A comprehensive analysis of blastocyst chromosome mosaicism in PGT-A cycle

Lluc Coll Luján

Doctoral Thesis

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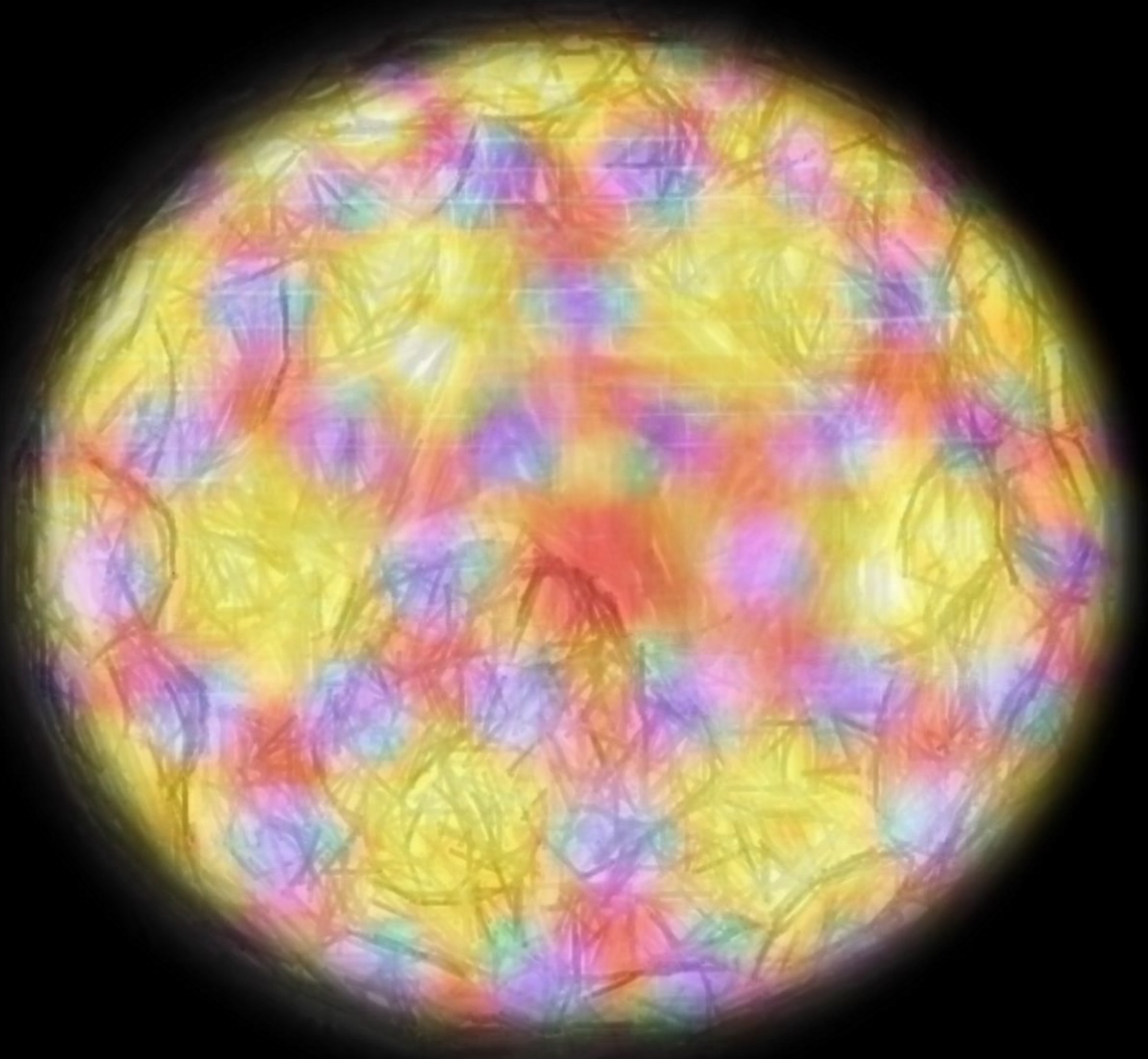
Lluc Coll Luján - April 2023



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A COMPREHENSIVE ANALYSIS OF BLASTOCYST CHROMOSOME MOSAICISM IN PGT-A CYCLES

A thesis submitted by Lluc Coll Luján for the PhD degree of Cellular Biology
(Faculty of Biosciences, Universitat Autònoma de Barcelona)

Lluc Coll Luján, M.Sc.

Barcelona, April 2023

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https://www.illesbalears.travel/experiencia/ca/mallorca/festa-de-la-llum-de-la-catedral-de-mallorca

About the cover: Twice a year, on February 2nd and November 11th, at Palma de Mallorca Cathedral, first sun rays go through the mosaic of 1.116 colored pieces of glass of the greater rose window (the largest of all European gothic cathedrals) projecting it on the inner wall of the main façade, just beneath the *minor* rose window, creating an "eight" shape of light and colour. This event is called "*Festa de la Llum*" (Festival of Light).

In the cover design I have merged a photograph of the greater rose reflection with an edited image of a blastocyst, fusing the architectonic and biological concepts of mosaicism. The cover is, therefore, a representation of a mosaic blastocyst.

As I was born in Mallorca, it is also a reference to my place of birth.

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We confirm that Lluc Coll Luján has conducted the Doctoral Thesis entitled "A comprehensive analysis of blastocyst chromosome mosaicism in PGT-A cycles" under our supervision. The present work has been conducted at the Reproductive Medicine Service of Dexeus University Hospital.

Barcelona, April 2023

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GLOSSARY

ART	Assisted Reproductive Technology
aCGH	Comparative Genomic Hybridisation array
BMI	Body mass index
CCS	Comprehensive Chromosome Screening
CVS	Chorionic villus sample/sampling
DNA	Desoxyribonucleic acid
ESHRE	European Society of Human Reproduction and Embryology
FISH	Fluorescence *in situ* hybridization
ICM	Inner cell mass
IUGR	Intrauterine growth retardation
ICSI	Intracytoplasmic sperm injection
IVF	*In vitro* fertilization
MET	Mosaic embryo transfer
MI	First meiotic division
MII	Second meiotic division
NIPT	Non-invasive prenatal testing
NGS	Next-generation sequencing
PBS	Phosphate buffered saline
(q)PCR	(quantitative) Polymerase Chain Reaction
PGDIS	Preimplantation Genetic Diagnosis International Society
PGT(-A)	Preimplantation Genetic Testing (for aneuploidies)
PVA	Polyvinyl alcohol
PZM	Post-zygotic mitotic

aSNP Single Nucleotide Polymorphism array

WGA Whole Genome Amplification

1 INTRODUCTION

Mosaic: a definition

Mosaic

/mə(ʊ)ˈzeɪɪk/

noun

a picture or pattern produced by arranging together small pieces of stone, tile, glass, etc.
"mosaics on the interior depict scenes from the Old Testament

BIOLOGY
an individual (especially an animal) composed of cells of two genetically different types.

verb

decorate with a mosaic.
"he mosaicked the walls, ceilings, and floors"

adjective
BIOLOGY

denoting an individual composed of cells of two genetically different types.

("Oxford Languages and Google - English | Oxford Languages,")

In the framework of the present thesis, the focus will be put on the biological meaning of the word.

More precisely, the term mosaic denotes an individual or tissue who has at least two populations of cells with distinct genotypes that are derived from a single fertilized egg (Martínez-González *et al.*, 2020). Throughout post-zygotic cell divisions any genotype alteration may result in a mosaic. The modification can occur from nucleotide to chromosomal scale with a wide range of possible consequences for the affected individual or tissue depending on which the modification is, as well as when and where it happens.

The present dissertation will focus on the study of chromosomal mosaicism in preimplantation embryos and more specifically at the blastocyst stage both focusing on its origins and consequences within the particular environment of Assisted Reproductive Techniques (ART) and Preimplantation Genetic Testing (PGT).

Chromosomal anomalies in human embryos

The presence of chromosomal anomalies in human embryos has been described as one of the main causes of adverse reproductive outcomes both in vivo and in vitro (Hassold and Hunt, 2001). Depending on the type of aneuploidy and the chromosome involved, an embryo may either fail to implant, or result in a miscarriage, or give rise to a viable affected pregnancy.

Aneuploidies in human embryos can either be from meiotic origin and, therefore, be already present in the gamete, or be originated after fertilization through mitotic errors.

Meiotic aneuploidy

Meiotic aneuploidies are those generated through chromosome segregation errors during gametogenesis. These errors can occur due to an existing risk factor in the progenitor, such as an altered karyotype, or may occur spontaneously "*de novo*".

The factors that may induce spontaneous chromosome missegregation have been widely studied. It seems that meiotic errors are a common feature in human species. Moreover, it has been observed that most aneuploidies have a maternal origin (Hassold *et al.*, 1996; Hassold and Hunt, 2001; Nagaoka *et al.*, 2012; Ottolini *et al.*, 2015; Capalbo *et al.*, 2017a; Gruhn *et al.*, 2019; Wartosch *et al.*, 2021).

Table 1: Summary of the origin of the main human trisomies. MI: meiosis I; MII: meiosis II; PZM: post-zygotic mitotic. Reproduced and adapted from Hassold *et al.*, 2007 and Hall *et al.*, 2007 (with permission).

Trisomy	*n*	Maternal		Paternal		PZM (%)
		MI (%)	MII (%)	MI (%)	MII (%)	
Acrocentrics						
13	74	56.6	33.9	2.7	5.4	1.4
14	26	36.5	36.5	0.0	19.2	7.7
15	34	76.3	9.0	0.0	14.7	0.0
21	782	69.6	23.6	1.7	2.3	2.7
22	130	86.4	10.0	1.8	0.0	1.8
Non-acrocentrics						
2	18	53.4	13.3	27.8	0.0	5.6
7	14	17.2	25.7	0.0	0.0	57.1
8	12	50.0	50.0	0.0	0.0	50.0
16	104	100	0.0	0.0	0.0	0.0
18	150	33.3	58.7	0.0	0.0	8.0
XXX	46	63.0	17.4	0.0	0.0	19.6
XXY	224	25.4	15.2	50.9	0.0	8.5

The specific characteristics of human female gametogenesis make it more prone to such errors. Ovarian reserve is generated and finalized through foetal development. Then, oocytes remain in a quiescent status until puberty when, in each cycle, a pool of follicles will be activated being finally ovulated the oocyte contained in the dominant follicle (Fritz and Speroff, 2011; Johnson, 2018). Therefore, female gametogenesis is not a continuous process in contrast to male gametogenesis (Figure 1). All the stopping and re-activation stages that need to happen during oogenesis increase the risk of an error.

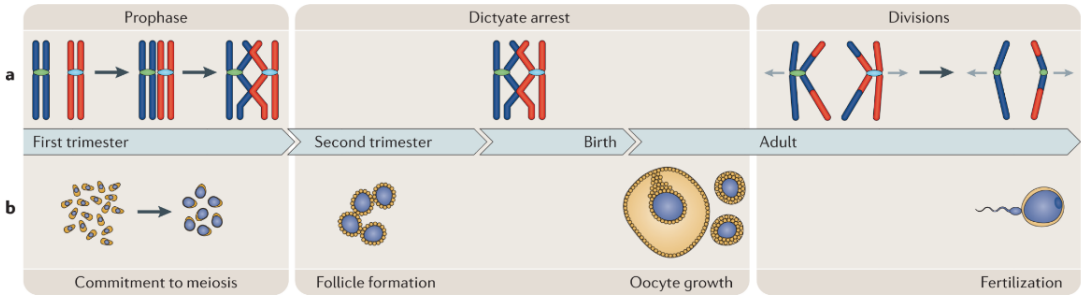

Figure 1: The female meiotic cycle (a) and oogenesis (b). Reproduced from Nagaoka *et al.*, 2012 (with permission).

Moreover, the probability of such errors to happen is high in teenagers, then it reduces in early twenties and increases again in late thirties, especially from the age of 37 (Gruhn *et al.*, 2019). The most important aneuploidy increase occurs with advanced female age (Figure 2). Although the reason why the inflexion point takes place at 37 is not clear, it has been hypothesized that the longest is the meiotic pause, the more errors occur: no turn over of cohesion-related proteins, destabilization of chiasms, deficient operation of division timings and check-points (Handyside, 2012). Moreover, as age increases, there may be a shift to a pre-menopausal endocrine ambient, a recruitment of the worst quality oocytes, and certain small chromosomes with few crossings may go past a critical point (Nagaoka *et al.*, 2012). Advanced female age has been the most associated factor to aneuploidy in human embryos.

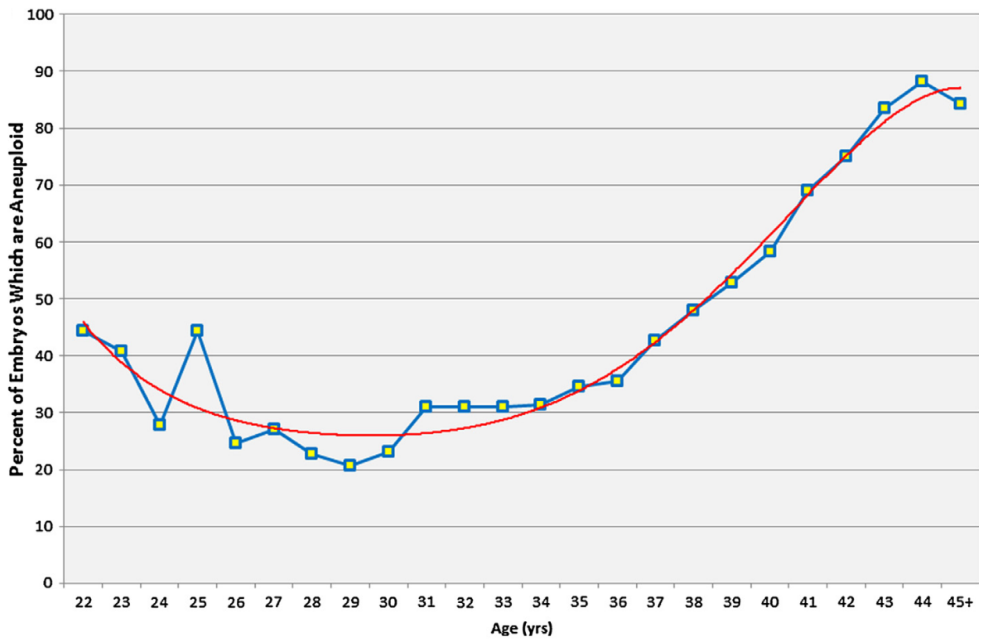

Figure 2: Aneuploidy rate in human blastocysts relative to female age. Reproduced from Franasiak *et al.*, 2014 (with permission).

In contrast to female gametogenesis, spermatogenesis has been proven to be less prone to errors (reviewed by Wartosch *et al.*, 2021). However, it has been reported that, in some infertile men, this rate of error can sometimes be increased as observed by fluorescence in-situ hybridization (FISH) studies in sperm (Egozcue *et al.*, 2000).

It has been reported that, although meiotic aneuploidies can be observed in preimplantation embryos affecting any chromosome, there are specific chromosomes that, due to their morphological and/or genetic characteristics, are clearly more prone to be involved in errors (Capalbo *et al.*, 2014; Nakhuda *et al.*, 2018)(Figure 3).

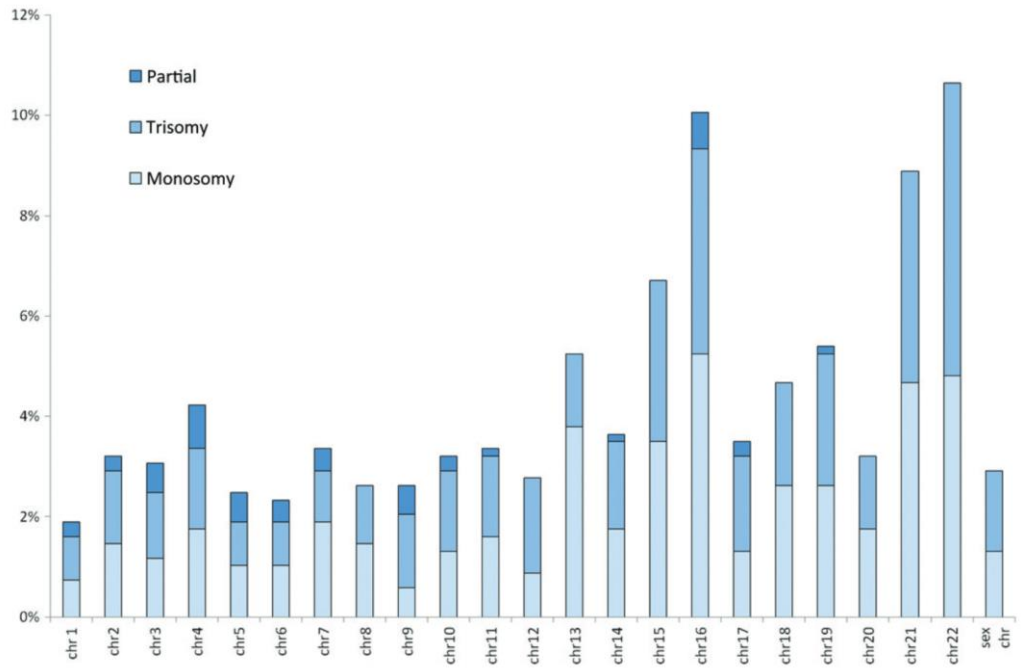

Figure 3: Percentage of aneuploidies observed for each chromosome in 956 screened blastocysts. Reproduced from Capalbo *et al.*, 2014.

In the context of *in vitro* fertilisation (IVF), oocyte meiotic anomalies can be increased due to suboptimal laboratory conditions, as meiosis will not finalize until fertilization in the laboratory. Moreover, it is possible that procedures used in ART, such as controlled ovarian hyperstimulation, may affect meiosis (Nagaoka *et al.*, 2012).

Mitotic aneuploidy

In contrast to meiotic aneuploidy, mitotic chromosome anomalies occur after fertilization and regardless of the chromosomal constitution of the gametes. Consequently, the resulting embryo will be a mosaic. Chromosomal mosaicism is defined as "a state in which there is more than one karyotypically distinct cell population arising from a single embryo" (Zegers-Hochschild *et al.*, 2017).

The phenomenon of embryo mosaicism was first reported in 1993 (Delhanty *et al.*, 1993; Munné *et al.*, 1993). Since then, it has been demonstrated that mitotic anomalies during preimplantation embryo development are a common feature in the human species (Vanneste *et al.*, 2009). The prevalence of mosaicism in cleavage stage embryos has been reported to be as high as 90% (Delhanty *et al.*, 1993; Munné *et al.*, 1993; van Echten-Arends *et al.*, 2011; Mertzanidou *et al.*, 2013) whereas at the blastocyst stage it is reduced to 5-45% (Northrop *et al.*, 2010; Fragouli *et al.*, 2011, 2017; Capalbo *et al.*, 2013; Novik *et al.*, 2014; Greco *et al.*, 2015; Ruttanajit *et al.*, 2016; Munné *et al.*, 2017; Nakhuda *et al.*, 2018; Popovic *et al.*, 2018). The wide ranges reported both at cleavage and blastocyst stage have been suggested to be due to several reasons. Studies have been performed with different patient populations in different settings, which could influence the prevalence of mitotic errors. Moreover, techniques used to detect mosaicism have been as diverse as their limitations. Finally, the nature of mosaicism itself makes it elusive to be detected if the whole embryo is not analyzed (reviewed by Popovic *et al.*, 2020).

Mosaic abnormalities, as meiotic aneuploidy, can be presented affecting the whole chromosome or just a segment of it (whole-chromosome mosaicism and segmental mosaicism).

The mechanisms through which a mitotic abnormality can occur have been extensively discussed and reviewed (Taylor *et al.*, 2014a). During a normal division, each chromosome replicates into a double-chromatid chromosome. Thanks to the mitotic spindle, each chromatid will be segregated to each of the sister cells, which will be identical in terms of chromosome constitution (Figure 4A). Any error occurring during this process may lead to an abnormal chromosome segregation. The main mechanisms through which sister cells with chromosome anomalies would be generated are mitotic non-disjunction, anaphase lagging and endoreduplication.

- In non-disjunction, sister chromatids fail to separate and both end up in one of the sister cells. Therefore, one cell will present a trisomy for the implicated chromosome while the other will present the complementary monosomy (Figure 4B).
- In anaphase lagging, a chromatid fails to attach to the mitotic spindle or to be incorporated to the nucleus. While one of the sister cells will have a normal chromosome complement, the other will present a monosomy for the implicated chromosome (Figure 4C).
- Endoreduplication implies a chromosome replication without cytokinesis due to a cell cycle error. The cell in which this error occurs will present a trisomy for the implicated chromosome (Figure 4D).

Other anomalies such as abnormal spindle formation or DNA replication without cell division can also lead to mosaicism (McCoy, 2017).

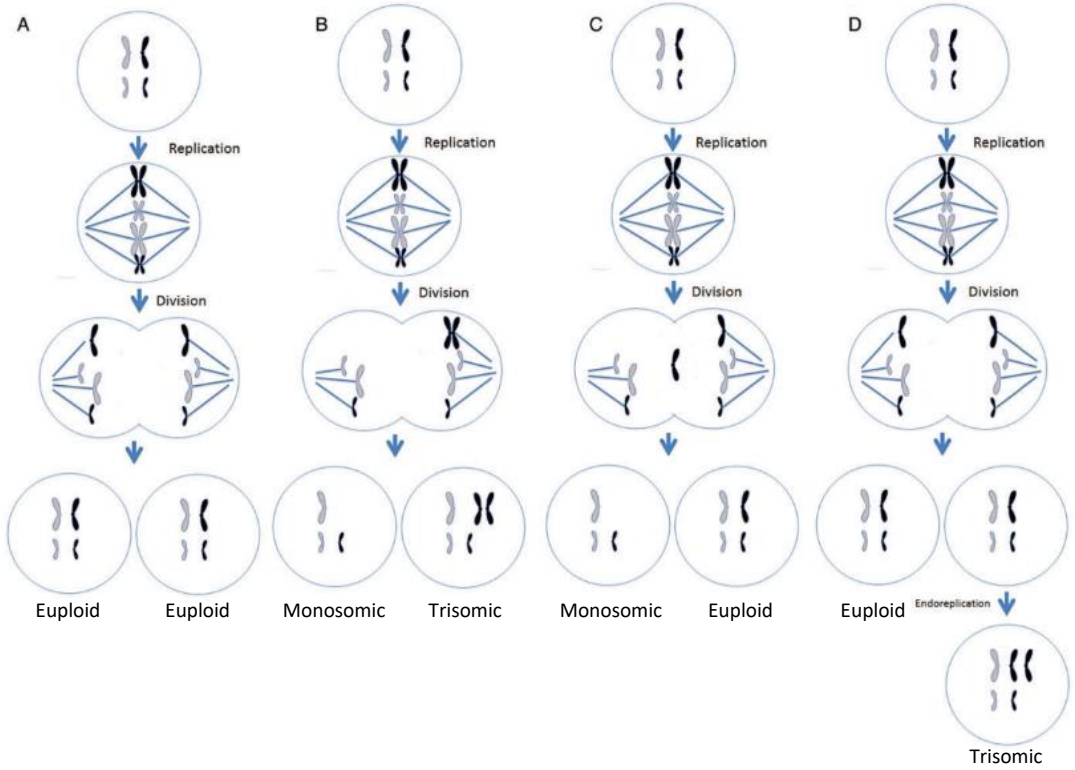

Figure 4: Different segregation patterns during mitotic divisions. Two chromosomes are represented in each figure. Grey chromosomes are maternal in origin and black chromosomes are paternal. A: normal segregation. B: Mitotic non-disjunction. C: Anaphase lagging. D: Endoreduplication of a paternal chromosome. Reproduced and adapted from Taylor *et al.*, 2014a (with permission).

Many authors have tried to discern which of the above-mentioned mechanisms would be the one more frequently associated to mosaic abnormalities in human preimplantation embryos. Most authors have reported that anaphase lagging would be the main mechanism followed by endoreduplication (Delhanty *et al.*, 1993; Coonen *et al.*, 2004; Ioannou *et al.*, 2012). However, others have reported contrarily (Munné *et al.*, 2002; Chow *et al.*, 2014).

Despite the fact that the general mechanisms for an abnormal chromosome segregation during mitosis are known, very little is known about the factors that cause such errors.

Mitosis in early embryos present several differences compared to later embryos and somatic cells (Figure 5). First embryo divisions are, comparatively, very rapid. It has been hypothesized that in order to allow them to be fast, mitotic check-points, such as spindle assembly check-point, may have to be compromised to some degree (Taylor *et al.*, 2014a; McCoy, 2017; Vázquez-Diez and FitzHarris, 2018) allowing errors to happen and persist. Moreover, other peculiarities of early embryo cleavage, such as spindle/cell size and the unusual lack of a centriolar structure (reviewed by Vázquez-Diez and FitzHarris, 2018), could make them more prone to errors. Problems with chromatin cohesion could also play an important role in mitotic anomalies (McCoy, 2017). With regards to segmental mosaicism, it has been extensively discussed that DNA double-strand breaks may contribute to its generation (Babariya *et al.*, 2017; Vera-Rodriguez and Rubio, 2017). Additionally, it could be hypothesized that, mirroring what happens with meiotic errors, certain chromosomes might be more susceptible to be involved with mitotic errors.

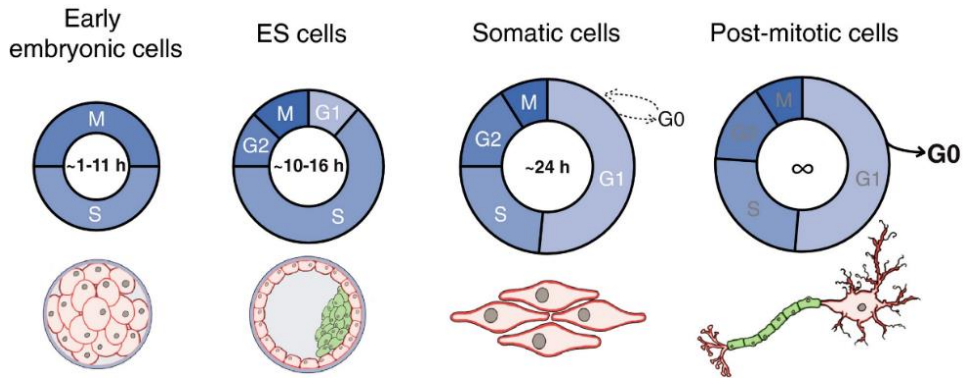

Figure 5: Differences in cell cycle changes throughout cellular differentiation. ES cells: embryonic stem cells. Reproduced from Padgett and Santos, 2020 (with permission).

Mosaic embryos can be categorized in different groups depending on the ploidy of the gametes, the number of different cell lines present within the embryo, and the moment when the mitotic abnormality happens; as summarized in Figure 6.

A euploid-aneuploid mosaic embryo presents a combination of euploid and aneuploid cells, and it is usually originated from chromosomally normal gametes. It can sometimes be a result of an aneuploidy rescue of a zygote derived from abnormal gametes (Taylor *et al.*, 2014a). It is important to mention that, from a reproductive point of view, the focus will be put on this kind of mosaic embryos as, having a euploid cell line, they have the potential to give rise to an ongoing pregnancy and healthy newborn.

An aneuploid-aneuploid mosaic embryo presents a meiotic aneuploidy together with at least one mitotic imbalance. It is usually originated from a chromosomally abnormal gamete with the resulting embryo undergoing at least one error during mitosis. It could also be originated from euploid gametes, occurring the mitotic error at the first embryo division through non-disjunction, which would result in two complementary abnormal cells. From a reproductive point of view, aneuploid-aneuploid embryos will behave, at least, as embryos just presenting the meiotic abnormality. Therefore, they will either not implant, or result in a miscarriage or give rise to an affected ongoing pregnancy.

Embryos with >3 cell lines are called complex mosaics.

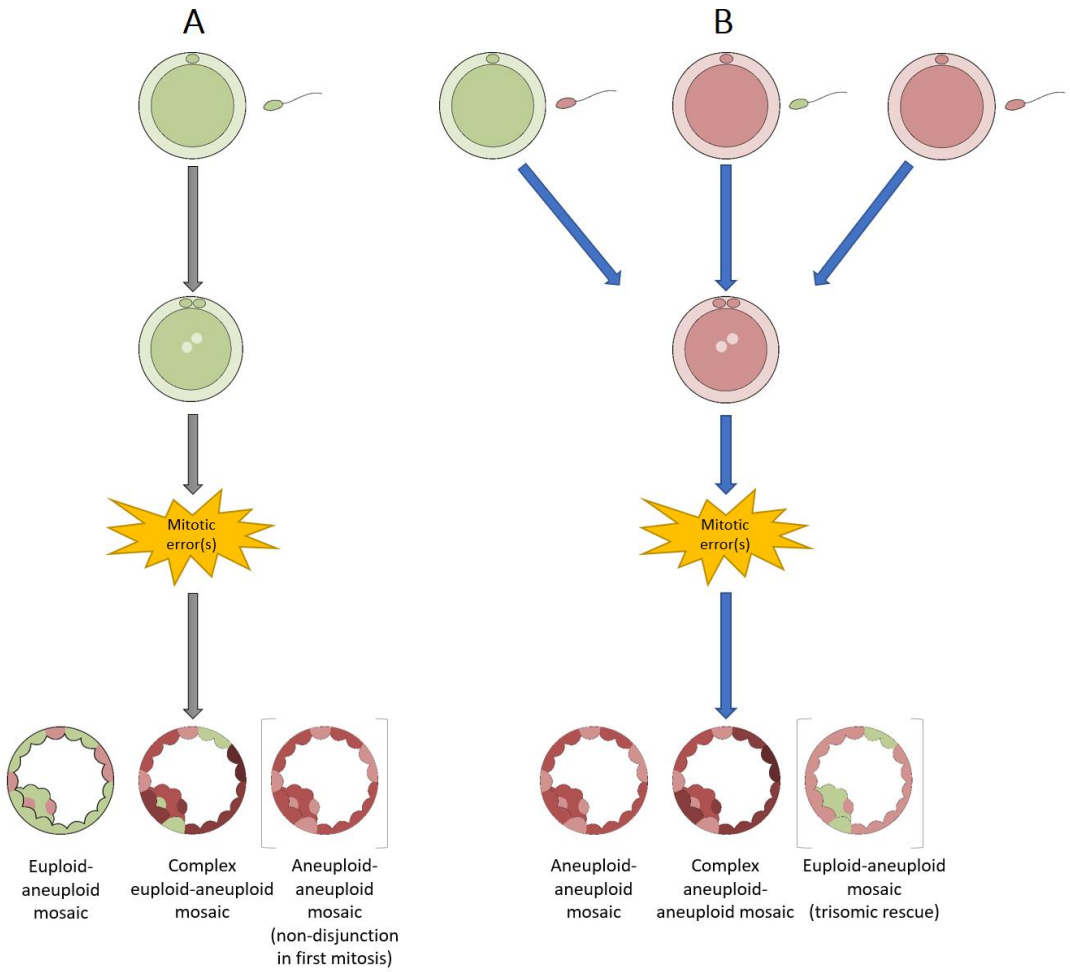

Figure 6: Outline of the different types of mosaic embryos and their origin. A: Possible mosaic embryos after fertilization with euploid gametes and mitotic error(s) during cleavage. B: Possible mosaic embryos after fertilization with aneuploid gamete(s) and mitotic error(s) during cleavage.

In the ART scenario, intrinsic and exogenous factors can compromise the proper function of cell cycle and cause mitotic errors that lead to mosaicism.

Intrinsic mosaicism

It is known that early embryo divisions are manly driven by oocyte-stored molecules (RNAs and proteins) (Lu *et al.*, 2017) and that mitochondria, which

are involved in cell division (Schon *et al.*, 2000; Wilding *et al.*, 2001), are maternally inherited. Moreover, as female age has been observed to be the main factor related to meiotic aneuploidies in human embryos, it would be reasonable to assume that female patients' characteristics may play a role in mitotic divisions.

On the other hand, sperm provides the embryo with the centrosome, which is key in the first mitotic divisions (Palermo *et al.*, 1997), making male factor a clear candidate to be associated to mosaicism. Therefore, abnormal sperm parameters might also be related to mosaicism.

Iatrogenic mosaicism

Although reported differences in mosaicism prevalence may be due to the study populations analysed or different diagnostic tools used, one additional reason might be differences in IVF laboratory conditions. Actually, the existence of iatrogenic mosaicism was first evidenced by a study with a controlled population (oocyte donation cycles) and a controlled single setting for genetic analysis, but with different IVF laboratories referring samples. The prevalence of mosaicism among laboratories was remarkably different (Sachdev *et al.*, 2016).

It should not be overseen that mosaicism might indeed be a normal feature in human embryos to some extent. Other mammals than human also present chromosomal mosaicism and some authors have discussed whether this could even be an evolutionary benefit (Vázquez-Diez and FitzHarris, 2018).

Nevertheless, as much as mosaicism seems to be usual in human embryos, its prevalence decreases through embryo and foetal development. Placental mosaicism

is detected in around 1-4% of prenatal chorionic villus samples (CVS) (Malvestiti *et al.*, 2015; Benn *et al.*, 2019; Li *et al.*, 2022). The likelihood of an abnormality also present in the foetus will depend on the chromosome involved, the type of aneuploidy, the percentage of abnormal cells and the tissue distribution (Grati *et al.*, 2018). In most cases mosaicism is confined to the placenta, being true foetal mosaicism detected in around 0.2-0.5% of pregnancies and it has been estimated to be present in less than 0.2% of live births (Spinner and Conlin, 2014; Benn, 2015; Malvestiti *et al.*, 2015; Li *et al.*, 2022). However, this figure may be an underestimation as mosaicism could be overseen in cases of low percentage or if confined to specific tissues (Figure 7).

Figure 7: Mosaicism prevalence through different developmental stages. Chorionic villi and amniotic fluid sampling images have been reproduced from https://ib.bioninja.com.au/standard-level/topic-3-genetics/33-meiosis/karyotyping.html.

It has been hypothesized that mosaicism may be corrected throughout embryo development, as demonstrated in mouse model, either by active apoptosis of abnormal cells or impaired development depending on the tissue. Moreover, an embryo would be able to repair mosaicism and avoid arrest, or not, depending on the percentage of cells with abnormality (Bolton *et al.*, 2016).

In contrast to meiotic aneuploidy, the effect of mosaicism on the developing pre, peri and post implantation embryo and baby-to-be are very difficult to predict, being unique for each case and dependent on when (which cell division cycle) and where (which tissue) it happens (Taylor *et al.*, 2014a)(Figure 8).

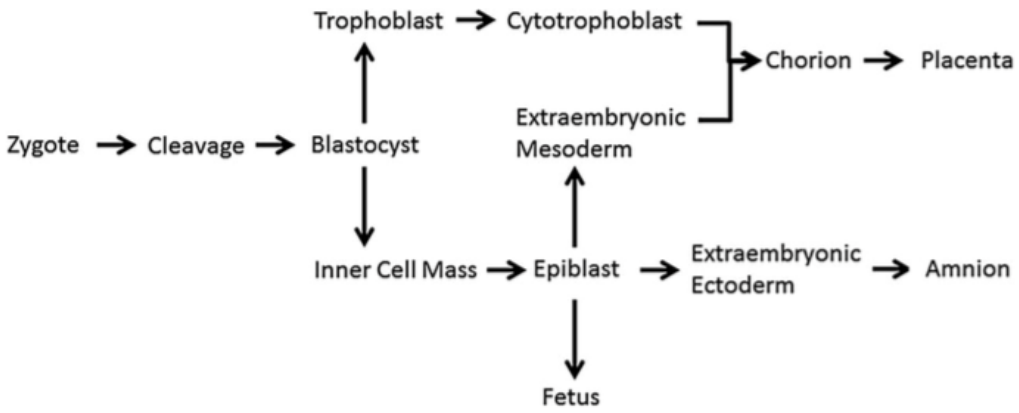

Figure 8: Cell lineage from zygote stage to the foetus. Reproduced from Taylor *et al.*, 2014a (with permission).

All trisomic anomalies are potentially viable in mosaic and some mosaic abnormalities can be harmless. For instance, it is known that some types of mosaicism are physiological in the human blastocyst such as trophectoderm cells polyploidy (Bielanska *et al.*, 2002). On the other hand, while it would not affect the embryo itself, confined placental mosaicism may produce intrauterine growth retardation (IUGR) due to placental insufficiency (Spinner and Conlin, 2014). The greatest potential risk would be when mosaicism is present in the foetus (true foetal mosaicism). In this scenario, the consequences on the foetus can be diverse from non-affected to severely compromised embryo and/or born child. Actually, some well-known conditions in human are often presented in mosaic, such as Turner syndrome (Zhong and Layman, 2012). Reports of different births affected by chromosome mosaicism with a wide spectrum of symptoms have also been

published (Spinner and Conlin, 2014). For all these reasons, all pre, peri and post-natal counselling in cases of a mosaic finding are really challenging.

Preimplantation Genetic Testing for Aneuploidy

Being embryo aneuploidy the most important factor leading to implantation failure or early pregnancy loss both in natural and IVF conceptions (Hassold and Hunt, 2001), Preimplantation Genetic Testing for Aneuploidy (PGT-A) arose in the early 90s as a revolutionary and promising technique (Munné *et al.*, 1993).

The rationale behind the technique was that, by selecting euploid embryos for transfer and conversely discarding those aneuploid, the implantation rate per transfer would considerably increase (Gianaroli *et al.*, 1997) while pregnancy loss and gestations with chromosomal syndromes would be minimized. Therefore, PGT-A promised to reduce the time to a healthy live birth diminishing the number of failures during the process (Figure 9).

PGT-A has traditionally been proposed to patients that, according to their characteristics, were candidates to produce a remarkable proportion of aneuploid embryos. The classic indications have been: advanced maternal age (>37 years), repeated implantation failure (≥3 good quality embryos transferred failing to implant), recurrent miscarriages (≥3 pregnancy losses within the first trimester), severe male factor with consequences on the cytogenetic constitution of sperm, and previous pregnancies affected of chromosome abnormality.

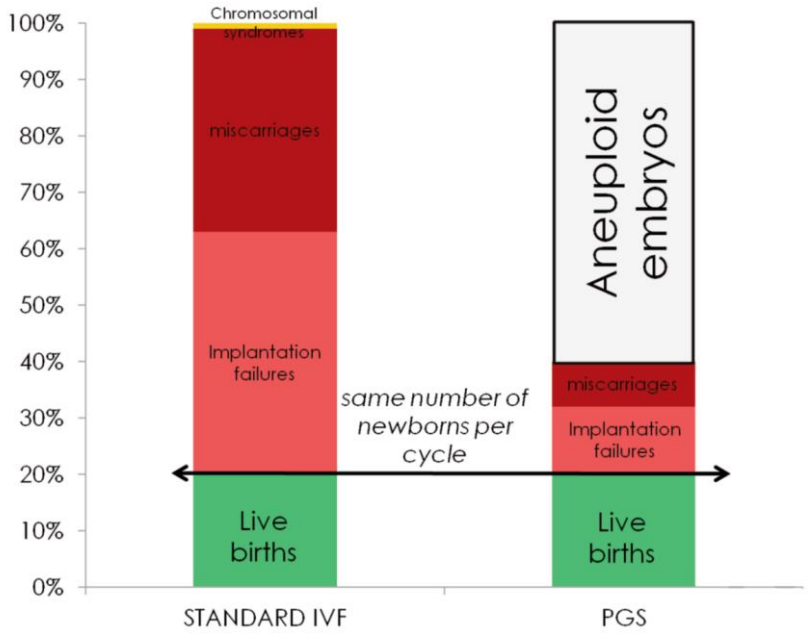

Figure 9: The rationale behind PGT-A. Reproduced from Capalbo *et al.*, 2015 (with permission).

Despite the promising premise of PGT-A, its history has been full of controversy and a constant path of evolution searching to improve the technique and prove its value.

PGT-A 1.0

Since the early times of PGT-A the most used approach was blastomere analysis by FISH (Figure 10). Although this was the main strategy used for a long time, it presented many limitations.

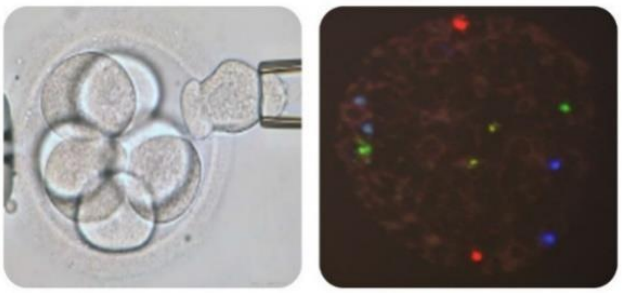

Figure 10: On the left, image of a blastomere biopsy on day 3 of embryo development. On the right, nucleus with a FISH hybridization for PGT-A.

On one hand, the biopsy of a blastomere from a day 3 cleaving embryo has been proven to be detrimental for embryo implantation (Scott *et al.*, 2013) regardless of previous data suggesting that embryo development would only be compromised if two blastomeres were extracted (De Vos *et al.*, 2009). Moreover, the analysis of a single blastomere was itself a limitation.

On the other hand, the main limitation of FISH methodology was the impossibility to analyse all the chromosomes as a consequence of a limited number of fluorescent probes and having only a single cell to analyse on which multiple hybridization rounds were not possible. Therefore, only a selected group of chromosomes were typically analysed (13, 15, 16, 17, 18, 21, 22, X, Y). However, aneuploidies have been reported to be notably present among all chromosomes (Capalbo *et al.*, 2014; Nakhuda *et al.*, 2018).

All the above-mentioned limitations together with the publication of PGT-A non-superiority trials (Staessen *et al.*, 2004, 2008) and even some randomized clinical trials evidencing a detrimental effect (Mastenbroek *et al.*, 2007, 2011; Hardarson *et al.*, 2008) opened a reflection period among scientific community with regards of PGT-A.

PGT-A 2.0

It was evident that several changes were needed in order to improve the effectivity of PGT-A making it a useful tool. There were several objectives that needed to be accomplished in order to do so. The use of Comprehensive Chromosome Screening (CCS) techniques together with the transition to the biopsy at the blastocyst stage allowed to overcome the limitations of PGT-A 1.0 and achieve better results (Dahdouh *et al.*, 2015)

Comprehensive Chromosome Screening Techniques

Aiming to overcome the limitations of FISH analysis, techniques of Comprehensive Chromosome Screening (CCS) were optimized and adapted to be used for PGT-A enabling a fast, reliable and cost-effective diagnosis from a single or few cells. Such techniques are able to detect aneuploidy in the whole chromosome set and each of them has its particularities (Wells *et al.*, 2008; Treff and Scott, 2012; Handyside, 2013). Most known and implemented CCS techniques are quantitative Polymerase Chain Reaction (qPCR), array of Single Nucleotide Polymorphisms (aSNP), and array of comparative genome hybridization (aCGH). The latter was the most used technique due to its feasibility and applicability in the clinical IVF context and its diagnostic reliability and reproducibility.

Nevertheless, in the recent years, the decrease in costs of genome sequencing has made this technique a real candidate for PGT-A analysis. Next-generation sequencing (NGS) has become the most predominant approach for PGT-A ousting aCGH (Fiorentino *et al.*, 2014; Yang *et al.*, 2015; Lai *et al.*, 2017). Among its advantages are its remarkable cost-efficiency, allowing the simultaneous analysis of a high number of samples, as well as an increased sensitivity and resolution for aneuploidy detection.

Trophectoderm biopsy

As early as in 1990, the use of a trophectoderm biopsy for PGT analysis was proposed in order to overcome the limitations of single-cell biopsy (Dokras *et al.*, 1990). However, many years of improvements in IVF protocols were needed for trophectoderm biopsy to finally be implemented in clinical practice (de Boer *et al.*, 2004). Optimisation of embryo culture to the blastocyst stage was key and was achieved by improvements in culture media, low oxygen tension (Bontekoe *et al.*,

2012; Gardner, 2016) and undisturbed culture conditions thanks to time-lapse technology (Yang *et al.*, 2014). Moreover, the irruption of the vitrification technique ensured excellent results of blastocyst cryopreservation (Cobo *et al.*, 2012; Taylor *et al.*, 2014b; Rienzi *et al.*, 2017). Finally, the development of high precision non-contact infrared laser technology for biopsy and its application was key for a harmless performance of trophectoderm biopsy (Veiga *et al.*, 1997; Boada *et al.*, 1998; Hartshorn *et al.*, 2005; Taylor *et al.*, 2010).

Trophectoderm biopsy offers a more reliable and robust diagnosis with less failures to obtain results (Forman *et al.*, 2012; Coll *et al.*, 2018) in comparison to single-cell biopsy thanks to the biopsy of multiple cells. Moreover, the chromosomal constitution of trophectoderm cells has been proven to be representative of the inner cell mass (ICM) (Fragouli *et al.*, 2008). Additionally, biopsy at the blastocyst stage does not seem to affect the reproductive potential of the biopsied blastocyst (Scott *et al.*, 2013). This may most likely be due to the biopsy being performed after genomic activation and the lower proportion of cells biopsied (5-10/150-300) in comparison to blastomere biopsy at cleavage stage (around 1/8). An additional advantage of trophectoderm biopsy is the fact that a selection by culture will be exerted before biopsy implying that only developing blastocysts being biopsied, thus reducing costs (Coll *et al.*, 2018).

However, blastocyst biopsy also presents some limitations. On one hand, in most settings, it will require of a deferred embryo transfer in order to have time to achieve diagnosis, although it may also be compatible with fresh transfer in some cases (Coates *et al.*, 2017). Despite the fact that no differences have been reported between one and another strategy, this could be an inconvenient for patients having to wait a longer time. On the other hand, while this would be rare at day 3 of embryo

development, it may happen that no blastocysts are available for biopsy (Franasiak *et al.*, 2014) and patients should be aware of this. Moreover, asynchrony in blastocyst formation may represent a logistics problem for the laboratory.

From the logistics point of view, there are different trophectoderm biopsy approaches that may be followed, each of them with its own advantages and limitations (Kokkali *et al.*, 2005; McArthur *et al.*, 2005; Capalbo *et al.*, 2014; ESHRE PGT Consortium and SIG-Embryology Biopsy Working Group *et al.*, 2020). However, to date, none has been clearly proven superior (Figure 11).

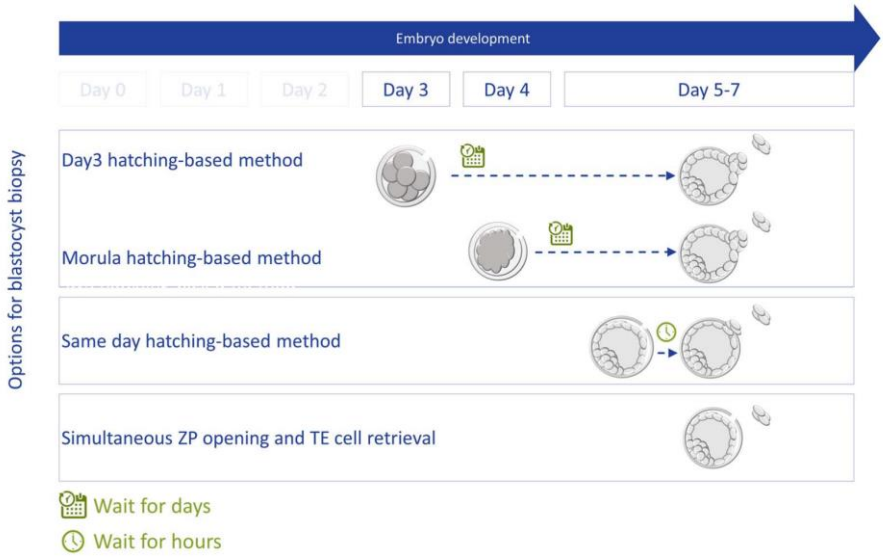

Figure 11: Methods for blastocyst biopsy. ZP: Zona Pellucida. TE: Trophectoderm. Reproduced from ESHRE PGT Consortium and SIG-Embryology Biopsy Working Group *et al.*, 2020 (with permission).

With regards to the trophectoderm biopsy itself, it should be the least invasive to ensure good results. Trophectoderm biopsy can be performed by combining the aspiration of the desired number of cells together with the appliance of laser pulses aimed at intercellular spaces. Then, by pulling the aspirated fragment it should release from the remaining blastocyst. In another approach for biopsy, pulling can

be substituted for flicking the aspirated fragment against the holding micropipette in order to release it (Figure 12).

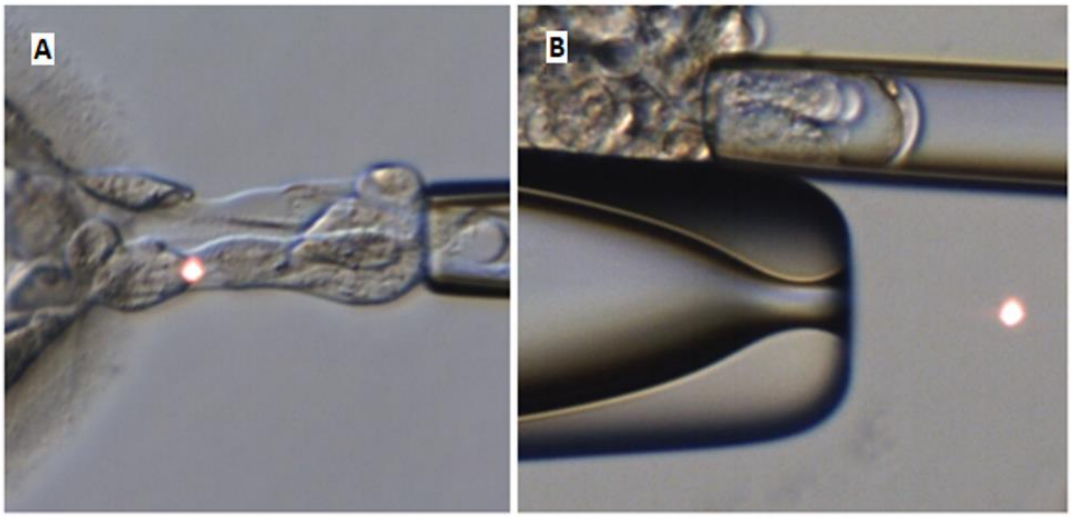

Figure 12: A: Image of a trophectoderm biopsy being obtained by pulling. B: Image of a trophectoderm biopsy being obtained by flicking. Reproduced from Coll *et al.*, 2022 (with permission).

After biopsy, the retrieved trophectoderm sample must be stored in a buffered solution before processing. The biopsy is usually isolated in a PCR tube after washings in microdroplets washing buffered solution. This procedure is key to ensure a clean sample for processing without exogenous DNA contamination.

For optimal laboratory logistics, the preferred strategy is to freeze all blastocysts after biopsy in order to have time for analysis, although, as previously stated, a fresh strategy can also be feasible in some occasions (Coates *et al.*, 2017).

Chromosomal mosaicism in the PGT-A context

Mosaicism: the eternal nemesis of PGT-A

From the origin of PGT-A, mosaicism has been a matter of concern, in many times suggested to compromise the usefulness of the technique itself. All PGT-A approaches have suffered either from the inability to detect mosaicism or from totally the opposite.

The analysis of polar body biopsies was initially proposed as a valuable PGT-A strategy. However, it was reported not to be an efficient option. Among its limitations, one was the fact that it could not detect post-zygotic errors (Verpoest *et al.*, 2018).

During the era of PGT-A 1.0 and cleavage-stage biopsy, only one blastomere was generally biopsied. Obviously, this approach was, conceptually, incompatible with mosaicism detection. Moreover, considering that mosaicism prevalence in day 3 embryos has been reported to be notably high, there was always a concern on the representativity of the biopsied cell and the possibility to wrongly categorize a mosaic embryo as either fully euploid or aneuploid (Mastenbroek *et al.*, 2011)(Figure 13).

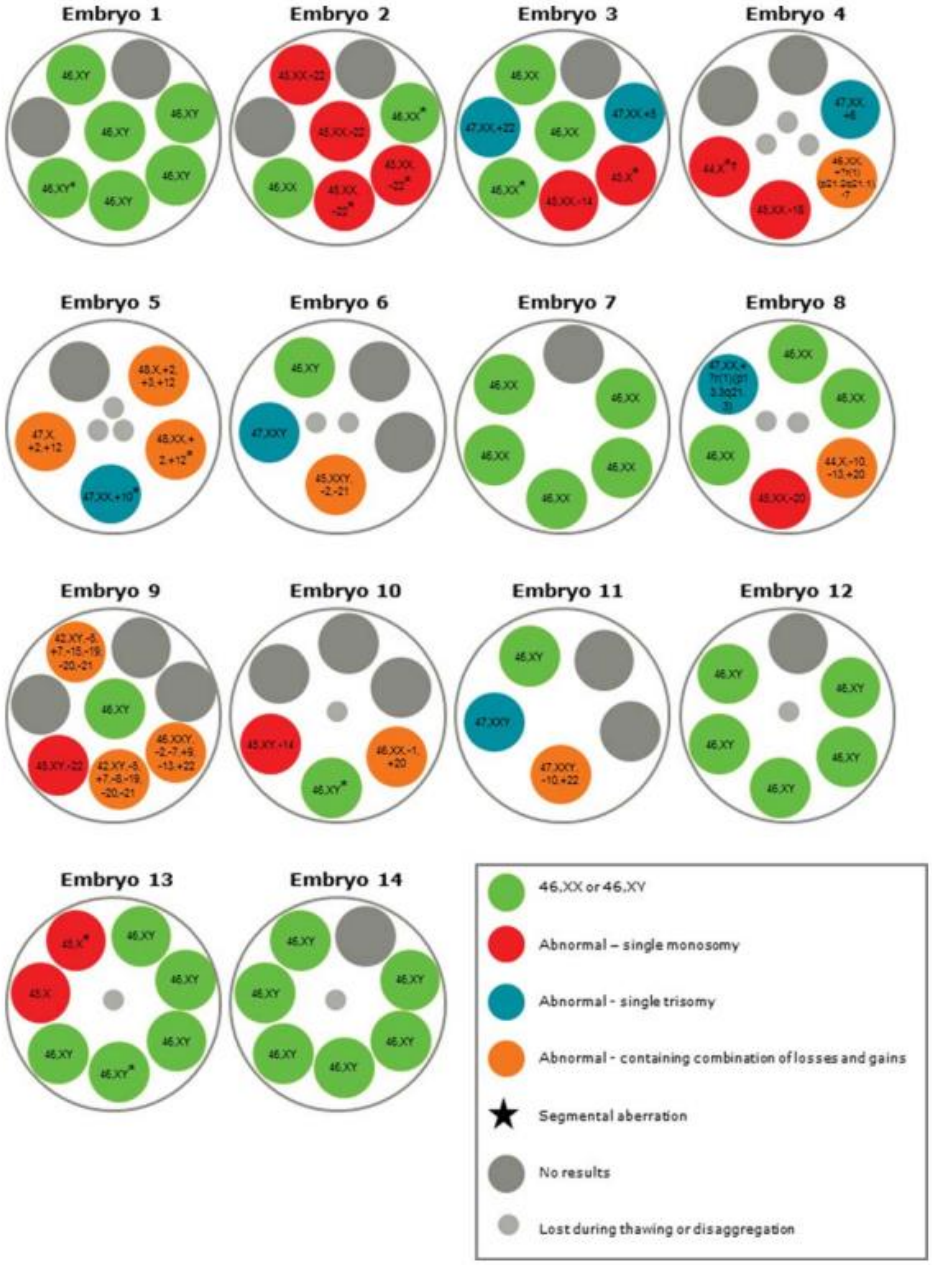

Figure 13: Chromosomal constitution of the blastomeres from 14 cleavage-stage embryos. Results evidence that mosaicism can compromise PGT-A when performing blastomere biopsy. Reproduced from Mertzanidou *et al.*, 2013 (with permission).

Later, with the change of paradigm of PGT-A 2.0, these issues seemed to be solved. Biopsies were performed at the blastocyst stage, when mosaicism prevalence was lower. Moreover, the combination of a multiple cell biopsy together with the development of more sensitive techniques for analysis, such as NGS, enabled mosaicism detection (Sermon *et al.*, 2016). However, the ability to detect mosaicism has been proven to be a double-edged sword, as the clinical management of euploid-aneuploid mosaic embryos is challenging. In addition, while early studies of trophectoderm biopsy representativity of the remaining embryo showed a very high accuracy (Evsikov and Verlinsky, 1998; Fragouli *et al.*, 2008), later concerns have arisen with regards of false positive and false negative diagnoses (Gleicher *et al.*, 2017; Popovic *et al.*, 2018).

Detection of mosaicism in trophectoderm biopsies

The most used technique for PGT-A in general, and mosaicism detection in particular, is, to date, NGS. While aCHG allowed to reliably detect mosaicism from around 50% (Mamas *et al.*, 2012), NGS has been reported to accurately detect mosaic abnormalities affecting from 20 to 80% of the biopsied cells (Maxwell *et al.*, 2016; Fragouli *et al.*, 2017; Munné *et al.*, 2017; Popovic *et al.*, 2018; Spinella *et al.*, 2018).

Typically, mosaicism is detected as an intermediate copy number variation between monosomy and disomy or disomy and trisomy (Figure 14).

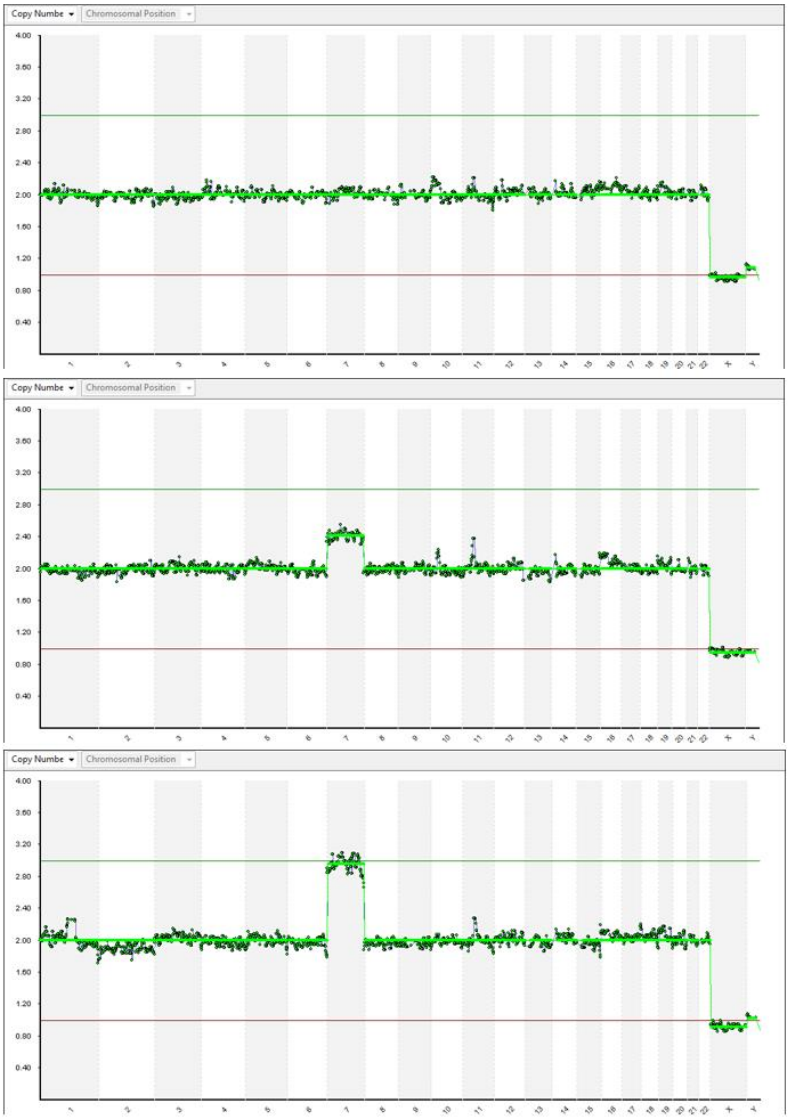

Figure 14: Example of mosaicism being detected as an intermediate copy number by NGS. From top to bottom: euploid male trophectoderm biopsy; mosaic trisomy 7; full trisomy 7.

One of the main problems of mosaicism is that, due to its nature, it can be either elusive or seem more severe than it actually is. Abnormal cells may not be homogeneously distributed in the blastocyst. Therefore, the result obtained from

the biopsy of 5-10 trophectoderm cells may not be representative of the remaining embryo. Moreover, the inner cell mass might be different from the trophectoderm in terms of mosaicism, potentially leading to false positives or negatives (Figure 15).

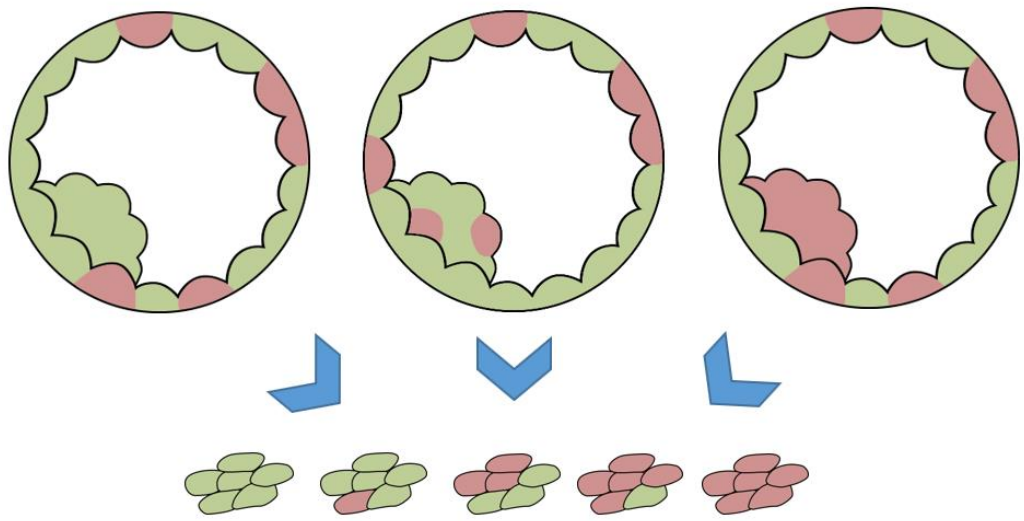

Figure 15: Example on how similar sets of biopsies might be obtained from completely different mosaic blastocysts.

In this sense, several authors evidenced that performing multiple trophectoderm (and ICM) biopsies from a single blastocyst, different results can be obtained (reviewed by Popovic *et al.*, 2020). Therefore, worries arose that false negatives and false positives could compromise the reliability of PGT-A, setting again the ground for the debate of the utility of this technique (Rosenwaks *et al.*, 2018). Specially concerning was the possibility that embryos with reproductive potential could be discarded considered as fully aneuploid. Despite important technical limitations compromising the study results, a publication of a paper ensuring that healthy births occurred after the transfer of embryos with a diagnostic of full aneuploidy had quite an impact (Gleicher *et al.*, 2016).

Artefactual or technical mosaicism

Despite the fact that an intermediate copy number result should be the consequence of a combination of euploid and aneuploid cells within a single biopsy, many authors have discussed that it could also be a consequence of a technical artefact (Capalbo *et al.*, 2017b; Goodrich *et al.*, 2017; Popovic *et al.*, 2020). In other words, a false mosaic result is obtained from a euploid biopsy as a consequence of the introduction of a technical artefact. This kind of "mosaic" finding has been typically called artefactual or technical mosaicism.

It has to be taken into account that, until the obtention of a result, the sample to be analysed undergoes several procedures that altogether or individually may lead to artefactual mosaicism.

First, the methodology through which the trophectoderm biopsy is obtained and the characteristics of such sample may play a key role in the generation of artefacts. It seems reasonable to think that if the sample is damaged, this may affect the results, as some authors have discussed (Munné and Wells, 2017; ESHRE PGT Consortium and SIG-Embryology Biopsy Working Group *et al.*, 2020; ESHRE PGT-SR/PGT-A Working Group *et al.*, 2020). Actually, the Preimplantation Genetic Diagnosis International Society (PGDIS) include in their mosaicism guidelines that the biopsy of too few cells as well as a suboptimal biopsy technique may lead to poorer quality results compatible with mosaicism (Leigh *et al.*, 2022). In this sense, it has been suggested that the use of laser pulses during the biopsy as well as the potential mechanical damage on the sample using different biopsy methodologies (flicking or pulling) may be related to artefactual mosaicism.

Then, the biopsy has to be isolated into a PCR tube containing a buffered solution: a procedure generally known as "tubing". During this procedure, exogenous DNA

contamination has to be avoided at all costs as, among other serious consequences, it could lead to artefactual results. After that, the sample needs to undergo whole genome amplification (WGA), which can generate artefacts too. Additionally, using PGT-A with NGS, inefficiencies during all the library preparation steps can occur. Last, but not least, the final result showing a profile in which the copy number for each chromosome is observed is, at the end, the result given by an algorithm interpreting the data. Therefore, artefactual mosaicism of bioinformatic origin is also plausible (Figure 16).

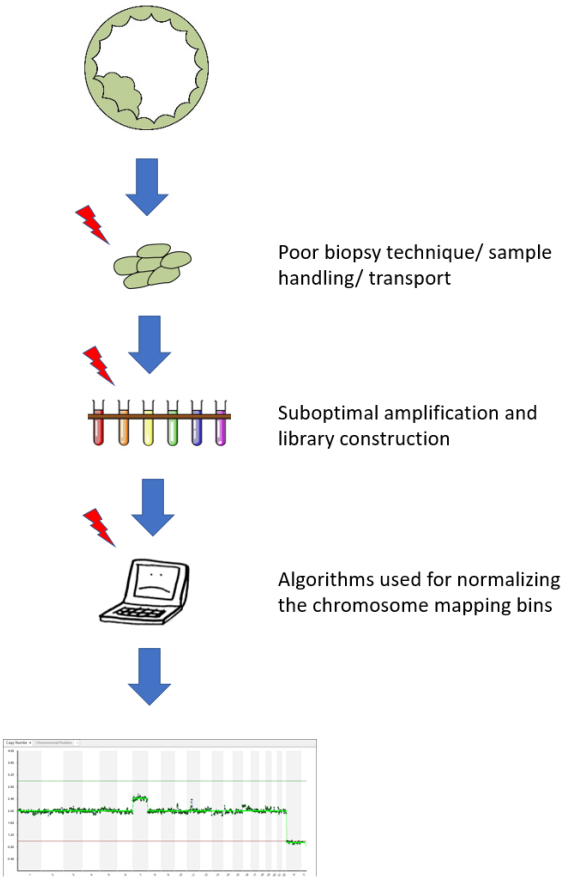

Figure 16: Graphical explanation of artefactual mosaicism and its potential origins.

It should also be taken into consideration that, on some occasions, artefactual mosaicism could be not originated by the technique itself but have a biological origin. A non-specific chromosome gain or loss might be detected due to cells being in S-phase (ESHRE PGT-SR/PGT-A Working Group *et al.*, 2020).

The challenging management of a mosaic diagnosis

As the technique surrounding PGT-A evolved, mosaicism began to be detected in trophectoderm biopsies. At the beginning, due to lack of experience and data, euploid-aneuploid mosaic embryos were usually considered non transferrable. However, in 2015, the report of live-births after the transfer of mosaic embryos, set a new scenario (Greco *et al.*, 2015). The paper showed the transfer outcomes of 18 mosaic embryos diagnosed by aCGH, reporting a healthy live birth rate of 33.3% (6/18) (Table 2).

Since that moment, several groups began to consider mosaic embryos for transfer as evidenced by the results from a survey including 102 IVF centres from 32 countries around the world (Weissman *et al.*, 2017)(Figure 17). The survey also evidenced discrepancies in the decision to report mosaicism and the cut-off values to call mosaicism. There was consensus in the need of more research, being the study of mosaicism distribution within the embryo and clinical data the topics of most interest.

Table 2: Details on the first mosaic embryos transferred and their outcomes. Reproduced from Greco *et al.*, 2015 (with permission). Copyright Massachusetts Medical Society.

Table 1. Clinical Outcomes of Single Mosaic Blastocysts Transferred.*

Patient No.	Chromosomal Constitution	Mosaicism†	Karyotype‡	Clinical Outcome
		percent		
1	arr(4)x1,(10)x1	40	46,XX	Baby healthy at birth
2	arr(6)x1,(15)x1	50	46,XX	Baby healthy at birth
3	arr(2)x1	40	46,XX	Baby healthy at birth
4	arr(2)x1	35	46,XY	Baby healthy at birth
5	arr(5)x1	50	46,XX	Baby healthy at birth
6	arr(5)x1,(7)x1	40	46,XX	Baby healthy at birth
7	arr(11)x1,(20)x3,(21)x3	30	NA	No pregnancy
8	arr(1)x1,(6)x3,(10)x3,(12)x3,(13)x3,(14)x3,(21)x3	50	NA	No pregnancy
9	arr(3)x1,(10)x3,(21)x3	35	NA	No pregnancy
10	arr(1)x3	50	NA	Biochemical pregnancy§
11	arr 9p21.2q34.3(26,609,645-140,499,771)x3	45	NA	Biochemical pregnancy§
12	arr(15)x3	30	NA	No pregnancy
13	arr(18)x1	50	NA	No pregnancy
14	arr(18)x1	50	NA	No pregnancy
15	arr(18)x1	40	NA	No pregnancy
16	arr(4)x1	50	NA	No pregnancy
17	arr(5)x3	40	NA	No pregnancy
18	arr 10q21.3q26.3(67,216,644-134,326,648)x3	50	NA	No pregnancy

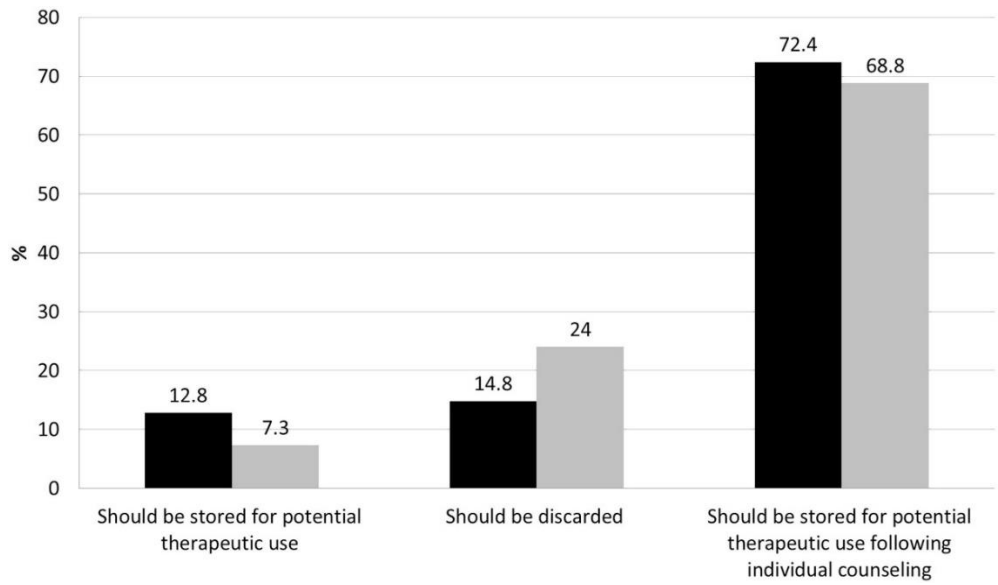

Figure 17: Opinions on the fate of mosaic embryos. Dark bars: centres performing PGT-A. Grey bars: centres not performing PGT-A. Reproduced from Weissman *et al.*, 2017 (with permission).

Consequently, as mosaic embryos began to be transferred, new data arose. A retrospective study reported, after sample reanalysis with NGS, that, among pregnancies with aCGH-analysed euploid embryos, those that miscarried were mosaic in a higher proportion than those ending up in a live birth (Maxwell *et al.*, 2016). Additional data has been published evidencing that mosaic embryos performe worse than euploid embryos with lower pregnancy rate and higher miscarriage rate. Moreover, some authors have tried to determine which mosaic embryos present a better prognosis according to the type of abnormality (monosomy/trisomy, whole-chromosome/segmental, simple/complex, high percentage/low percentage) with different results among them (Fragouli *et al.*, 2017; Munné *et al.*, 2017; Spinella *et al.*, 2018; Munné *et al.*, 2019b; Victor *et al.*, 2019b). As new data have been made available, scientific societies have developed and updated guidelines for mosaic diagnosis and mosaic embryo transfer (MET) (COGEN, 2018; Cram *et al.*, 2019; Leigh *et al.*, 2022).

Despite the fact that ongoing pregnancies after MET seem to end up in healthy births, there has always been a concern with regards to a potential adverse effect to the foetus or the newborn. It is true that data seem to point to the fact that either the aneuploid line prevails and the embryo fails to implant or miscarries, or the euploid line prevails and the embryo results in the birth of a healthy baby. However, there is a potential risk that the presence of an abnormal cell line could result in abnormalities in the foetus, the baby, or even in adulthood.

Mosaic abnormalities of specific chromosomes may be more prone to affect an ongoing pregnancy. Some authors proposed a risk categorization of chromosomes according to their implication in anomalies found in products of conception (Grati *et al.*, 2018) (Figure 18). Monosomies and trisomies should be equally considered for

transfer as a monosomy can be the complement of a trisomy originated by mitotic non-disjunction.

- Mosaic trisomies 1, 3, 10, 12 and 19 have a composite score of 0 and have the highest priority for transfer because of a very low risk of any of these adverse outcomes.
- Mosaic trisomies 4 and 5 and 47,XYY have a composite score of 1 and are the second group to be considered for transfer, albeit with a disclaimer of a slightly increased likelihood of miscarriage (**Tables 4 and 5**) or a viable aneuploidy (47,XYY).
- Mosaic trisomies 2, 7, 11, 17 and 22 have a composite score of 2 and are the third group to be considered for transfer, having a slightly higher risk of miscarriage or a relatively low risk for UPD (trisomies 7 and 11).
- Mosaic trisomies 6, 9 and 15 have a composite score of 3 due to increased risk of miscarriage, UPD or viable aneuploidy. The possibility for transfer should be considered with caution and only after detailed discussion with the prospective parents.
- Mosaic trisomies 8, 20, 47,XXX and 47,XXY have a composite score of 4–5, due to high risk of fetal involvement and a slightly increased risk for miscarriage and viable aneuploidy. The possibility for transfer could be considered after extensive discussion with prospective parents regarding the possible clinical manifestations thereof.

The remaining mosaic aneuploidies are best avoided: trisomies 13, 14, 16, 18, 21 and 45,X.

Figure 18: Chromosome risk groups for mosaicism according to Grati's categorization. Reproduced from Grati *et al.*, 2018.

The figure of the genetic counsellor has become of capital importance in an ART setting for many reasons. Counselling before MET has become one of them. They are essential to provide patients with all the updated information available with regards to the prognosis of a MET in order to allow an informed decision on transferring or not such kind of embryos (Besser and Mounts, 2017). The lack of data and the

uncertainty with regards to MET outcomes has made the decision very difficult for patients.

For all these reasons, the recommendation of performing prenatal diagnosis after any established pregnancy following PGT especially applies after a MET. Most guidelines and recommendations agree that prenatal testing options should be offered to patients disclosing the advantages and limitations of each approach (NIPT, CVS, Amniocentesis). With regards to mosaicism, the most representative analysis would be amniocentesis. (Besser and Mounts, 2017; COGEN, 2018; Leigh *et al.*, 2022).

2 HYPOTHESIS AND OBJECTIVES

HYPOTHESIS

There are intrinsic and extrinsic factors related to chromosomal mosaicism in human blastocysts from Preimplantation Genetic Testing cycles.

OBJECTIVES

1. To investigate the chromosomal constitution of mosaic blastocysts.

2. To identify the factors that may be associated with chromosomal mosaicism in preimplantation human blastocysts.

3. To better understand the mechanisms of mosaicism.

4. To assess the impact of chromosomal mosaicism diagnosis on PGT-A patients.

    a. Prevalence
    b. Decision making
    c. Pregnancy follow-up

3 WORK PLAN

The objectives of the present thesis have been accomplished through **3 different studies** that have been published in international journals in reproductive medicine and are presented in the following section.

The **first study** was developed to improve the knowledge of mosaicism at the blastocyst stage in the IVF and PGT-A context. We retrospectively analysed the data of our PGT-A programme in order to assess the prevalence of mosaicism, the type of mosaic abnormalities found, the chromosomes affected by mosaic abnormalities, and, most importantly, to identify whether specific patients' ad IVF cycles' characteristics were associated with mosaicism.

As no published data were available with regards to the role of the trophectoderm biopsy on the generation of artefactual mosaic results, we put in place a **second study** with prospective data collection to assess whether different biopsy techniques were related to different mosaicism prevalences.

Finally, from the beginning of the thesis project, it was evident that patients were importantly affected by the diagnosis of mosaicism in their embryos and had to face important decisions with regards to the possibility of MET. That is why, throughout the whole development of the project, we collected data for a **third study** to assess mosaicism from patients' perspective, bring light to the complicated MET decision-making process, and analyse the outcomes of METs in our setting.

4 RESULTS

Prevalence, types and possible factors influencing mosaicism in IVF blastocysts: results from a single setting

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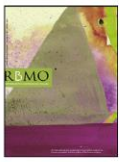
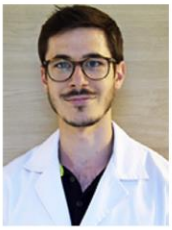


ARTICLE

Prevalence, types and possible factors influencing mosaicism in IVF blastocysts: results from a single setting



BIOGRAPHY

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KEY MESSAGE

To the best of our knowledge, this is the first study to comprehensively assess embryo mosaicism and the factors affecting it within a single set-up. Paternal age was the only factor showing an association with mosaicism. Further research on the role of male factor in embryo mosaicism is needed.

ABSTRACT

Research question: Are intrinsic or extrinsic factors associated with embryo mosaicism prevalence in IVF cycles?

Design: Retrospective cohort study of preimplantation genetic testing for aneuploidy (PGT-A) cycles carried out at a university-affiliated IVF clinic between October 2017 and October 2019. Trophectoderm biopsies were analysed by next generation sequencing. Mosaicism prevalence, type of anomaly and the chromosomes involved were analysed. Intrinsic and extrinsic factors potentially inducing mosaicism were studied: maternal and paternal age, antral follicle count, cumulus–oocyte complexes retrieved, female body mass index, PGT-A indication, sperm concentration, total dosage of gonadotrophins, embryo quality and day of blastocyst formation, single-step commercial media used and biopsy operator.

Results: Overall prevalence of mosaicism in our PGT-A setting was 13.9%. In segmental mosaicism, larger chromosomes tended to be more affected, which was not observed in whole-chromosome mosaicism. Additionally, segmental mosaicism was mostly observed in monosomy (69.6%; $P < 0.01$) compared with whole-chromosome mosaicism (49.7% monosomies versus 50.3% trisomies; $P = 0.83$). Although a high inter-patient variability was observed, only paternal age showed a positive association with mosaicism (adjusted OR 1.26, 95% CI 1.02 to 1.54) among the analysed variables.

Conclusions: Our results suggest remarkable differences in the mechanisms generating segmental and whole-chromosome mosaicism, indicating that they may deserve different consideration when studying them and when prioritizing them for transfer. Male factor seems to be associated with mosaicism and may be worthy of specific assessment in future studies.

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KEYWORDS

Biopsy
Blastocyst
Mosaicism
PGT-A

INTRODUCTION

Mosaicism is biologically defined as the presence of more than one cell lineage with different cytogenetic composition within the same tissue or organism. Although this phenomenon was first reported in preimplantation human embryos as early as 1993 (*Delhanty et al., 1993*), this has recently become a matter of concern among the assisted reproductive technology (ART) scientific community after the analysis of the results obtained after from the transfer of mosaic embryos.

Traditionally, preimplantation genetic testing for aneuploidies (PGT-A) has allowed the cytogenetic characterization of preimplantation embryos as either euploid or aneuploid. Nevertheless, improvements in PGT-A methodology involving the analysis of multiple cells at the blastocyst stage and the use of high sensitivity diagnostic techniques (*Sermon et al., 2016*) have allowed the identification of a third embryo category: the mosaic embryo. Mosaicism can be found in embryos as a coexistence of euploid and aneuploid cells (usually called a euploid–aneuploid mosaic embryo) or as a mixture of aneuploid cells with no presence of a euploid cell line (an aneuploid–aneuploid mosaic embryo). In the context of ART, it is the euploid–aneuploid mosaic embryo that is clinically relevant owing to its reproductive potential.

The ability to detect mosaicism in PGT-A cycles is a double-edged sword. *Greco et al. (2015)* first reported that mosaic embryos can result in healthy live births. Despite the prognosis of mosaic embryo transfer (MET) being worse than the one expected from a euploid embryo for implantation potential and miscarriage (*Maxwell et al., 2016*; *Fragouli et al., 2017*; *Munné et al., 2017*; *2019*; *Spinella et al., 2018*; *Victor et al., 2019*), all babies born from a mosaic embryo seem to be healthy so far. The potential consequences of mosaicism in such embryos, however, cannot be dismissed and, to date, the available data on babies born from MET are limited. The clinical effect of chromosome mosaicism cannot be predicted, as each event can result in a totally different outcome depending on its characteristics and the distribution of the different cell lines within the embryonic and fetal tissues (*Taylor et al., 2014*). Therefore, most efforts are currently focused on collecting further data from MET to establish the risks and outcomes of different kinds of mosaicism. Moreover, the clinical management of mosaic embryos is a matter of concern. As a result, scientific societies such as PGDIS and COGEN have proposed guidelines to prioritize such embryos for transfer (*COGEN, 2018*; *Cram et al., 2019*), and an evidence-based scoring system founded on prenatal testing results of regular pregnancies has been published (*Grati et al., 2018*).

It is known that, in contrast to constitutional aneuploidy, which results from meiotic errors during oocyte or sperm formation, mosaicism is generated through a mitotic error in the cleaving embryo. Meiotic errors in gametes have been studied in detail (*Hassold and Hunt, 2001*); however, little is known about the origins of embryo mosaicism. Different mechanisms originating mitotic errors in preimplantation embryos have been reported (*Taylor et al., 2014*). First cleavages during embryo development are known to be rapid. To allow that to happen, it has been postulated that cell cycle checkpoints may need to be less strict. In addition, some factors can increase the risk of mitotic errors, such as aberrations in the centrosome, mitotic spindle or defects in chromatid cohesion (*McCoy, 2017*). This allows mitotic errors to occur and persist.

With this idea in mind, intrinsic mosaicism in human embryos may exist. Available evidence highlights huge variations in the prevalence of mosaicism in human blastocyst, ranging from around 5–45% (*Northrop et al., 2010*; *Fragouli et al., 2011*; *2017*; *Capalbo et al., 2013*; *Novik et al., 2014*; *Greco et al., 2015*; *Ruttanajit et al., 2016*; *Munné et al., 2017*; *Nakhuda et al., 2018*; *Popovic et al., 2018*). Although these differences could be attributed to different study methodologies and techniques used, data on mosaicism prevalence among trophectoderm biopsies using the same technique still demonstrate remarkable variation (*Ruttanajit et al., 2016*; *Sachdev et al., 2016*; *Munné et al., 2017*; *Simon, 2017*; *Nakhuda et al., 2018*). All these data suggest that, although an intrinsic prevalence of mosaicism may exist in human preimplantation embryos, extrinsic factors could also induce it.

The existence of extrinsic factors generating mosaicism was reported for the first time in a study analysing the differences in mosaicism prevalence among different centres despite the fact that it was conducted on a controlled patient population (egg donors) and the PGT-A was carried out in the same laboratory (*Sachdev et al., 2016*). As mentioned above, virtually any extrinsic factor that can affect mitotic divisions may induce mosaicism (iatrogenic mosaicism). In this sense, many investigators have suggested several factors that may cause such a phenomenon during an IVF cycle: from ovarian stimulation, to culture media, as well as different laboratory and culture conditions (*Fragouli et al., 2010*; *Morbeck et al., 2017*; *Katz-Jaffe et al., 2018*; *Swain, 2019*). Moreover, some technical inefficiencies during the biopsy and sample processing for analysis and the algorithms used by different diagnostic platforms may lead to the detection of artefactual or technical mosaicism (*Munné and Wells, 2017*; *Fragouli et al., 2019*). All these factors have been extensively discussed; however, few studies have assessed its effect within an IVF and PGT-A setting.

Given the limited available data on the outcomes of mosaic embryo transfers and even more limited data on factors affecting mosaicism published to date, the potential factors that may affect embryo mosaicism in a PGT-A setting were investigated.

MATERIALS AND METHODS

Study design and population

A retrospective analysis of the chromosomal constitution through next generation sequencing (NGS) of trophectoderm biopsies from 482 PGT-A cycles was conducted in a private centre between October 2017 and October 2019. The prevalence of mosaicism, the chromosomes involved as well as intrinsic and extrinsic factors that may correlate with mosaicism were analysed. Indications for PGT-A included advanced maternal age (>37 years), recurrent miscarriages (three or more), male factor (severe male factor, altered fluorescence in-situ hybridization in spermatozoa, altered sperm DNA fragmentation) and repeated implantation failure (three or more transfers without implantation or five or more optimal quality transferred embryos failing to implant).

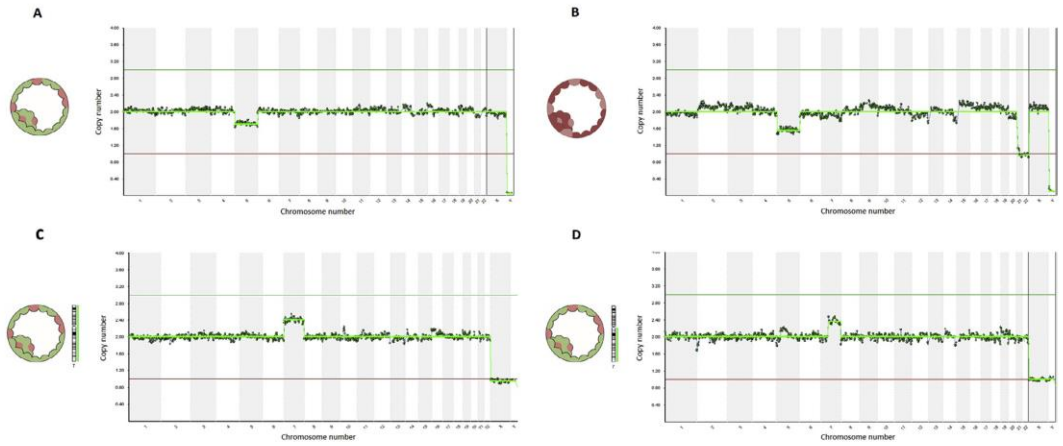

FIGURE 1 Copy-number variation diagrams showing different types of mosaicism. (A) An euploid-aneuploid mosaic as no meiotic anomalies are observed (mosaic monosomy in chromosome 5); (B) an aneuploid–aneuploid mosaic (mosaic monosomy in chromosome 5 with aditional imbalances from meiotic origin); (C) a whole-chromosome mosaicism in trisomy in chromosome 7; (D) a segmental trisomy in mosaic in the same chromosome (q arm gain).

IVF cycle

Ovarian stimulation was carried out according to established standard protocols (*Martinez et al., 2016*). Briefly, gonadotrophins were used in a flexible GnRH antagonist protocol. The stimulation protocol and gonadotrophin dose were chosen depending on age, body mass index (BMI) and ovarian reserve tests. Gonadotrophin releasing hormone (GnRH) agonist (triptorelin 0.2 mg) was used for ovulation stimulation when at least three follicles measuring wider than 17 mm were observed on ultrasound (according to ovarian response and clinician discretion).

Oocytes were retrieved 36 h after ovulation was triggered. Recovered cumulus–oocyte complexes (COC) were washed and stored in fertilization media. Denudation was conducted before intracytoplasmic sperm injection 4 h after collection. Inseminated oocytes were cultured in a single-step culture media using a time-lapse incubator (Geri®) (Merck, Darmstadt, Germany) with low oxygen tension (5%). Either LifeGlobal® (CooperSurgical Inc, Trumbull, CT, USA) media or Vitrolife® (Vitrolife, Göteborg, Sweden) media (G series) were used for gamete and embryo handling and culture during the period analysed.

Preimplantation genetic testing for aneuploidy procedure

Zona opening was carried out on normally developing day-3 embryos (66 ± 2 h after insemination) using laser thermolysis (OCTAX Navilase™) (Olympus, Tokyo, Japan) while embryos were kept in the culture dish. Culture was resumed until day 5, 6 or 7. Hatching or fully hatched blastocysts showing a well-defined inner cell mass and a trophectoderm with multiple cells were eligible for biopsy. Three to eight trophectoderm cells were obtained through laser thermolysis and micromanipulation. Biopsied blastocysts were immediately vitrified and kept stored until diagnosis was achieved.

Trophectoderm samples were placed in 0.2 ml polymerase chain reaction tubes with 1.5 µl of 1 x phosphate saline buffered (PBS) after thorough washings in droplets of 1 x PBS with 0.1% polyvinyl alcohol. Tubed samples were centrifuged at 202 g for 3 min and then stored in a –80°C freezer until processing.

Whole-genome amplification was achieved using the Sureplex Amplification System (Illumina®, USA). The cytogenetic analysis of the biopsies was performed by NGS using the VeriSeq™PGS - MiSeq® platform (Illumina, San Diego CA, USA) following the manufacturer's protocols and guidelines.

Embryos were diagnosed as euploid, aneuploid or mosaic. An anomaly was reported to be mosaic when the deviation from the normal copy number was 30% or more and less than 70%.

Mosaic anomalies were sub-classified in different groups. Mosaic embryos were classified as euploid–aneuploid (embryos with at least one mosaic anomaly and no meiotic aneuploidy) and aneuploid–aneuploid (embryos with at least one mosaic anomaly together with at least one meiotic aneuploidy). Whole-chromosome mosaicism refers to a mosaic alteration of an entire chromosome, whereas segmental mosaicism defines a mosaic alteration affecting only a fragment of a chromosome (FIGURE 1).

Statistical analysis

An analysis of the mosaicism prevalence, type of anomaly and chromosomes involved was conducted. For the assessment of the factors that could induce mosaicism, embryos were divided in two groups: mosaic embryos (euploid–aneuploid and aneuploid–aneuploid) and embryos with no mosaicism (euploid and aneuploid). Intrinsic patient and embryonic factors that could influence embryo mosaicism were studied: maternal and paternal age, antral follicle count, female BMI, PGT-A indication, sperm concentration (oligozoospermia versus normal), embryo quality and day of blastocyst formation. Factors associated with the IVF procedure were also analysed as potential sources of mosaicism: the number of COCs retrieved, the total dose of gonadotrophins, and the use of two different culture media (Global® Total® [Cooper Surgical, Trumbull, CT, USA] versus G-TL™ [Vitrolife, Göteborg Sweden). Finally, artefactual mosaicism potentially caused by different biopsy operators was assessed.

TABLE 1 CYCLE CHARACTERISTICS

	Mean ± SD
Female age	39.5 ± 3.1
Male age	40.7 ± 4.9
Antral follicle count	12.1 ± 6.2
Female body mass index	23.0 ± 3.9
Ovarian stimulations per PGT-A cycle	1.2 ± 0.5
Total dose of gonadotrophins	2699.9 ± 828.7
Biopsied embryos per PGT-A cycle	3.7 ± 2.6

PGT-A, preimplantation genetic testing for aneuploidy.

For the analysis of mosaicism prevalence per chromosome, three mixed models were adjusted according to the type of mosaicism (total, whole and segmental), with the chromosome as fixed effect and the embryo within a same cycle and within a same patient as a random effect.

Continuous variables were described with mean and SD, whereas categorical variables were described with number and percentage. Chi-squared test was used to compare categorical variables, and t-test or Wilcoxon Mann–Whitney test were used to compare continuous variables according to the necessary assumptions.

A logistic mixed multivariable model with random intercepts was applied to estimate the odds for every end point. Patient and biopsy procedure were treated as random effects to control the correlated observations effect and the operator random sample effect. Continuous variables were standardized (subtracting the mean and divided by SD) to help the model converge. Mixed model was adjusted using linear and nonlinear mixed effects models package (nlme) (*Pinheiro et al., undated*). Evaluation of collinearity was through examination of variance inflation factor, with none of the predictors presenting a value higher than 3.

IBM© SPSS© Statistics v 22 and R software (*R Core Team, 2018*) was used for statistical analyses. For all comparisons, a statistical significance was set at $P < 0.05$. Packages [nlm4] and [MASS] were used to adjust the mixed models (*Venables and Ripley, 2002*; *Bates et al., 2015*) .

Ethical approval

This study was approved by our Institutional Review Board on 18 July 2018 (Reference number 13201807186).

RESULTS

A total of 1708 blastocysts from 482 PGT-A cycles were included. The characteristics of each cycle are presented in TABLE 1.

A euploid result was observed in 596 embryos (34.9%), whereas 875 embryos (51.2%) were aneuploid; 237 (13.9%) showed at least one mosaic anomaly independently of their ploidy. A total of 111 blastocysts were mosaic euploid–aneuploid (6.5%) and 126 (7.4%) mosaic aneuploid–aneuploid (FIGURE 2).

Mosaicism and chromosomes

Most mosaic embryos presented whole-chromosome mosaicism (140/237 [59.1%]), whereas 87 (36.7%) were segmental mosaics. Fewer embryos (10/237 [4.2%]) showed a mixture of segmental and whole-chromosome mosaicism within the same embryo. Most of the embryos had only one altered chromosome (194/237 [81.9%]). Two chromosomes were involved in 28 cases (11.8%) and 15 mosaic embryos (6.3%) showed mosaicism in three or more chromosomes.

The distribution of mosaicism among chromosomes was uneven in whole-chromosome mosaicism and segmental mosaicism. In segmental mosaicism, however, larger chromosomes generally tended to be more affected for such phenomenon, which was not observed in whole-chromosome mosaicism (FIGURE 3). Chromosomes 1, 5 and 9 were found to be remarkably affected by segmental mosaicism.

Overall, from 291 mosaic events detected in 237 mosaic embryos, 126 were observed as a trisomy (43.3%) compared with 165 observed as monosomy (56.7%). For whole-chromosome mosaicism, monosomy and trisomy were equally observed (49.7% versus 50.3%). Conversely, for segmental mosaicism, the percentage of embryos with mosaic monosomy was significantly higher than mosaic trisomy (69.6 versus 28.4 %, $P < 0.001$).

Factors potentially affecting mosaicism

None of the analysed factors were found to be associated with mosaicism prevalence when studied individually (TABLE 2 and TABLE 3).

A multivariable model was applied. Antral follicle count was excluded from the model to avoid multicollinearity as it was observed to be highly correlated with female age. The analysis showed a positive association between mosaicism prevalence and paternal age (adjusted OR 1.25, 95% CI 1.02 to 1.53) (TABLE 4 and FIGURE 4). Although no further associations with specific patient factors were found, huge variation occurred in the prevalence of mosaicism among them (FIGURE 5A). Neither the variables regarding iatrogenic mosaicism nor biopsy operator as a potential source of artefactual mosaicism (FIGURE 5B) were observed to be related to such phenomenon.

DISCUSSION

The present study is, to our knowledge, the first to investigate in depth laboratory and clinical factors that may be associated with embryo mosaicism. According to our results, paternal age seems to be the only factor that significantly and independently increases the prevalence of mosaicism. On the contrary, none of the other clinical or laboratory factors tested seem to have any effect on mosaicism prevalence.

The observed overall prevalence of mosaicism in the present study was 13.9%, which is in the lower range of mosaicism prevalence reported in trophectoderm biopsies using NGS technology (*Ruttanajit et al., 2016*; *Munné et al., 2017*; *Simon, 2017*; *Nakhuda et al., 2018*). This could be attributed to specific characteristics of our IVF setting but also to a narrower threshold for mosaicism calling (30–70%). Although many validations by cell-mixing experiments have shown that NGS-based PGT-A can detect mosaic anomalies reliably from 20–80%, some investigators have discussed that cell-

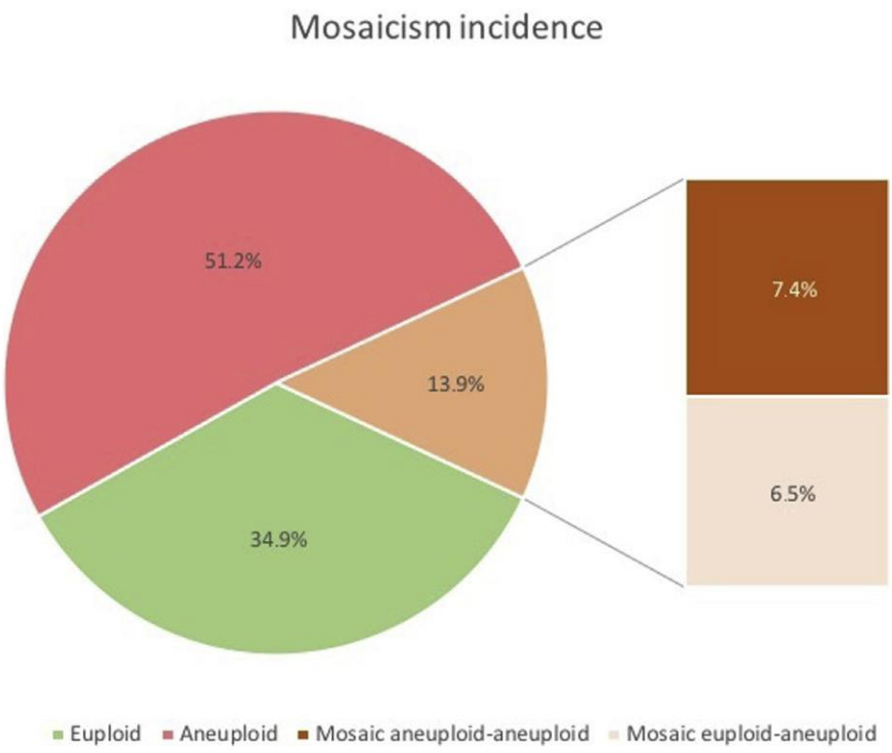

FIGURE 2 Mosaicism prevalence.

mixing experiments may not properly represent a trophectoderm biopsy sample and detection may not be as accurate when analysing such material (*Capalbo et al., 2013*; *Munné and Wells, 2017*; *Treff and Franasiak, 2017*; *Fragouli et al., 2019*). Therefore, background noises or typical described artefacts could be easily confused for a 20% mosaicism.

Most mosaicism events were observed affecting the whole chromosome. More than one-third of mosaic alterations, however, were segmental, in agreement with other published reports (*Munné et al., 2017*; *Nakhuda et al., 2018*). Conflicting information on the clinical outcomes of segmental mosaicism has been published (*Fragouli et al., 2017*; *Munné et al., 2017*; *Victor et al., 2019*). Consequently, no specific position has been taken on segmental mosaicism in the published guidelines for mosaic embryo transfer (*COGEN, 2018*; *Cram et al., 2019*). More evidence on the cause of segmental aneuploidies will be essential to make the best clinical decision concerning embryos with such anomalies.

As observed in meiosis (*Franasiak et al., 2014*; *Nakhuda et al., 2018*), our results show that mitotic errors also seem to be distributed unevenly among chromosomes. In agreement with previously published data (*Munné et al., 2017*), larger chromosomes were observed to be more affected by segmental mosaicism than smaller ones, a finding that was not observed for whole-chromosome mosaicism. Owing to their size, such chromosomes might be more prone to breaks, leading to segmental aneuploidies. Remarkably, chromosomes 1, 5 and 9 showed an exceptionally high frequency of segmental mosaicism. Presence of heterochromatin blocks can lead to breakages as they are challenging for DNA replication (*Saksouk et al., 2015*). Conveniently, both chromosomes 1 and 9 are rich in such regions. Moreover, other break hot-spots unrelated to heterochromatin have been reported (*Durkin and Glover, 2007*), with chromosome 5 presenting several of such regions. As whole-genome amplification is required for PGT-A intervention, artefactual segmental mosaicism may also be generated because of the above-mentioned regions, challenging this additional and extensive replication procedure.

Several investigators have suggested anaphase lagging as the main mechanism for the generation of mitotic errors in preimplantation embryos (*Coonen et al., 2004*; *Delhanty, 2005*; *Ioannou et al., 2012*). Our results are not in agreement with such findings as no differences were observed in mosaic trisomy and mosaic monosomy prevalence for whole-chromosome mosaicism. *Munné et al. (2002)* reported chromatid non-disjunction to be the main mechanism for mitotic aneuploidy, whereas *Chow et al. (2014)* stated that the three possible mechanisms (selective endoreduplication, anaphase lagging and mitotic non-disjunction) would all happen with the same frequency. As both hypotheses would result in a balanced trisomy–monosomy rate, our findings could be explained by either hypotheses. On the other hand, in segmental mosaicism, monosomies were significantly more frequent than trisomies, in agreement with previous reports (*Babariya et al., 2017*; *Nakhuda et al., 2018*). In this case, the most frequent mechanism would be

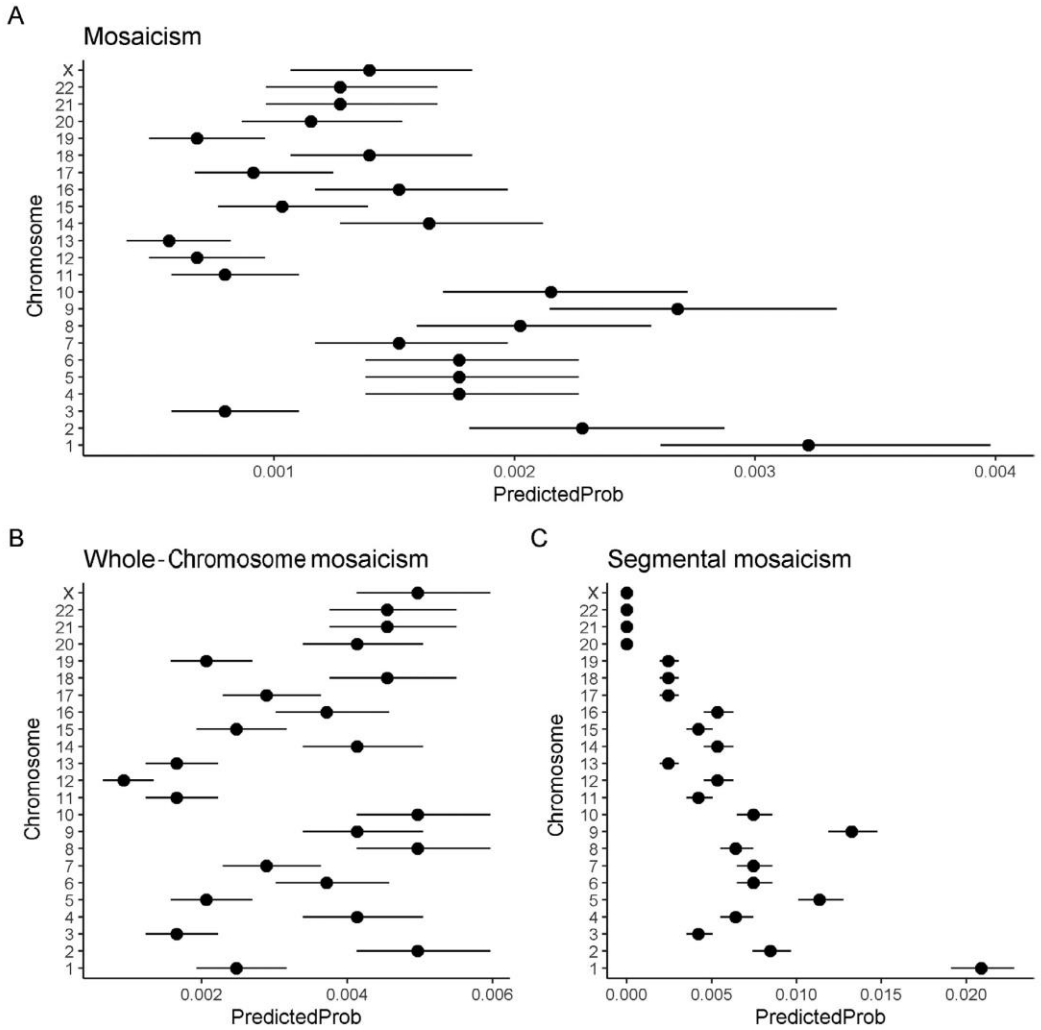

FIGURE 3 Predicted prevalence of mosaicism per chromosome for (A) total mosaicism; (B) whole-chromosome mosaicism; and (C) segmental mosaicism.

the loss of the acentric fragment resulting from chromosome breakage.

Paternal age has been the only studied factor showing a significant positive association with mosaicism prevalence. Paternal age has been associated with a decay in sperm quality (*Johnson et al., 2015*) and an increase in de-novo mutations (*Kong et al., 2012*). Moreover, some studies have shown a correlation between advanced paternal age and increased sperm DNA fragmentation (*Belloc et al., 2014*; *Yatsenko and Turek, 2018*). Taking into account the role

TABLE 2 ASSOCIATION OF CONTINUOUS VARIABLES WITH MOSAICISM

	Mosaicism, yes (n = 237)	Mosaicism, no (n = 1471)	P-value
Female age (mean ± SD)	38.9 ± 3.1	39.1 ± 3.1	0.40
Male age (mean ± SD)	40.8 ± 4.9	40.2 ± 4.6	0.07
Antral follicle count (mean ± SD)	13.1 ± 5.4	13.5 ± 7.0	0.45
COCs retrieved	14.2 ± 7.0	14.4 ± 7.2	0.77
BMI (mean ± SD)	22.8 ± 4.2	23.0 ± 4.0	0.64
Total dose of gonadotrophins (mean ± SD)	2612.8 ± 871.7	2654.2 ± 834.4	0.49

BMI, body mass index; COC, cumulus–oocyte complex.

TABLE 3 ASSOCIATION OF DISCRETE VARIABLES WITH MOSAICISM

	Mosaicism prevalence (%)	*P*-value
Sperm concentration		
Oligozoospermia	31/291 (10.7)	0.1
Normal	206/1417 (14.5)	
PGT-A indication		
AMA	165/1229 (13.4)	0.9
Recurrent miscarriage	28/185 (15.1)	
Male factor	7/48 (14.6)	
Implantation failure	21/130 (16.2)	
No indication	16/116 (13.8)	
Blastocyst formation day		
5	127/864 (14.7)	0.1
6	91/714 (12.7)	
7	19/130 (14.6)	
Blastocyst quality		
Excellent/good	147/1034 (14.2)	0.4
Fair/poor	90/674 (13.3)	
Culture media		
G-TL®	182/1291 (14.1)	0.9
Global total®	55/417 (13.2)	
Biopsy operator		
1	37/389 (9.5)	0.1
2	12/86 (14.0)	
3	58/378 (15.3)	
4	36/247 (14.6)	
5	28/177 (15.8)	
6	66/431 (15.3)	

AMA, advanced maternal age; PGT-A, preimplantation genetic testing for aneuploidy.

played by double-strand breakages in the generation of segmental mosaicism (*Babariya et al., 2017*), these reports further support our findings in mosaicism association with advanced male age. Contrary to a recently published study (*Tarozzi et al., 2019*), we did not observe an increase in mosaicism prevalence in embryos from oligozoospermic sperm samples compared with normal concentration specimens. As limited number of embryos from oligozoospermic samples were available, however, and several confounding factors are at stake when analysing sperm characteristics, no compelling conclusions can be reached in this regard. In any case, male factor seems to be a subject worthy of more in-depth and accurate study regarding embryonic mosaicism.

On the other hand, our data show that mosaicism prevalence is not related to any specific female factor. Advanced maternal age was not seen to be correlated with a higher prevalence of mosaicism, unlike what is observed regarding meiotic origin aneuploidies (*Hassold and Hunt, 2001*). Other investigators reported similarly (*Munné and Wells, 2017*; *Nakhuda et al., 2018*; *Popovic et al., 2018*). Additional maternal factors, such as BMI and ovarian reserve, were also studied in relation to mosaicism prevalence with no associations observed, as previously reported in relation to meiotic aneuploidies (*Morin et al., 2018*; *Sermondade et al., 2019*).

According to our results, mosaic blastocysts have the same quality and reach the blastocyst stage on the same day than non-mosaic ones. *Popovic et al. (2018)* did not find any association between embryonic mosaicism and trophectoderm or inner cell mass quality (*Popovic et al., 2018*). As only blastocysts were assessed, an association between mosaicism and compromised embryo development at early stages cannot be excluded. *Lee et al. (2019)* recently reported altered morphokynetic parameters in mosaic embryos. These data point to the hypothesis that compromised embryo cleavage at early stages could induce mosaicism generation.

As early embryo cleavage is driven by the oocyte, factors involved in its maturation could induce errors in chromosome segregation (*Fragouli et al., 2010*). Therefore, ovarian stimulation, which has been widely studied in relation to aneuploidy with controversial results (*Baart et al., 2007*; *Barash et al., 2017*), is a first clear candidate. The data obtained in the present study, applying standardized stimulation protocols for PGT-A cycles, show that total dosage of gonadotrophins is not related to mosaicism in PGT-A cycles. Moreover, higher response was not observed to be associated with higher prevalence of mosaicism. It remains to be assessed whether specific stimulation protocols could affect mosaicism prevalence.

Culture media composition can be widely different among manufacturers with diverse effects on blastocyst development (*Morbeck et al., 2017*). In the present study, two different single-step media could be compared with no differences observed in mosaicism prevalence. Data available are controversial and most studies have limitations as they involve a large amount of confounding variables (*Swain, 2019*). Because of the standardized conditions in our laboratory, the effect of other laboratory factors on mosaicism could not be assessed. According to published data, suboptimal oxygen concentration and pH may generate embryonic mosaicism (*Katz-Jaffe et al., 2018*). In any case, it seems evident that a high-standard IVF setting ensuring the best conditions during gamete handling and embryo culture together with the application of optimal standardized operating procedures may be essential to keep mosaicism prevalence low.

Biopsy procedure has been proposed to have an effect on embryo mosaicism (*Munné and Wells, 2017*). Our data show that, within a same set-up, biopsy operator does not influence mosaicism rate. All the embryologists of the team,

TABLE 4 LOGISTIC MIXED MULTIVARIABLE MODEL TO ESTIMATE THE ODDS FOR EVERY END POINT

Mosaicism prevalence	Adjusted OR	95% CI	*P*-value
Female age[a]	0.80	0.60 to 1.06	0.11
Male age[a]	1.25	1.02 to 1.53	0.03
BMI[a]	0.97	0.80 to 1.18	0.79
COCs retrieved[a]	0.99	0.97 to 1.02	0.67
Total dose of gonadotrophins[a]	1.00	0.81 to 1.22	0.94
Sperm concentration			
Normal concentration	1	n/a	
Oligozoospermia	0.64	0.38 to 1.08	0.09
PGT-A indication			
Recurrent miscarriage	1	n/a	
AMA	0.89	0.45 to 1.80	0.76
Male factor	0.95	0.31 to 2.86	0.30
Implantation factor	0.87	0.39 to 1.95	0.73
No indication	0.62	0.25 to 1.54	0.92
Blastocyst formation day			
5	1	n/a	
6	0.80	0.55 to 1.15	0.23
7	1.24	0.62 to 2.47	0.54
Blastocyst quality			
Excellent/good	1	n/a	
Fair/poor	1.23	0.85 to 1.78	0.26
Culture media			
G-TL®	1	n/a	
Global total®	0.87	0.56 to 1.33	0.50
Random effects (variance)			
Patient (intercept)	0.43	n/a	
Biopsy procedure (intercept)	0.002	n/a	

Patient and biopsy procedure were treated as random factor to control the correlated observations effect.
[a] Standardized variables.
AMA, advanced maternal age; BMI, body mass index; COC, cumulus–oocyte complex; PGT-A, preimplantation genetic testing for aneuploidy; n/a, not applicable.

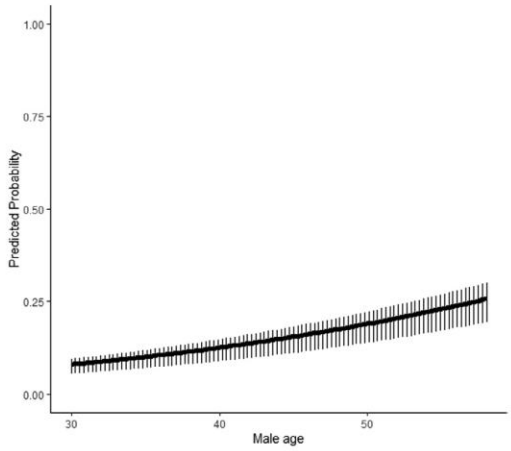

FIGURE 4 Predictive model on mosaicism prevalence regarding male age.

however, took embryo biopsies through the same procedure. Continuous learning and evaluation is essential to ensure standardization. It is possible that specific biopsy parameters unaddressed in this study (number of laser impacts, flicking versus pulling methodology) might be related to the generation of artefactual mosaicism. The biopsy of too few cells has been related to increased artefactual mosaicism (*Cram et al., 2019*; *Fragouli et al., 2019*). Unfortunately, the potential effect of the number of analysed cells could not be assessed in the present study.

To the best of our knowledge, this is the first report that aims to analyse embryo mosaicism in IVF cycles extensively and in-depth. An important strength of the study is that all data come from a single IVF unit with an in-house PGT-A facility, which ensures that all cases analysed have followed the same standard operating procedures, carried out by equally trained professionals. Moreover, in many of the published or presented studies assessing the cause of mosaicism, the mosaic group includes only euploid–aneuploid mosaics. In the present study, the mosaic group included any embryo presenting a mosaic anomaly, given that the mechanism for the generation of a mosaic event ought to be independent of the presence of constitutional aneuploidy. On the contrary, results were based on single trophectoderm biopsy for each embryo, retrospectively analysed and with a significant but limited sample size. Therefore, results should be considered with caution. It cannot be excluded that results could be different among specific types of mosaic embryos.

The present study highlights for the first time that paternal age is associated with embryo mosaicism prevalence. Although we cannot confirm a cause–effect relationship, these data could be used for patient counselling and explain, in several cases, surprisingly high mosaicism rates among specific women undergoing PGT-A. Furthermore, our findings warrant future research and replication studies in order to further elucidate the actual biological background behind embryo mosaicism.

In conclusion, the distribution of mosaic anomalies among the affected chromosomes and the implicated mechanisms behind this event seem to be different in segmental mosaicism

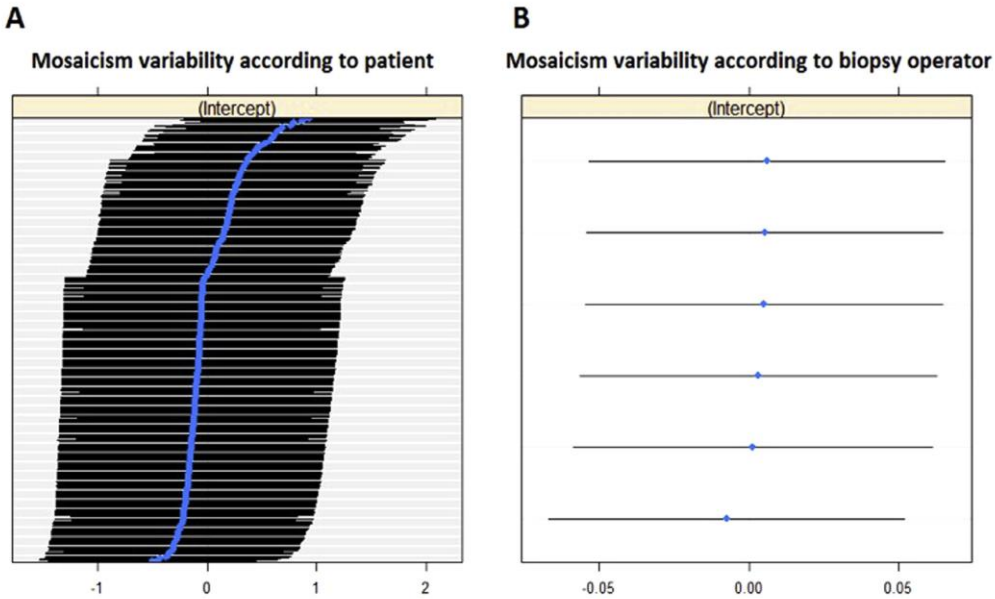

FIGURE 5 (A) Random intercepts according to patient; (B) random intercepts according to biopsy operator.

compared with whole-chromosome mosaicism. Segmental mosaicism occurs more frequently in monosomy affecting large and specific chromosomes, whereas no differences are observed in whole-chromosome mosaicism.

Embryonic mosaicism is a patient-related phenomenon. Paternal age was identified as a specific intrinsic factor related to mosaicism. Considering the high variation in mosaicism prevalence among different patients, however, other patient related factors not analysed in the present study should be investigated.

In a single and standardized IVF and PGT-A setting, embryonic mosaicism is not related to different interventions during the ART treatment. Therefore, mosaicism prevalence among set-ups with similar patient profiles might not be due to the specific approaches used, but to the general suboptimal culture or environmental conditions and standardized operating procedures. Optimization and standardization of clinical and laboratory procedures are essential to minimize the generation of embryonic mosaicism.

To the best of our knowledge, this is the first study assessing the factors affecting embryo mosaicism in a comprehensive way within a single set-up. It would be of great importance that other groups present mosaicism data in their own settings so that results could be compared, and more compelling conclusions could be reached.

ACKNOWLEDGEMENTS

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The effect of trophectoderm biopsy technique and sample handling on artefactual mosaicism

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ASSISTED REPRODUCTION TECHNOLOGIES

The effect of trophectoderm biopsy technique and sample handling on artefactual mosaicism

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Abstract

Purpose To determine whether embryo mosaicism prevalence in preimplantation genetic testing for aneuploidy (PGT-A) cycles is associated with the trophectoderm biopsy technique used (a. number of laser pulses or b. the use of flicking or pulling) or the time to tubing.

Methods Prospective observational study performed in a single IVF-PGT-A setting from May 2019 to May 2021. Trophectoderm biopsies were analysed by next-generation sequencing. Mosaicism was analysed in relation to the biopsy methodology (number of laser pulses and pulling vs flicking), time elapsed from biopsy to tubing (min), and time of sample cryostorage from tubing to amplification (days). As a secondary objective, the number of laser pulses and biopsy methodology were studied in relation to clinical outcomes of transferred euploid blastocysts.

Results None of the analysed variables were associated to mosaicism prevalence. Multivariable regression analysis demonstrated that mosaicism prevalence was comparable either when > 3 laser pulses were used as compared to ≤ 3 (13.9% vs 13.8%, aOR = 0.8726 [0.60–1.28]) and pulling compared to flicking (13.1% vs 14.0%, aOR = 0.86 [0.60–1.23]). Moreover, neither the number of laser pulses during biopsy (> 3 vs ≤ 3) nor the technique used (pulling vs flicking) were associated with clinical pregnancy after the transfer of frozen-thawed euploid blastocysts (54.9% vs 55.2%, aOR = 1.05 [0.53–2.09]; 61.1% vs 52.9%, aOR = 1.11 [0.55–2.25], respectively).

Conclusion Our results suggest that, as long as the biopsy and tubing procedures are performed following standardized high quality procedures, no specific approach would increase the generation of artefactual mosaicism as a result of trophectoderm biopsy. Trophectoderm biopsies should be performed regardless of the methodology but always aiming on minimising blastocyst manipulation.

Keywords PGT-A · Blastocyst · Biopsy · Tubing · Mosaicism · Artefactual

Introduction

Embryo chromosomal mosaicism detection has been for a long time a bump in the road of preimplantation genetic testing programs. The transfer of mosaic embryos with a euploid cell line in coexistence with at least one aneuploid line has been proven to give rise to healthy pregnancies and live births [1, 2]. However, there are still many concerns regarding the associated potential risks of replacing mosaic embryos, making it difficult to provide counselling to patients that most of the times decide not to transfer them [3].

Mitotic errors during early preimplantation development are at the origin of this phenomenon and have been known to occur in in vitro preimplantation embryos for a very long

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time [4]. The molecular mechanisms that origin chromosomal mosaicism in human embryos have been widely studied and discussed [5, 6]. The fact that embryo mosaicism prevalence may vary among different patients and that it might be associated to specific patients' characteristics has been assessed and discussed [7–9]. This kind of mosaicism has been referred to as intrinsic mosaicism. Furthermore, a wide variability in the prevalence of chromosomal mosaicism among different IVF laboratories has been reported. Interventions during the performance of an IVF cycle, such as ovarian stimulation and culture conditions, may increase the occurrence of chromosomal mosaicism as they may be affecting mitotic division. Some authors have actually evidenced that suboptimal culture conditions are associated with a higher prevalence of mosaicism [10, 11]. Therefore, a certain percentage of mosaicism observed in in vitro preimplantation embryos may be iatrogenic and could be reduced.

Some studies analysing the concordance of a mosaic trophectoderm biopsy with the results obtained from additional portions of the same embryo (including ICM) have evidenced that it is not unusual not to observe it in the latter samples [8, 12–14]. While this can be explained by low degree mosaicism being confined in a specific region of the embryo, some authors have speculated that mosaicism is actually not present in the embryo and an artefactual result caused by technique may be sometimes diagnosed [15–17]. This has been called artefactual or technical mosaicism.

In this sense, it has been discussed that the quality and integrity of the trophectoderm biopsy obtained may be related to artefactual mosaicism [18, 19]. Actually, the Preimplantation Genetic Diagnosis International Society (PGDIS) statement on mosaic embryo transfer warns that poor biopsy technique causing excessive cell damage or the biopsy of too few cells may affect chromosome profiles [20]. Therefore, the biopsy operator and its expertise may be key to minimize this issue. Moreover, the use of laser itself and the amount of laser pulses applied to obtain the biopsy has also been discussed as a potential source of technical mosaicism [21].

Different methodologies for trophectoderm biopsy have been described [19]. On one hand, the biopsy can be obtained by a combination of aspiration and pulling the cells to be biopsied while applying a few intercellular laser pulses to separate them from the rest of the embryo. Alternatively, the biopsy can be retrieved from the embryo by aspirating the cells to be biopsied inside the biopsy pipette and flicking this pipette with the holding pipette to detach the cells. This method can be complemented by the use of laser pulses before flicking to ease the biopsy. These different approaches may lead to trophectoderm biopsies of different characteristics and DNA integrity that may, in turn, lead to different kinds of results in terms of artefactual mosaicism. Notably, the lapse of time between biopsy and tubing and between tubing and whole-genome amplification (WGA) have been paid very little attention, though they could also influence DNA quality and therefore artefactual mosaicism.

The aim of the present study was to evaluate in depth the potential role of different interventions during trophectoderm biopsy and tubing prior to WGA in the generation of technical mosaicism. As a secondary objective, we also assessed whether the biopsy technique influences clinical outcomes after euploid blastocyst transfer.

Material and methods

This is a prospective observational study performed in a single IVF-preimplantation genetic testing (PGT) setting from May 2019 to May 2021 analysing the chromosomal constitution of 1341 biopsied blastocysts. The prevalence of mosaicism detected in trophectoderm biopsies was analysed in relation to the number of laser pulses used for biopsy, the biopsy technique (flicking vs pulling), the time lapsed from biopsy to tubing, and time of sample storage (from tubing to WGA). Additionally, the number of laser pulses and biopsy technique were analysed with regard to clinical outcomes.

All cycles included underwent comprehensive chromosome screening for PGT-A for one of the following reasons (advanced maternal age, recurrent miscarriages, severe male factor, repeated implantation failure, no indication).

Study design was approved and reviewed by our centre's institutional review board.

IVF cycle

Standardized protocols were used for ovarian stimulation [22]. Pituitary suppression was achieved using gonadotrophin-releasing hormone analogues (agonists or antagonists) and multiple follicular recruitment, and growth was performed under gonadotrophins at clinician's discretion according to patient's characteristics.

Oocyte retrieval was performed 36 h after ovulation triggering. Denudation was conducted before insemination of metaphase-II oocytes by ICSI 40 h after triggering. Injected oocytes were left in culture in single-step media (G-TL™, Vitrolife®, Göteborg, Sweden) in a time-lapse incubator (Geri®) (Merck, Darmstadt, Germany) with low oxygen tension (5%). All media used for gamete and embryo handling and culture were G-series media (Vitrolife®, Göteborg, Sweden).

Biopsy procedure

Zona pellucida opening was performed on day 3 developing embryos using laser thermolysis [23]. Embryos

reaching the blastocyst stage were biopsied between day 5 and day 7. Only hatching or fully hatched blastocysts with a defined inner cell mass and a cohesive multiple-cell trophectoderm epithelium were considered suitable for biopsy.

Trophectoderm biopsy was performed by a combination of intercellular laser pulses and pulling and aspiration [24], or by laser and flicking. Laser pulses were performed with OCTAX Navilase™ (Olympus, Tokyo, Japan) using trophectoderm biopsy mode. Flicking or pulling methodology (Fig. 1) was performed for each embryo biopsy at operator's discretion and aiming to minimize manipulation according to the following criteria. Pulling was the preferred strategy for biopsy and was generally performed to blastocysts with few hatched cells and when biopsy could be obtained without needing an excessive number of laser pulses and excessive manipulation. In cases of half hatching blastocysts or more, flicking was preferred to avoid induced full hatching of the blastocyst. Fully hatched blastocysts were always biopsied by flicking. Holding pipette used for procedure was a 35° angled Small Holding (CooperSurgical®, Connecticut, USA), and biopsy pipette was 35° angled, flat, and polished with an inner diameter of 13–15 µm (CooperSurgical®, Connecticut, USA). Characteristics of the biopsy procedure (operator, number of laser pulses, time of biopsy) were annotated by the operator immediately after biopsy.

Tubing of the sample was performed with UV-sterilized material with sterile gown and sterile gloves. Biopsied sample was thoroughly washed in 1 × phosphate-buffered saline (PBS) with 0.1% polyvinyl alcohol (PVA) droplets before isolation in 0.2 ml PCR tubes with 1.5 µl 1 × PBS. A 80-µm capillary was used for sample handling. Placement of the biopsy into the tube was confirmed through stereomicroscope. Isolated samples were centrifuged at 1200 rpm during 3 min and immediately stored at − 80 °C freezer.

Biopsied blastocysts were vitrified as previously reported [25].

PGT protocol and results

WGA was achieved using Sureplex Amplification System (Illumina®, California, USA). The cytogenetic analysis of the biopsies was performed by NGS using the VeriSeq™PGS-MiSeq® platform (Illumina, California, USA) following the manufacturer's protocols and guidelines.

Embryos were diagnosed as euploid, aneuploid, or mosaic. A mosaicism event was called when a deviation from the normal copy number of 30 to 70% was observed. In the context of the present study and analysis, mosaic embryo group includes embryos with at least one mosaic anomaly regardless of them being euploid or aneuploid for the remaining chromosomes.

Euploid embryo transfer

Euploid embryos were transferred in a deferred cycle. Hormonal replacement for endometrial preparation and frozen embryo transfer was performed following the established protocols in our centre [26]. Embryo transfer of warmed surviving blastocysts was conducted under ultrasound guidance [27]. Luteal phase support treatment plan has been previously reported [28].

Statistical analysis

Continuous variables were described with mean and SD, and categorical variables were described with number and percentage. Chi-squared test was used to compare categorical variables, and *t*-test or Wilcoxon Mann–Whitney test was used to compare continuous variables according to the necessary assumptions.

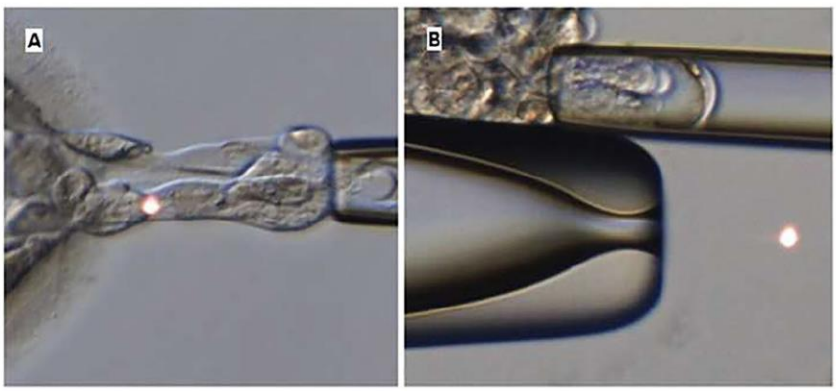

Fig. 1 **A** Image of a blastocyst being biopsied by pulling technique. **B** Image of an embryo being biopsied by flicking technique

A logistic mixed multivariable model with random intercepts was applied to estimate the association between mosaicism and different interventions during trophectoderm biopsy and tubing adjusted by female and male age, as well as embryo quality. Patient, IVF cycle, and biopsy operator were treated as random effects.

To evaluate the association between clinical pregnancy and the interventions during biopsy, a logistic regression model was adjusted.

R software [29] was used for statistical analyses. For all comparisons, a statistical significance was set at $P < 0.05$. Package MASS [30] was used to adjust the mixed model.

Results

Patients' demographics and cycles' characteristics

A total of 474 cycles undergoing PGT with comprehensive chromosome screening from May 2019 to May 2021 were included. Patients' demographics and cycles' characteristics are shown in Table 1. Indications for PGT were advanced maternal age (70.0%; 332/474), recurrent miscarriages (9.9%; 47/474), repeated implantation failure (9.5%; 45/474), PGT-A with no indication (9.3%; 44/474), and severe male factor (1.3%; 6/474).

A trophectoderm biopsy was performed in 1341 developing blastocysts, achieving a conclusive diagnosis for 1318 of them (98.3%). The distribution of diagnostic rate was similar among the studied variables (Supplementary Table 1). A euploidy rate of 40.2% (530/1318) was observed, while 45.9% (606/1318) of embryos were aneuploid, 6.5% (86/1318) aneuploid with at least one additional mosaic anomaly (aneuploid-aneuploid mosaic), and 7.3% (96/1318) were euploid-aneuploid mosaic.

From 182 mosaic blastocysts (86 aneuploid-aneuploid mosaic + 96 euploid-aneuploid mosaic), 89 (48.9%) exhibited whole-chromosome mosaicism, 87 (47.8%) segmental mosaicism, and 6 (3.3%) presented a combination of both.

Table 1 Patients' demographics and cycles' characteristics

	Mean ± SD
Female age (y)	38.4 ± 4.4
Male age (y)	41.0 ± 5.3
Antral follicle count (AFC)	12.8 ± 6.9
Female body-mass index (BMI)	23.5 ± 4.7
Ovarian stimulations per PGT-A cycle	1.1 ± 0.3
Inseminated oocytes per PGT-A cycle	9.3 ± 5.4
Biopsied embryos per PGT-A cycle	3.0 ± 2.1

Biopsy technique, sample handling, and mosaicism

Trophectoderm biopsies were performed by 5 different senior operators and were distributed as follows: 34.1% Operator 1 (449/1318), 28.4% Operator 2 (375/1318), 20.5% Operator 3 (270/1318), 9.8% Operator 4 (129/1318), and 7.2% Operator 5 (95/1318). The number of laser pulses applied was ≤ 3 in 79.2% of biopsies with results (1044/1318), while > 3 pulses were applied in the remaining cases. The mean number of pulses was 3.24 ± 0.55 with a range from 2 to 8. Additionally, flicking methodology was more frequently applied than pulling (74.6% vs 25.4%). With regard to the tubing procedure, the mean time lapsed from biopsy was 53.8 ± 40.4 min. Samples were cryostored 13.4 ± 19.1 days before amplification.

The univariate analysis of the biopsy and tubing procedure characteristics in relation to mosaicism prevalence observed in the biopsies did not evidence any statistically significant association (Table 2).

When a subanalysis was performed differentiating between embryos with whole-chromosome mosaicism ($n = 89$) and those with segmental mosaicism ($n = 87$), no differences in the parameters analysed were observed either (Supplementary Table 2).

The multivariable analysis in a mixed-model including the biopsy related variables with patient, cycle, and biopsy operator as random effects and adjusted for female and male age, and embryo quality, confirmed the results observed in the univariate analysis (Table 3).

Biopsy technique and clinical outcomes

From all embryos included in this study, to date, 213 euploid blastocysts have been warmed for transfer. Survival rate was 95.3% (203/213). No differences were found in the survival rate with regard to the number of laser pulses used for biopsy, nor to the biopsy technique (Table 4).

Currently, we have data on the clinical pregnancies from 194 single euploid blastocyst transfers with a clinical pregnancy rate of 55.1% (107/194). Our data did not evidence differences regarding how the biopsy was performed (Table 5). A multivariable analysis adjusting the results for embryo quality, biopsy day, and hatching status of the embryo (hatching vs hatched) showed results in the same line (Table 6).

To date, data on pregnancy follow-up is available for 104 pregnancies. Three cases are lost to follow-up. No significant differences were observed in miscarriage rate neither between the use of ≤ 3 pulses vs > 3 pulses (13.2%, 10/66 vs 25.0%, 7/21; $p = 0.229$) nor between the use of flicking vs pulling (14.1%, 10/61 vs 21.2%, 7/26; $p = 0.52$).

Table 2 Univariate analysis of mosaicism association with biopsy and tubbing procedures

	Mosaicism NO ($n=1136$)	Mosaicism YES ($n=182$)	OR (95%CI)	p-value
Biopsy operator:				0.798
Operator 1	392 (87.5%)	56 (12.5%)	Ref	
Operator 2	320 (85.1%)	56 (14.9%)	1.22 (0.82;1.83)	
Operator 3	229 (84.8%)	41 (15.2%)	1.25 (0.81;1.93)	
Operator 4	113 (87.6%)	16 (12.4%)	1.00 (0.53;1.77)	
Operator 5	82 (86.3%)	13 (13.7%)	1.12 (0.56;2.09)	
Number of laser pulses:				1.000
≤3	900 (86.2%)	144 (13.8%)	Ref	
>3	236 (86.1%)	38 (13.9%)	1.01 (0.68;1.47)	
Biopsy technique:				0.747
Flicking	845 (86.0%)	138 (14.0%)	Ref	
Pulling	291 (86.9%)	44 (13.1%)	0.93 (0.64;1.33)	
Time to tubing (min):				0.215
(1,22)	284 (85.3%)	49 (14.7%)	Ref	
(22,45)	292 (85.4%)	50 (14.6%)	0.99 (0.65;1.52)	
(45,71)	267 (84.5%)	49 (15.5%)	1.06 (0.69;1.64)	
(>71)	293 (89.6%)	34 (10.4%)	0.67 (0.42;1.07)	
Time to amplification (d):	13.5 (19.1)	13.3 (20.3)	1.00 (0.99;1.01)	0.90

Table 3 Multivariable analysis of mosaicism association with biopsy and tubbing procedures. *Odds ratios adjusted for female and male age and embryo quality with patient, cycle, and biopsy operator as random effects

	aOR*	95% CI
Number of laser pulses		
≤3	Ref	Ref
>3	0.87	0.60–1.28
Biopsy technique		
Flicking	Ref	Ref
Pulling	0.86	0.60–1.23
Time to tubing	1.00	0.99–1.00
Time to amplification	1.00	0.99–1.01

Discussion

The potential diagnosis of artefactual mosaicism has become an important concern among PGT scientific community. Some authors have suggested that the true incidence of mosaicism in trophectoderm biopsies might be much lower than what is actually diagnosed [14]. The fact that we might be overestimating the prevalence of mosaicism in trophectoderm biopsies is an important issue to be addressed as diagnosis of mosaicism in preimplantation embryos leads, in most cases, to patients finally discarding such embryos for transfer [3]. The trophectoderm biopsy procedure has been suggested as a clear candidate for technical mosaicism generation [7, 21, 31]. However, to date, data actually assessing this issue are scarce. Therefore, in this study, we have performed a comprehensive analysis around the role that biopsy and tubing procedure may have on the generation of artefactual mosaicism.

The experience of the biopsy operator has always been a matter of concern regarding PGT results. The biopsy operator skills could affect the viability of embryos due to a detrimental biopsy, but could also be inducing artefactual mosaicism by altering the sample. The results obtained in this study confirm our previous reports [9] and agree with

Table 4 Warming results according to the biopsy methodology used

	Survival NO ($n=10$)	Survival YES ($n=203$)	OR (95%CI)	p-value
Number of laser pulses				0.270
≤3	6 (3.75%)	154 (96.2%)	Ref	
>3	4 (7.55%)	49 (92.5%)	0.47 (0.13;2.00)	
Biopsy technique				0.725
Flicking	7 (4.46%)	150 (95.5%)	Ref	
Pulling	3 (5.36%)	53 (94.6%)	0.80 (0.21;4.05)	

Table 5 Univariate analysis showing the association of biopsy methodology with clinical outcomes

	Clinical pregnancy NO ($n=87$)	Clinical pregnancy YES ($n=107$)	OR (95%CI)	p-value
Number of laser pulses				1.000
≤3	64 (44.8%)	79 (55.2%)	Ref	
>3	23 (45.1%)	28 (54.9%)	0.99 (0.52;1.89)	
Biopsy technique				0.136
Flicking	66 (47.1%)	74 (52.9%)	Ref	
Pulling	21 (38.9%)	33 (61.1%)	1.40 (0.74;2.68)	

Table 6 Multivariable logistic model for biopsy methodology adjusted for embryo quality, biopsy day, and hatching status of the embryo (hatching vs hatched)

	aOR	95% CI
Number of laser pulses		
≤3	Ref	Ref
>3	1.05	0.53–2.09
Biopsy technique		
Flicking	Ref	Ref
Pulling	1.11	0.55–2.25

other authors that senior biopsy operators equally trained do not differ in their results [32]. However, the situation might change in the case of inexpert operators [33].

The implementation of laser-assisted trophectoderm biopsy has so far been proven safe and harmless for the embryo [34, 35]. However, it is not clear whether DNA damage on the biopsied cells may be induced by the use of laser and result in technical mosaicism at diagnosis. Our results do not evidence an increased prevalence of mosaicism in biopsies that had been obtained by using > 3 laser pulses compared to ≤ 3 (mean of 3.24 laser pulses). It should be taken into account that most of the biopsies were obtained by applying 3 or 4 pulses and that the range of pulses applied is quite narrow as it was always intended to minimize manipulation during biopsy. We cannot rule out that the use of different number of pulses and intensities might compromise sample quality, although this has not been observed in previous reports [36, 37]. While all these studies have compared different laser methodologies, recent data have pointed to differences in mosaicism rates when comparing biopsies obtained with and without laser, with less mosaicism prevalence in the latter [38]. More data would be needed to confirm these results. Performing a biopsy without laser use should be considered with caution as it should be confirmed that it does not induce more damage to both the embryo and the sample.

Additionally to the use of laser, two biopsy methods have been proposed: flicking and pulling. Both methods have been used depending on centres' or practitioners' criteria. However, little is known about the results obtained with such methodologies and, to date, no published studies have addressed this issue. Our results regarding mosaicism prevalence using each of these strategies do not evidence any difference. It would seem that flicking is more rough and could lead to more cell damage than pulling strategy where sampling is more gentle. In fact, some authors have reported poorer quality of biopsy samples obtained by flicking [39]. However, while flicking may be inducing more cell damage, according to our data, this does not seem to be related to artefactual mosaicism.

Concerning laser pulses and biopsy technique, we considered important to assess whether they were related to clinical outcomes. An increased number of laser pulses or the use of a less precise technique such as flicking may be harming the embryo and thus affecting its implantation ability. Our data do not show any association between the methodology used with the survival rate after warming, the clinical pregnancy rate, or the miscarriage rate. As this was a secondary aim of the study, results should be considered with caution due to the limited sample size.

Tubing procedure is a key step in PGT. While the importance of a proper and standardized tubing procedure is evident [19], this step has been neglected with regard to artefactual mosaicism generation. The tubing of damaged cells may lead to inconclusive results, but also to artefactual mosaicism. This can be minimized throughout extensive washings. We hypothesised that extended time from biopsy to tubing may be helpful to detach such cellular debris from the biopsy sample and reduce artefacts that could be interpreted as mosaicism. However, this hypothesis was not confirmed by our results, as there is no association between mosaicism prevalence and time from biopsy to tubing. Additionally, storage at − 80 °C is known to be the optimal strategy for maintenance of DNA quality [40]. In tune with that, the length of time the sample was stored at − 80 °C until amplification was not associated to mosaicism either.

As previously reported, the aetiology of mosaicism seems to be different when considering separately segmental and whole-chromosome mosaicism [9]. In this sense, we wanted to confirm whether a determinate kind of mosaicism was more influenced by the biopsy procedure than the other. Due to its nature, segmental mosaicism may be more prone to be artefactually generated through the biopsy procedure. Our results did not evidence differences regarding the type of mosaicism either.

Even though our results show that no biopsy methodology is related to mosaicism when biopsies are performed by experienced operators, this does not exclude that artefactual mosaicism can be generated during biopsy or tubing.

Consequently, it is key to keep the biopsy and tubing operations at the highest quality standards. Moreover, it has to be considered that aneuploidy calling in most PGT-A protocols is based on DNA obtained after whole-genome amplification (WGA) and processing, and results are obtained through algorithms. All this steps could also, at some point, generate artefacts that may be interpreted as mosaicism [17, 18]. While most artefacts originated at this point are technique-related and difficult to avoid, it is still important to work under optimal conditions [19]. However, even taking all precautions, artefactual mosaicism might always be present in some cases and artefacts from biological origin due to cells being in S-phase will be impossible to prevent [18].

Data from this study are of great interest as they shed light on one of the most discussed yet less assessed topics around mosaicism: the biopsy technique. The fact that our results are obtained in a single centre ensures standardized conditions. However, sample size is limited and data should be considered with caution. Moreover, being a prospective observational study, the choice of flicking or pulling was not randomized. Results regarding clinical outcomes should be considered as preliminary and must be confirmed after more data are available.

Conclusions

To the best of our knowledge, this is the first study to assess in detail the role of biopsy methodology and sample handling on the diagnosis of mosaicism. Our results evidence that under standardized high-quality procedures, neither the use of flicking nor pulling nor the number of laser pulses are related to mosaicism incidence.

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Author contribution LC and MP conceived and designed the study. All the authors analyzed and interpreted the data. LC, MP, and BC contributed to data collection. IR performed the statistical analysis. All authors revised the article for important intellectual content. LC and MP wrote the article. All the authors approved the final version of the manuscript.

Declarations

Conflicts of interest The authors declare no competing interests.

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Do reproductive history and information given though genetic counselling influence patients' decisions on mosaic embryo transfer?

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Do reproductive history and information given through genetic counselling influence patients' decisions on mosaic embryo transfer?

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Abstract

Objective: To assess patients' and embryonic characteristics that may have an influence on the decision to transfer a mosaic embryo.

Method: Single centre retrospective cohort study including 1247 PGT-A cycles. Demographic and clinical factors associated with a decision to transfer a mosaic embryo were studied. Female age, number of previous cycles, previous availability of euploid embryos, history of miscarriages and parity as well as percentage of mosaicism, type of anomaly and chromosome risk were studied in relation to decision-making. Outcomes after mosaic embryo transfer were assessed.

Results: To date, in 7.9% of cycles (99/1247), patients have had to make a decision on the fate of their mosaic embryos. In 23.2% of cycles (23/99), patients decided to transfer. In most cases (79.8%; 79/99), patients underwent genetic counselling before the decision. None of the variables analysed were associated with the patients' decision, although parity and the high-degree mosaicism (>50%) seemed to be negatively associated with the decision to transfer (18.2% vs. 29.8%, $p = 0.294$; 10% vs. 32.2%, $p = 0.052$).

Conclusions: Neither reproductive history nor information on mosaic embryo characteristics through counselling seems to be determinative for patients when deciding to transfer a mosaic embryo. Promising and increasing data on clinical outcomes after mosaic embryo transfer will be of utmost importance to soften risk perception regarding mosaic embryos and give a better, simplified and more evidence-based counselling.

Key points

What is already known?

- Mosaic blastocysts have reproductive potential and can give rise to healthy children. However, there are potential risks after achieving a pregnancy from a mosaic blastocyst. Counselling and decision-making regarding mosaic embryo transfer are both challenging for genetic counsellors and patients, respectively, and very few studies have assessed this process.

What does this study add?

- This study focuses not only on clinical outcomes achieved after mosaic embryo transfer but on the effect and impact that mosaicism diagnosis has on PGT-A patients by analysing the mosaic embryo transfer decision-making process and the influence that patients' background and genetic counselling may have on their decision.

1 | INTRODUCTION

Euploid–aneuploid mosaic embryos are those showing a result compatible with the coexistence of a normal and an abnormal cell line. The fact that these embryos have a euploid cell line makes them potentially able to give rise to a healthy live birth as proven by Greco and co-workers.[1] However, the presence of abnormal cells implies a potential risk for the foetus and the future child. Taken together, this creates a dilemma around the decision of transferring mosaic embryos obtained after Preimplantation Genetic Testing for aneuploidy (PGT-A) cycles.

Since the first report by Greco, many groups started considering euploid–aneuploid mosaic embryos suitable for transfer. Data on mosaic embryo transfers evidenced a lower performance of mosaic embryos in terms of clinical outcomes compared to euploid ones.[2–5] Reduced clinical pregnancy and increased miscarriage rates have been observed. As a result, some authors have aimed to identify which percentage of mosaicism and type of chromosome abnormalities can be considered less detrimental.[6,7] Accordingly, scientific societies have developed guidelines to help in prioritising mosaic embryos for transfer[8,9] that have changed over time with growing scientific evidence.

It has been reported that the aneuploid cell lines would be negatively selected throughout embryo and foetal growth[10] and the observed prevalence of mosaicism detected in later stages of pregnancy is much lower than the one observed in trophectoderm biopsies.[6,11] In light of the published data, if the transfer of a mosaic embryo results in a pregnancy, either it is an ongoing pregnancy with the birth of a healthy child or it ends in a miscarriage. However, there is a theoretical scenario in which the presence of an abnormal cell line could result in abnormalities in the foetus, the baby or even in adulthood.

The role of genetic counsellors has become a key element in In Vitro Fertilisation (IVF) programmes in general and for PGT-A in particular. They are essential to provide patients with all the available information regarding mosaicism so that they can make an informed decision on transferring or not their mosaic embryos.[12] There is a considerable amount of available data on the reproductive potential of mosaic embryos.[2–5,7] Nevertheless, the uncertainty regarding the actual potential effect of mosaicism on a newborn's health still makes both the counselling and the decision challenging for both genetic counsellors and for patients. Furthermore, depending on the degree and tissue(s) affected, mosaicism could be missed at prenatal testing, which is always recommended after mosaic embryo transfer.

Regardless of this, the counselling and decision-making process has hardly been assessed. To the best of our knowledge, only Besser and co-workers evaluated the decision-making process for the transfer of mosaic embryos[13] although this study did not assess the impact that the specific mosaic anomaly observed might have on patients' decisions.

The aim of this study is to provide a global overview on patients' perspectives on their decision to transfer a mosaic embryo or not, by assessing both patients' and embryonic characteristics that may influence transfer decision-making.

2 | METHODS

2.1 | Study design and population

This is a retrospective study including 1247 PGT-A cycles performed from October 2017 to March 2021 at a single centre analysing the impact of mosaicism on PGT-A cycles and patients. The incidence of cycles with mosaic embryos, the patients' decision-making process with regard to mosaic embryo transfers and the outcomes and pregnancy follow-up were assessed.

This study was approved after evaluation by our Institutional Review Board (IRB). Ethical approval by an ethics committee was not required due to the retrospective nature of the study without patients' identifiers.

2.2 | IVF cycle and PGT-A procedure

Ovarian stimulation was achieved following the protocols in our centre.[14] Oocyte pick-up was performed 36h after ovulation triggering, and intracytoplasmic sperm injection (ICSI) of metaphase II (MII) denudated oocytes was carried out 40h post-trigger. Inseminated oocytes and embryos were cultured in time-lapse incubators (Geri®, Merck) with 5% oxygen and using single-step culture media. All culture and handling media used during the IVF procedure were G-series™ media from Vitrolife®.

PGT-A protocol has been previously described.[15] Briefly, zona opening was performed on all developing day 3 embryos and trophectoderm biopsies were conducted from day 5 to day 7 on hatching or hatched blastocysts with a multicellular trophectoderm and a well-defined inner cell mass. Five to 10 cells were obtained from the trophectoderm through the use of laser and either pulling or

flicking methodology. Biopsied blastocysts were immediately vitrified afterwards.[16] Genetic results were achieved using the reagents, equipment and protocols for the VeriSeq™PGS - MiSeq® Kit from Illumina®. Embryos were diagnosed as euploid, aneuploid or mosaic (30%–70% mosaicism). In the context of the present study, the concept "mosaic embryo" will make reference to euploid–aneuploid mosaics as they are the ones with the potential to be transferred. Euploid and mosaic embryos were kept stored while fully aneuploid embryos were discarded after diagnosis as specified in the informed consent. Euploid embryos were always prioritised for transfer. The decision to keep stored and/or transfer mosaic embryos was taken by the patients after a genetic counselling consultation.

2.3 | Information for patients and genetic counselling

In our clinical institution, a clinical embryologist performed the pre-test counselling to all couples undergoing IVF with PGT (A, SR or M). During this consultation, the process of IVF and PGT, benefits and limitations, possible results (euploid, aneuploid, mosaic embryos or the inability to obtain a result) and implications are discussed.

After availability of PGT-A results, the clinician reports the results to the patients. Those with mosaic embryos were offered a genetic counselling session with an experienced genetic counsellor.

During genetic counselling consultation, the genetic counsellor explained the definition of a mosaic embryo, its biological cause and technical limitations and discussed in detail the possible outcomes of transferring a mosaic embryo as well as the criteria used for prioritising mosaic embryos based on updated available scientific evidence. Prenatal diagnosis and cytogenetic and molecular tests are also discussed in detail. Following current guidelines and scientific evidence, they took into account the percentage of mosaicism, the type of alteration (segmental or whole chromosome), the number of chromosomes involved and the specific chromosome(s) involved. Mosaics embryos were considered a suitable option to transfer. However, patients made an informed decision after the genetic counselling session on the fate of their mosaic embryo (transfer, preserve or discard). Free consultation with a psychologist specialised in reproductive medicine was also offered to patients struggling with the decision.

The items discussed both in pre and post test sessions were approached following available guidelines[17] and modified accordingly based on updated scientific evidence.

2.4 | Decision making-analysis

For the decision-making analysis, only patients undergoing genetic counselling consultation for which their only option for transfer was a mosaic embryo were included.

For patients undergoing genetic counselling, reproductive history including maternal age, number of previous IVF cycles, previous availability of euploid embryos, history of miscarriages and parity were studied in relation to the decision to transfer. Similarly, the characteristics of the mosaic anomaly observed including percentage, type and chromosome involved according to Gratti's risk groups[6] were also analysed. When considering the characteristics of the detected mosaic anomaly, in the case of more than one mosaic embryo being available, the reference was always the lowest risk embryo according to the current guidelines. In contrast, when an embryo presented more than one mosaic anomaly, the reference was the one with the highest reproductive risk.

2.5 | Mosaic embryo transfer

Frozen-thawed mosaic embryo transfer protocol and endometrial preparation as well as luteal phase support were performed as with euploid embryos, as previously described.[18]

Prior to the transfer, patients signed a specific informed consent confirming that they understood the risks and limitations of transferring a mosaic embryo.

Surviving mosaic embryos after warming were transferred under sonographic control, as previously reported.[19]

2.6 | Statistical analysis

Continuous variables were expressed as mean and standard deviation, whereas categorical variables were expressed as percentage and number.

Categorical variables were compared with a Chi Squared test, and a Mann–Whitney *U* test was used to compare the continuous variables between categorical variables such as decision-making or reproductive outcomes. All tests were 2 tailed, and $p < 0.05$ was considered statistically significant. Statistical analyses were performed with IBM© SPSS© Statistics v 22.

3 | RESULTS

A total of 1247 PGT-A cycles were included. Cycles' characteristics are included in Table 1.

3.1 | Mosaicism diagnosis in PGT cycles

Out of 1247 cycles, 21.7% (270/1247) ended with at least one mosaic embryo suitable for transfer. In cycles with euploid and mosaic embryos, the mean number of euploid embryos was 2.7 ± 1.6. At the time of analysis, in 7.9% of cycles (99/1247), patients had to make a decision about the fate of their mosaic embryos, either because only mosaic embryos were available (5.4%; 67/1247) or because all euploid embryos had already been transferred and only mosaic embryos were left.

3.2 | Decision making

After receiving information about the PGT-A result from a clinician, patients of all 99 cycles having to make a decision on mosaic embryo transfer were referred to a genetic counsellor.

In 20 cycles, patients declined a genetic counsellor consultation (20.2%). Eighteen of them (90%) did not consider transfer as an option and decided to discard their mosaic embryos. On the other hand, two patients accepted mosaic embryo transfer and refused genetic counselling.

In 79 cycles (79.8%, 79/99), patients consulted with a genetic counsellor, from which 26.6% (21/79) decided to transfer and 73.4% (58/79) decided not to. Of them, 56.9% (33/58) opted for maintaining their mosaic embryos in case they changed their mind in the future and 43.1% (25/58) chose to discard them. The decision to transfer after genetic counselling consultation among the years of study period was 0% (0/2) in 2017, 23.5% (4/17) in 2018, 21.2% (7/33) in 2019, 35.0% (7/20) in 2020% and 42.9% (3/7) in 2021 (NS).

A total of 23.2% (23/99) of cycles with only mosaic embryos available patients decided to transfer their mosaic embryos (Figure 1).

3.3 | Reproductive history and decision-making

Female age was similar between the group of patients deciding to transfer and those that decided not to (39.0 ± 4.8 vs. 39.5 ± 2.9, $p = 0.818$) and so was the mean number of previous IVF cycles (1.1 ± 1.6 vs. 0.9 ± 1.2, $p = 0.827$). Similarly, the decision on mosaic embryo transfer was not different with regard to previous availability of euploid embryos, history of miscarriages or parity (Table 2).

TABLE 1 Cycles' characteristics

	Mean ± SD
Female age	39.9 ± 3.4
Ovarian stimulations per PGT cycle	1.2 ± 0.5
Biopsied blastocysts per PGT cycle	3.6 ± 2.6
Euploid blastocysts per PGT cycle	1.3 ± 1.5
Aneuploid blastocysts per PGT cycle	1.7 ± 1.6
Mosaic euploid blastocysts per PGT cycle	0.3 ± 0.5

TABLE 2 Decisions on mosaic embryo transfer regarding previous availability of euploid blastocysts, history of miscarriages and parity

	Patients deciding to transfer	*p*-value
Euploid embryos available in the cycle		
YES	7/25 (28.0%)	0.846
NO	14/54 (25.9%)	
History of miscarriages:		
YES	11/39 (28.2%)	0.747
NO	10/40 (25.0%)	
Parity:		
YES	4/22 (18.2%)	0.294
NO	17/57 (29.8%)	

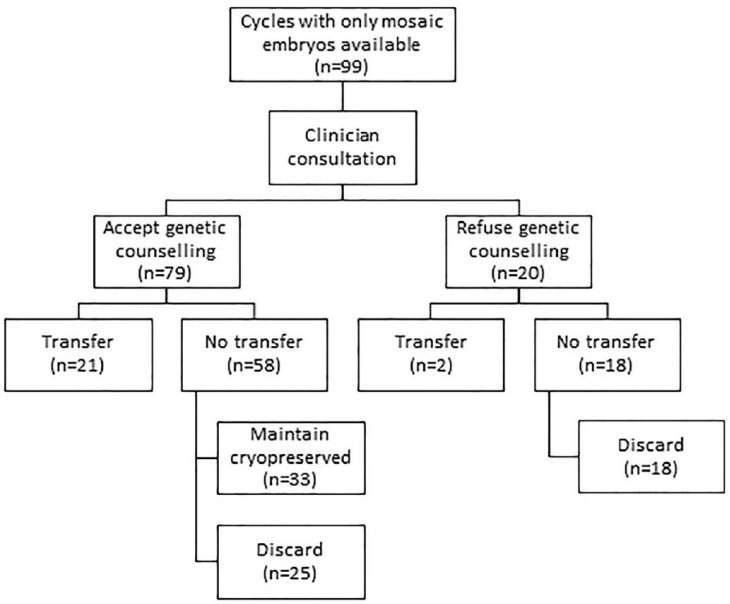

FIGURE 1 Mosaic embryo transfer decision tree

3.4 | Mosaicism characteristics and decision-making

After genetic counselling, a negative association between the percentage of mosaicism detected in the biopsy (<50% vs. ≥50%) and the decision to transfer was observed, although no statistically significant differences were detected (32.2% vs. 10.0%, $p = 0.052$). Additionally, neither the type of mosaicism (whole-chromosome, segmental or complex), nor the risk score of the chromosome(s) implicated in the mosaic anomaly(ies) according to Gratti[6] were related to the decision (Table 3).

3.5 | Transfer outcomes and pregnancy follow-up

As of the date of completion of this study, a total of 24 mosaic embryo transfers had been programmed. In 2 cases, transfer could not be performed due to embryos not surviving warming. Twenty two transfers were finally performed to 22 patients with 23 mosaic embryos transferred. Twenty transfers were single embryo transfers while 2 were double. Remarkably, one patient decided to transfer 2 mosaic embryos while the other opted for transferring a euploid embryo and a mosaic embryo at the same time (not included in the decision-making analysis), although in both cases, these options were not recommended. Implantation data could be obtained from all embryo transfers. Clinical outcomes are detailed in Table 4.

Detailed characteristics of transferred mosaic embryos can be found in Suppl. Table 1.

Of the 13 patients with an ongoing pregnancy after mosaic embryo transfer, we found that only six (46.1%) of them followed the pregnancy follow-up recommendations including thorough ultrasound evaluations and invasive prenatal testing through amniocentesis. For all of them, cytogenetic results were normal. Five patients (38.5%) did not perform any additional prenatal testing, while 2 underwent noninvasive prenatal testing. All patients underwent combined first trimester screening with the measurement of pregnancy-associated plasma protein A and free beta-human chorionic gonadotrophin (free βHCG) in maternal blood, nuchal translucency measurement and maternal age. For all term pregnancies, babies born have so far been reported as normal at birth after regular neonatal screening.

TABLE 3 Decision to transfer according to the characteristics of the mosaic anomaly(ies) observed

	Patients deciding to transfer	*p*-value
% of mosaicism		
<50%	19/59 (32.2%)	0.052
≥50%	2/20 (10.0%)	
Type of mosaicism:		
Whole-chromosome	9/35 (25.7%)	0.932
Segmental	8/31 (25.8%)	
Complex	4/13 (30.8%)	
Risk score:		
0	6/14 (42.9%)	0.207
1	1/14 (7.1%)	
2	4/14 (28.6%)	
3	4/10 (40.0%)	
4–5	3/8 (37.5%)	
6	3/19 (15.8%)	

4 | DISCUSSION

In the present study, we analysed both the influence of patients' reproductive history and mosaic characteristics on their decision to transfer a mosaic embryo. Data obtained may be useful for a better understanding of patients' perceptions throughout the process and to improve counselling.

We observed that the percentage of cycles in which patients had to make a decision on transferring a mosaic embryo is as low as 7.9%, despite mosaic embryos being reported in 21.6% of cycles. As the mean number of euploid embryos available is 2.7 ± 1.6 in cycles with mosaic and euploid embryos, the majority of patients will probably achieve term pregnancy without needing to face the decision of transferring a mosaic embryo. These figures may be variable depending on the prevalence of mosaicism in each setting. In our centre, mosaicism prevalence is around 13% (7% mosaic euploid–aneuploid) (Coll et al., 2021). It has been discussed that mosaicism may be iatrogenically generated through IVF due to inefficient protocols[20,21] and some mosaic anomalies can be technical artefacts.[22,23] In this sense, an optimised the IVF-PGT setting will be key to minimising the percentage of patients that will have to face the decision of transferring a mosaic embryo. Additionally, arbitrary threshold values may have significant clinical implications, as they determine whether an embryo is perceived acceptable for clinical use and how it is prioritised. Genetic counselling is thoroughly recommended in international guidelines for mosaic embryo transfer decision-making as it is a complex scenario for patients.[8,9] However, surprisingly, a significant percentage of patients (20.4%) refused genetic counselling. Transferring a non-euploid embryo was not acceptable for most of

TABLE 4 Clinical outcomes per transfer and per mosaic embryo transferred

	Clinical pregnancies	Miscarriages	Livebirths
Clinical outcomes per transfer	14/22 (63.6%)	1/14 (7.1%)	13/22 (59.1%)
Clinical outcomes per mosaic embryo	15/23 (65.2%)	2/15 (13.3%)	13/23 (56.5%)

them (18/20) and in two cases, the possibility of a healthy livebirth was enough to pursue transfer.

Information provided by a genetic counsellor may change their decision even when it seems clear, and in such a delicate and complex scenario, full disclosure of information and nondirective genetic counselling are important to prevent future regrets.[24] Moreover, the importance of psychological support throughout IVF treatments has been extensively discussed[25] and, although not addressed in this study, in our centre, it has proven very valuable for patients both through the decision-making process and after the transfer of a mosaic embryo.

Our results show evidence that a limited percentage of patients (23.5%) decided to transfer their mosaic embryos. This figure is in line with other authors[13] which points to the fact that general risk perception with regard to mosaic embryos may be similar among different settings. Although available data on livebirths after the transfer of mosaic embryos do not show evidence of adverse outcomes, an altered PGT result could be perceived by patients as the most determinant factor influencing their decision. Moreover, a potential adverse mosaicism consequence on the foetus or baby cannot be completely ruled out even with exhaustive pregnancy follow-up and prenatal testing. Although we could not obtain compelling conclusions from our results on the decision to transfer a mosaic embryo among years, it would seem that in the latter years, patients have been more inclined to transfer. As more evidence has been published, we have been able to shift from only discussing risks to being able to also discuss probability. A study assessing more than 2759 mosaic embryo transfers reported only one mosaic pregnancy giving an outcome of a mosaic pregnancy of 0.04%.[23] To date, only 2 mosaic pregnancies and births have been reported after the transfer of a mosaic embryo.[26,27] Therefore, when we describe the risk of an affected mosaic pregnancy, we clearly state that based on current evidence, this seems like the most unlikely scenario.

Although many patients deciding not to transfer choose to keep their mosaic embryos stored, many of them will actually end up not using and discarding them. The option to repeat a PGT-A cycle may be a good option for some patients with a good prognosis as other authors have discussed.[13] However, cost-effectiveness should be assessed for each case.

We observed that neither female age nor the number of previous cycles showed an association with the decision to transfer. Other authors have reported to the contrary.[13] While it would seem reasonable that older patients having undergone more cycles may be more inclined to mosaic embryo transfer as a last opportunity, in our population, this does not seem to be a determining factor in the decision.

With regard to patients' reproductive history influence on their decision, it could have been speculated that patients having had euploid embryos would be more inclined to try a new cycle and not transfer a mosaic embryo. However, our results do not show evidence of an association with previous availability of euploid embryos and decision-making. Also, it may seem that patients with a history of miscarriages or parity might be more reluctant to try mosaic embryo transfer due to fear of a new adverse result or parenthood already accomplished. In our results, couples who have previously accomplished parenthood show a trend towards not transferring mosaic embryos when compared to the ones without offspring.

Beyond reproductive history, risk assessment of the characteristics of the observed mosaic anomaly may be important for the decision-making. In this sense, we observed that patients with a high degree mosaic anomaly (≥50%) were more reluctant to transfer their mosaic embryos, although the difference was not statistically significant (32.8% transfer vs. 10.0% transfer, $p = 0.053$). Neither the type of mosaicism (whole-chromosome vs. segmental) nor the chromosome involved seemed to influence their decisions. Actually, the percentage and type of mosaicism have been extensively reported to be related to clinical outcomes[3,7] but not to pregnancy anomalies. The involved chromosome is the main risk marker of foetal abnormality.[6] In this sense, it is important when providing the information to clearly differentiate between reproductive potential of the mosaic embryo and foetal abnormality risk.

Our results on mosaic embryo transfer outcomes in this series of data are similar to the results from our euploid embryo transfer programme.[28] However, it must be taken into account that sample size is limited and results should be interpreted with caution. Most transfers were low-level mosaic embryos, which other reports with a larger and more compelling data set showed to give rise to good clinical outcomes, although still inferior compared to euploid embryo transfers.[7] Therefore, despite pregnancy and neonatal risks appearing to be low, low-level mosaicism reporting can still be useful for embryo transfer prioritisation and proper counselling.

In terms of pregnancy follow-up, a significant percentage of patients that undergo mosaic embryo transfer and achieve an ongoing pregnancy do not follow pregnancy monitoring and current prenatal testing recommendations. Previous reports also showed similar results.[13] It may be reasonable to think that the complexity and difficulty in achieving a pregnancy might explain their reluctance to undergo an invasive prenatal procedure. Moreover, a low-risk first trimester pregnancy screening result may lead patients to a false sense of security.

Despite the value of the presented data, one important limitation of this study is that, as the impact of mosaicism in our centre is quite low, sample size is limited. Moreover, as few patients decided to transfer a mosaic embryo, information on clinical outcomes did not allow for a deeper analysis and should be assessed with caution. As attitudes towards PGT-A vary across different regions and policy on mosaic embryo transfer may vary among different IVF centres, the results observed in the present study may not be extended to other circumstances.

Due to the retrospective nature of the present study, we could only assess quantifiable variables in relation to patients' decisions. However, given the results obtained, it might be of much interest for future studies to include information on patients' subjective perceptions through either questionnaires or interviews in order to better assess and understand their needs and decisions, being able to offer better counselling.

In general, it seems evident that mosaic embryo transfer decision path is a complex scenario both for professionals and patients. Professionals have had limited evidence-based data on mosaic embryo transfer outcomes, and risk assessment has been mainly made based on theoretical estimates, extrapolating data from prenatal diagnosis. Moreover, patients have had to deal with very complex information for the general public. Our results in this area together with recent published data analysing the outcomes from mosaic embryo transfers provide evidence that pregnancies achieved do not show a significant increase in foetal abnormality.[7,23] Results on clinical outcomes of mosaic embryo transfers and pregnancy follow-up are beginning to be compelling and reassuring, paving the way forward both for genetic counsellors and patients when facing the mosaic embryo transfer dilemma.

5 | CONCLUSION

Neither reproductive history nor information on mosaic embryo characteristics through counselling seem to be determinative for patients when deciding to transfer a mosaic embryo. In light of the results obtained, it seems that although receiving genetic counselling is still important, information received would not be the most determining factor involved in the decision-making process.

Until very recently, the information given was based on theoretical risks and a small amount of data. An increasing amount of promising data on clinical outcomes after the transfer of mosaic embryos will be of utmost importance to soften risk perception regarding mosaic embryos and to give better, simplified and more evidence-based counselling to patients that will hopefully lead to fewer potentially viable embryos being discarded and more patients achieving their reproductive goals.

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CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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Supporting information

	Type of mosaicism	% of mosaicism	Anomaly	Embryo quality	Pregnancy	Miscarriage	Adverse pregnancy outcomes	Live birth
Embryo 1	WC	30	-6	D5 5CC	N	-	-	-
Embryo 2	Segmental	30	+1q24.3qter	D7 5CC	N	-	-	-
Embryo 3	WC	30	+3	D6 6CC	N	-	-	-
Embryo 4	WC	30	-18	D6 5BB	Y	N	N	Y
Embryo 5	WC	30	-20	D5 5BB	Y	N	N	Y
Embryo 6	WC	35	+7	D5 5CB	Y	N	N	Y
Embryo 7	Segmental	35	-10q24qter	D5 5BB	Y	N	N	Y
Embryo 8	WC	40	-10	D5 5BB	Y	N	N	Y
Embryo 9	WC	40	-2	D5 5BB	Y	N	N	Y
Embryo 10	WC	30	+14	D7 5CC	Y	N	N	Y
Embryo 11[a]	Segmental	35	-9q21.13qter	D6 5AA	Y	Y	-	N
Embryo 12[a]	Complex	50	+14,+15,+19	D5 5BB	Y	Y	-	N
Embryo 13	WC	40	+19	D6 5BB	Y	N	N	Y
Embryo 14	Complex	40	+2pterp13.3, -8q22.2qter	D6 5AA	Y	N	N	Y
Embryo 15	Segmental	65	-12q13.13qter	D5 5CC	Y	N	N	Y
Embryo 16	Segmental	35	+4pterq24	D6 5CC	N	-	-	-
Embryo 17[b]	Complex	35	+7,+9	D5 5CC	Y	N	Preterm (36w), Pre-eclampsia	Y
Embryo 18	WC	40	-2	D5 5CC	N	-	-	-
Embryo 19	WC	30	46,XX/47,XXX	D6 5CC	Y	N	N	Y
Embryo 20	Segmental	45	+9q33.1qter	D5 6CC	N	-	-	-
Embryo 21	WC	30	+17	D5 5BB	Y	N	N	Y
Embryo 22	Segmental	30	+9q22.3qter	D7 6BB	N	-	-	-
Embryo 23	Complex	40	-2,+4	D6 5CC	N	-	-	-

Supplementary Table 1: Detailed information on transferred mosaic embryos. WC: Whole-chromosome. Y: Yes. N: No. [a] Embryos transferred together in a double mosaic embryo transfer. [b] Embryo transferred with a euploid embryo.

5 DISCUSSION

The present doctoral thesis is being presented as a compendium of publications. Therefore, extensive discussion of results has already been done in the discussion section of each article. For this reason, this section has been addressed as a summarized discussion incorporating the most recent findings after the articles' publication. The focus will be put on answering the questions that were raised at the beginning of the project and during its progress. Thanks to the knowledge acquired both from our research and other researchers' studies, we are now in position to try to respond to many questions for which we had no answers at the beginning.

What is the chromosomal constitution of mosaic blastocysts?

We observed that most of the mosaic abnormalities found in human blastocysts affect a whole chromosome, while around one third of them correspond to segmental abnormalities (Coll *et al.*, 2021). These results are in concordance with previous publications (Munné and Wells, 2017; Nakhuda *et al.*, 2018) and have been corroborated in later studies (Palmerola *et al.*, 2022).

With regards to the distribution of mosaic abnormalities among chromosomes we found that it is heterogeneous (Coll *et al.*, 2021), which has also been confirmed in other studies (Nakhuda *et al.*, 2018; Capalbo *et al.,* 2021; Wu *et al.*, 2021). Therefore, some chromosomes seem to be more sensitive to be involved in mitotic errors than others. For the sub-group of whole-chromosome mosaicism, we observed that, unlike in meiotic aneuploidy, the uneven distribution of anomalies showed no specific pattern that allowed to understand why some chromosomes were more affected than others (Coll *et al.*, 2021). Still, anomalies in chromosomes 18, 21 and 22 were at the highest frequencies also in mosaicism, as similarly observed by other authors (Nakhuda *et al.*, 2018; Capalbo *et al.*, 2021; Wu *et al.*, 2021). Regarding segmental mosaicism, we concluded that larger chromosomes are more prone to be affected by segmental mosaicism (Coll *et al.*, 2021) in agreement with previous findings (Munné and Wells, 2017). Remarkably, we observed chromosomes 1, 5 and 9 to be especially affected.

Our results in whole-chromosome mosaicism evidenced that gain and loss frequencies are similar. However, there are conflicting published data in this regard. While some authors have published results in accordance with ours (Munné *et al.*, 2002; Chow *et al.*, 2014) or even reported trisomies to be more frequent (Nakhuda *et al.*, 2018), others have observed monosomies to be more common (Coonen *et al.*,

2004; Delhanty, 2005; Ioannou *et al.*, 2012). We observed that mosaic segmental monosomies, on the contrary, were remarkably more frequent than trisomies (69.6 vs 28.4%) (Coll *et al.*, 2021) in agreement with other reports (Babariya *et al.*, 2017; Nakhuda *et al.*, 2018).

The fact that the characteristics of the chromosomal constitution between whole-chromosome and segmental mosaics are widely different, strongly suggests that they arise from different causes and should be treated separately.

Which are the mechanisms behind mosaicism?

With regards to segmental mosaic abnormalities, our results are in agreement with the hypothesis that the most frequent mechanism through which it occurs would be chromosome breakage and loss of the acentric fragment, since most segmental anomalies are found as monosomies (Coll *et al.*, 2021). In fact, other authors have reached the same conclusion (Babariya *et al.*, 2017; Nakhuda *et al.*, 2018; Palmerola *et al.*, 2022). Actually, hot-spots for chromosome breakage have been reported to exist in low gen-density regions rich in heterochromatin blocks (Durkin and Glover, 2007; Saksouk *et al.*, 2015; Palmerola *et al.*, 2022). Accordingly, the three chromosomes we found to be most affected by segmental mosaicism (1, 5 and 9) are especially rich in such regions (Coll *et al.*, 2021).

Contrarily, data are not so conclusive regarding whole-chromosome mosaicism. Our results were compatible with either the hypothesis that non-disjunction is the main mechanism behind it or that anaphase lagging and endoreduplication occur at similar frequencies (Coll *et al.*, 2021). This is conflicting with the most extended idea that anaphase lagging is the main mechanism involved in mosaicism (Coonen *et al.*, 2004; Delhanty, 2005; Taylor *et al.*, 2010; Ioannou *et al.*, 2012). It should be noted that FISH was the technique used in these early studies and, considering its limitations, (including limited probes, hybridization failure, or unspecific hybridization), these results ought to be considered with caution. In this sense, later analysis performed by CCS techniques showed results in agreement with ours (Chow *et al.*, 2014; Nakhuda *et al.*, 2018). However, a recent study inducing replication errors in a murine model at cleavage stage showed that the prevalence of chromosome loss doubled that of gain (Palmerola *et al.*, 2022).

Trying to discern the mechanisms underlying mosaicism through trisomy and monosomy rates is extremely complex for several reasons. First, mosaic embryos may end up with an arrested development depending on the mechanism that has originated the mosaicism (McCoy, 2017; Vázquez-Diez and FitzHarris, 2018; Palmerola *et al.*, 2022). Therefore, certain mechanisms may be under or overrepresented at advanced developmental stages such as the blastocyst. Second, regardless of the mechanism, trisomic cells may be more proliferative than monosomic cells leading to a bias in sampling when biopsy is performed at later stages (Munné and Wells, 2017). Finally, technical limitations and the use of different diagnostic platforms may also lead to conflicting results.

Does "a prevalence of mosaicism in preimplantation embryos" exist?

As many factors may affect the occurrence of mosaicism and mosaicism reporting varies among centres, it is extremely challenging to reach a consensus on the prevalence of mosaicism in human blastocysts and, actually, "a prevalence of mosaicism" may not even exist. It might be more accurate to talk about the prevalence of a mosaic diagnosis according to a specific environment.

Our results with regards to mosaicism prevalence have evidenced it to be 14%, with around 7% blastocysts showing a euploid-aneuploid mosaic result (Coll *et al.*, 2021, 2022a). Although the reported prevalence of mosaicism has been different among clinics, most settings with NGS-based analysis of a single trophectoderm biopsy have a prevalence of euploid-aneuploid mosaic ranging from around 5 to 15%, which is in concordance with our results (Ruttanajit *et al.*, 2016; Munné *et al.*, 2019a; Popovic *et al.*, 2020; Rodrigo *et al.*, 2020; Leigh *et al.*, 2022).

Our data for euploid-aneuploid mosaicism prevalence is among the lowest reported. Among other factors, the fact that the range used to call mosaicism was established at 30-70% may have been important to avoid overdiagnosis. Cell-mixing experiments demonstrate that NGS-based PGT-A can identify mosaicism from 20-80% (Maxwell *et al.*, 2016; Fragouli *et al.*, 2017; Munné *et al.*, 2017; Popovic *et al.*, 2018; Spinella *et al.*, 2018). However, most authors currently agree that those experiments may not properly represent a trophectoderm sample (Munné and Wells, 2017; Treff and Franasiak, 2017; Fragouli *et al.*, 2019), and narrowing the range may be very helpful in order to avoid false-positive mosaic results (Treff and Marin, 2021; Wu *et al.*, 2021; Capalbo *et al.*, 2022).

Moreover, being strict with regards to quality parameters after sequencing as well as to ensure the expertise of the person performing the diagnosis will be crucial to avoid mosaicism overdiagnosis.

Are patients', IVF cycle, or biopsy procedure characteristics associated with mosaicism?

Patients

We only identified paternal age as a patient-related factor to be independently associated with mosaicism prevalence (Coll *et al.*, 2021). We hypothesize that this may be an indirect finding pointing towards DNA fragmentation, as it has been associated both with paternal age (Belloc *et al.*, 2014; Yatsenko and Turek, 2018) and segmental mosaicism (Babariya *et al.*, 2017). Actually, some authors have observed an increase in segmental aneuploidy in embryos from males above 50 years (Dviri *et al.*, 2020), which further supports our hypothesis (Babariya *et al.*, 2017; Victor *et al.*, 2019a). Our results did not show any association between an altered spermiogram and an increase in mosaicism prevalence (Coll *et al.*, 2021), in contrast to other reports (Tarozzi *et al.*, 2019; Huang *et al.*, 2022). In summary, the male factor seems to play an important role in mosaicism.

On the contrary, none of the female characteristics analysed in our study were associated with mosaicism (Coll *et al.*, 2021), which is in agreement with previous data from other authors (Munné and Wells, 2017; Popovic *et al.*, 2018, 2020) and has been corroborated by later research (Xiong *et al.*, 2021). Therefore, the female factor does not seem to affect mosaicism, in contrast with meiotic aneuploidy.

We found that the indication for PGT-A is not associated with mosaicism (Coll *et al.*, 2021). This has also been confirmed by other authors (Xiong *et al.*, 2021).

Despite the fact that we only identified male age to be related to mosaicism, we observed a high interpatient variability regarding mosaicism prevalence (Coll *et al.*, 2021). This suggests that other patient related factors that have not been analysed might be affecting mosaicism prevalence and deserve further investigations.

IVF cycle

Our results suggested that ovarian stimulation variables are not related to mosaicism, neither the total dose of gonadotropins nor the ovarian response (Coll *et al.*, 2021). Later research has corroborated our findings and further supported this idea by analysing more variables (Cascales *et al.*, 2021; Xiong *et al.*, 2021).

Some authors have suggested that oocytes inseminated by conventional IVF give rise to more mosaic embryos compared to intracytoplasmic sperm injection (ICSI) derived embryos (Palmerola *et al.*, 2019; Huang *et al.*, 2022). The biological explanation is still unclear and artefactual results due to DNA contamination (sperm and granulosa cells) cannot be completely ruled out. We could not have data in this regard as we followed the good-practice guidelines recommending ICSI for PGT cases (ESHRE PGT Consortium and SIG-Embryology Biopsy Working Group *et al.*, 2020)

Previously published data have evidenced that suboptimal culture conditions can affect mosaicism prevalence (Katz-Jaffe *et al.*, 2018; Swain, 2019). As our culture conditions were standardized for optimal culture to the blastocyst stage, we could not test the effect that altered culture conditions may have on mosaicism prevalence. We could test, nevertheless, the effect that two different continuous culture media (G-TL®, Vitrolife and Global total®, CooperSurgical) may have on mosaicism, observing no differences in its prevalence (Coll *et al.*, 2021). Results from other authors are controversial, highlighting the diversity of manufacturers and culture media that can affect embryo development (Morbeck *et al.*, 2017; Swain, 2021).

In summary, as IVF cycle characteristics may have an effect on mosaicism prevalence, it is recommended that centres investigate both their laboratory and PGT-A methodologies in cases of high prevalence of mosaicism (Leigh *et al.*, 2022).

In our results, mosaicism was not associated with blastocyst quality (Coll *et al.*, 2021). These findings were in agreement with other authors' (Popovic *et al.*, 2018; Rodrigo *et al.*, 2020). However, it cannot be ruled out that embryos with mosaicism may present a compromised embryo development during early stages (McCoy, 2017; Vázquez-Diez and FitzHarris, 2018; Lagalla *et al.*, 2017).

Biopsy and tubing

Our results confirmed that the prevalence of mosaicism is the same among distinct senior biopsy operators (Coll *et al.*, 2021, 2022a). This finding has been corroborated by other authors (Mizobe *et al.*, 2022). However, results may not be the same in the case of inexperienced practitioners (Yap *et al.*, 2021).

With respect to the use of laser pulses to obtain the biopsy, we observed no differences in mosaicism prevalence when applying ≤3 pulses vs >3 (Coll *et al.*, 2022a), which is in agreement with previous reports showing no differences even in cases of extreme exposure to laser (Kelk *et al.*, 2017; Johnson *et al.*, 2019). However, other authors report contrarily (Whitney *et al.*, 2018). Some authors have suggested that the use of laser *per se,* regardless of the number of pulses, would induce more mosaic artefactual results compared to mechanical retrieval of the biopsy (Yelke *et al.*, 2021). This finding should be confirmed and considered with caution as not laser-assisted biopsy might be more harmful both for the embryo and the sample.

Our results confirmed that neither pulling nor flicking led to an increase in mosaicism (Coll *et al.*, 2022a). Contemporary published research confirmed our findings (Mizobe *et al.*, 2022). Therefore, despite flicking leads to the obtention of more damaged samples (Benavent *et al.*, 2019), this does not seem to have an effect on results. Importantly enough, we observed that the reproductive potential of biopsied embryos is not affected by the biopsy methodology (Coll *et al.*, 2022a).

Some authors have reported that sequential and simultaneous biopsy strategies may lead to different prevalence of mosaicism, possibly as a result of differences in sample quality leading to differences in artefactual results (ESHRE PGT Consortium and SIG-Embryology Biopsy Working Group *et al.*, 2020; Xiong *et al.*, 2021).

An important fact to ensure low artefactual mosaicism seems to be the number of collected cells. Both, a low number of biopsied cells and too many, can increase the risk to observe artefacts compatible with mosaicism (Treff and Marin, 2021; Leigh *et al.*, 2022; Mizobe *et al.*, 2022). Therefore, the recommended number of cells to be biopsied both for minimizing artefactual mosaicism and not compromising embryo development is 5 to 10 (ESHRE PGT Consortium and SIG-Embryology Biopsy Working Group *et al.*, 2020; Leigh *et al.*, 2022).

With regards to tubing and sample storing, our data evidenced that neither the time from biopsy to tubing nor the time the sample is stored at -80ºC have an effect on the prevalence of mosaicism (Coll *et al.*, 2022a). Therefore, there is no reason to delay tubing expecting debris and lysed cells to be more easily washed to reduce artefacts.

What is the impact of mosaicism diagnosis in patients?

Recent evidence has clearly demonstrated that the accuracy of full aneuploidy calling is almost 100% (Victor *et al.*, 2019a; Tiegs *et al.*, 2021; Capalbo *et al.*, 2022; Kim *et al.*, 2022). Therefore, discarding aneuploid embryos for transfer will not have an effect on cycles' cumulative live births. However, the scenario is different with regards to mosaic embryos. As such embryos can potentially lead to healthy pregnancies, if patients refuse their transfer, cumulative live-birth rate per cycle will be compromised (Capalbo *et al.*, 2021; Armstrong *et al.*, 2022).

Cycles affected by mosaicism

In our setting, while at least one euploid-aneuploid mosaic embryo is detected in 21% of cycles, the percentage of cycles in which patients only had mosaic embryos as an option for transfer was as low as 5% (Coll *et al.*, 2022b). During the study period, this figure raised up to 8% when also considering cases in which all euploid embryos had been transferred. Therefore, despite all the concerns with respect to the diagnosis of mosaic embryos and its effect on PGT-A outcomes, it may actually impact into a limited number of patients who will have to make a decision on the transfer of a mosaic embryo. In this sense, ensuring a reduced rate of both iatrogenic and artefactual mosaicism is key for a good PGT-A program (Treff and Marin, 2021; Wu *et al.*, 2021; Capalbo *et al.*, 2022; Leigh *et al.*, 2022).

Patients' decisions on MET

We observed that around 75% of patients refuse MET (Coll *et al.*, 2022b), which is in agreement with previous data (Besser *et al.*, 2019; Coll *et al.*, 2022b). This has most certainly been due to the uncertainty and lack of data with regards to the prognosis of such embryos. Actually, we have observed a change in patients' decision making

in relation to the increase in data availability throughout 2021 (Capalbo *et al.*, 2021; Treff and Marin, 2021; Viotti *et al.*, 2021)(Figure 19).

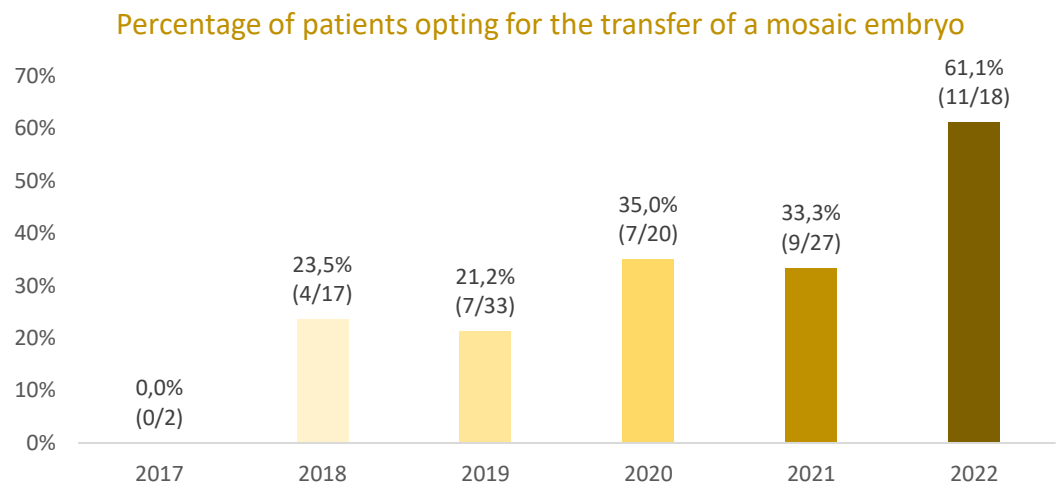

Figure 19: Percentage of patients opting for the transfer of a mosaic embryo along the years (Coll *et al.*, 2022b; Dexeus Dona, unpublished data).

We observed that patients' decision is not strongly modified by their reproductive history (female age, number of previous cycles, history of miscarriages, the fact of having had euploid embryos) (Coll *et al.*, 2022b), although a previous study reported otherwise in a different population. Authors found that patients with a poorer prognosis (advanced female age and high number of previous unsuccessful treatments) were more favorable to MET (Besser *et al.*, 2019). Moreover, our results suggested that already having children made patients more reluctant to face the potential risks of a MET.

We reported for the first time data on patients' decision in relation to the characteristics of the mosaic finding as explained in genetic consultation. Patients were not influenced neither by the type of abnormality (whole-chromosome, segmental, complex) nor by the chromosome involved. Only the level of mosaicism

(<50% vs >=50%) may affect the decision to transfer (Coll *et al.*, 2022b). These data may reflect that the information received may be overwhelming and too complex for patients to understand.

Clinical outcomes after MET

We observed outcomes after MET to be similar to those obtained after euploid embryo transfer (Coll *et al.*, 2022b). Updated data to December 2022, including the transfer of 50 mosaic embryos, produced the results shown in Table 3 (Dexeus Dona, unpublished data). Our data agree with larger studies reporting results close or even equivalent to those obtained with euploid embryos, specifically with regards to low-range mosaicism (≤50%), with no increase of affected pregnancy risk (Capalbo *et al.*, 2021; Treff and Marin, 2021; Viotti *et al.*, 2021).

Table 3: Clinical results of mosaic embryo transfers from January 2018 to December 2022.

		Clinical pregnancies	Miscarriages	Ongoing pregnancies
Outcomes per transfer		22/49 (44.8%)	3/22 (13.6%)	19/49 (38.7%)
Outcomes per mosaic embryo transferred	Total	23/50 (46.0%)	4/23 (17.4%)	19/50 (38.0%)
	≤50% (low-range)	20/41 (48.8%)	2/20 (10.0%)	18/41 (43.9%)
	>50% (high-range)	3/9 (33.0%)	2/3 (66.7%)	1/9 (11.1%)

Getting to know the clinical value of a mosaic diagnosis. A new era for PGT-A and mosaicism?

The story of mosaicism detection at the blastocyst stage in PGT-A cycles is full of ups and downs and unexpected turns.

All in all, at the beginning, this finding in trophectoderm biopsies was received with lots of caution, and many embryos were discarded deemed to be incompatible with a healthy live birth. Luckily, soon enough, healthy births with such kind of embryos were reported (Greco *et al.*, 2015). This made evident that they could not simply be discarded. Research was conducted to better know how to deal with mosaic embryos, and it seems that, after years of research and transfer follow-ups, we have reached the conclusion that mosaicism consequences should be reconsidered.

On one hand, studies have demonstrated that the true prevalence of mosaicism in preimplantation embryos may be much lower than reported, being in many occasions a result of a biological or technical artefact (Capalbo *et al.*, 2021; Treff and Marin, 2021; Wu *et al.*, 2021; Kim *et al.*, 2022). Moreover, while true mosaicism exists in preimplantation embryos (Capalbo *et al.*, 2017b; Kahraman *et al.*, 2020; Wu *et al.*, 2021; Schlade-Bartusiak *et al.*, 2022; Greco *et al.*, 2023), evidence show that in many cases it resolves through embryo development (Bolton *et al.*, 2016; Coorens *et al.*, 2021; Yang *et al.*, 2021; Griffin *et al.*, 2023).

On the other hand, clinical outcomes of more than 2700 METs have been published evidencing lower clinical pregnancy rate and higher miscarriage rate compared to euploid embryo transfers, but yet still yielding remarkable results, especially in the case of low-range mosaics (Viotti *et al.*, 2021). Actually, a prospective non-selection clinical trial evidenced that results of low-range mosaics are equivalent to those obtained with euploid embryos (Capalbo *et al.*, 2021). With regards to high range

mosaics (>50%) data are more limited and clinical outcomes are not so good (Viotti *et al.*, 2021). Additionally, the risk of a mosaic ongoing pregnancy has not been observed to be superior to the one observed and estimated for the general population and, although true foetal mosaicism occurs and should be carefully considered, it still seems to be a very rare exception (Spinner and Conlin, 2014; Benn, 2015; Malvestiti *et al.*, 2015; Kahraman *et al.*, 2020; Treff and Marin, 2021; Schlade-Bartusiak *et al.*, 2022; Greco *et al.*, 2023). Moreover, a purely euploid TE biopsy result cannot totally prevent mosaicism in the pregnancy either (ESHRE Working Group on Chromosomal Mosaicism *et al.*, 2022).

Therefore, both the diagnostic and the clinical value of a mosaic result are poor. With this conclusion in mind, the new European Society of Human Reproduction and Embryology (ESHRE) guidelines on good practice recommendations on managing chromosomal mosaicism imply a change of paradigm (ESHRE Working Group on Chromosomal Mosaicism *et al.*, 2022). Embryos diagnosed as low-range mosaics should be considered just as equals to euploid embryos, which makes diagnosing and reporting low-range mosaicism meaningless. On the contrary, no conclusions are reached with regards to high-range mosaics recommending that, in case of transfer, previous genetic counselling and extensive pregnancy follow up should be performed.

In summary, at present, low-range mosaics are proposed to be considered equal to euploid embryos and high-range mosaics are still considered risky embryo transfers. With regards to the latter, some authors have evidenced that they are, in many cases, completely or almost completely abnormal, suggesting that they should be considered as aneuploid and not mosaic (Capalbo *et al.*, 2021; Wu *et al.*, 2021). However, clinical pregnancies with such embryos have been reported, which should also be taken into consideration (Viotti *et al.*, 2021; Coll *et al.*, 2022b). Further data

will be needed to elucidate whether the transfer of high-range mosaics is worth the risk. Luckily, they seem to be the least among PGT-A results, implying around 2% of the diagnoses (Capalbo *et al.*, 2021).

All in all it seems that, after all the struggle with the colorful mosaic of possible embryo diagnosis, we may now be in the way back to a black and white scenario.

6 CONCLUSIONS

1. The distribution of mosaic abnormalities among chromosomes is uneven. While no clear distribution pattern is observed for whole-chromosome mosaicism, segmental mosaicism is more prone to occur to larger chromosomes and chromosomes with fragile sites.

2. The mechanisms behind whole-chromosome and segmental mosaicism are different and they should be treated differently.

3. Within an experienced, optimised and standardised IVF and PGT setting, the prevalence of mosaicism is low.

4. Advanced male age is associated with an increased mosaicism prevalence in human blastocysts.

5. Detected mosaicism is equivalent among equally trained senior biopsy operators.

6. The number of laser pulses applied to obtain a trophectoderm biopsy is not associated with detected mosaicism.

7. Detected mosaicism is equivalent among embryos biopsied using pulling vs flicking.

8. Patients' reproductive history and information given in counselling pre-MET do not influence patients' decisions.

9. Although most patients have been refusing the transfer of embryos with a diagnosis of mosaicism, the availability of empirical data on MET outcomes has brought more patients to transfer their mosaic embryos

10. Low-range mosaic embryos can yield similar outcomes compared to euploid embryos.

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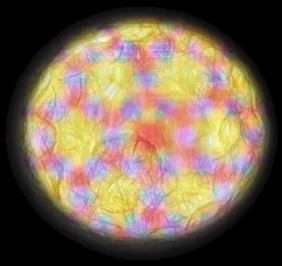

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