transfer of mosaic embryos could be followed simply. Furthermore, since NIPT has been shown to be capable of detecting many fetal mosaics involving rare trisomies (Scott et al., 2018) the opportunity to follow up the original
trophectoderm mosaicism result is now available. Collection of this data, and where possible placental tissue at birth, will help us better understand the safety of transferring mosaic embryos. At the research level, genetic analysis of donated non-transferred mosaic embryos through NGS analysis of the remaining blastocyst will continue to shed light on the significance of the initial biopsy measurement and give valuable information about the genetic constitution of mosaic embryos.

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