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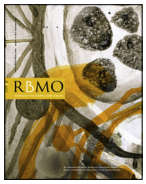

ARTICLE

Chromosomal mosaicism in human blastocysts: a cytogenetic comparison of trophectoderm and inner cell mass after next-generation sequencing

BIOGRAPHY

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KEY MESSAGE

A cytogenetic comparison of trophectoderm and inner cell mass of human embryos shows a high incidence of chromosomal mosaicism and a low confirmation rate, while a TE biopsy does predict whether the ICM consists mostly of normal or abnormal cells. Thus, the current findings support the notion that reporting the level of mosaicism is clinically relevant.

ABSTRACT

Research question: What is the incidence of chromosomal mosaicism in human blastocysts and can a single trophectoderm (TE) biopsy accurately predict the chromosomal constitution of the inner cell mass (ICM)?

Design: Observational study in 46 surplus cryopreserved preimplantation embryos of unknown chromosomal constitution. For each embryo, a TE biopsy was performed and the ICM was collected separately. Both samples underwent next-generation sequencing (NGS) for cytogenetic analysis and were classified as chromosomally normal, abnormal or mosaic. Mosaic samples were classified as low or high mosaic, based on the majority dominance of either normal or abnormal cells in the biopsied sample. Findings within each embryo were compared.

Results: Chromosomal mosaicism was detected in 59% (n = 27/46) of the embryos, with a cytogenetic concordance rate between TE and corresponding ICM of 48% (n = 22/46). Concordance was higher from a clinical perspective: in 86% of embryos with a high-mosaic or abnormal TE, the ICM was also high-mosaic or abnormal. In 88% of the blastocysts with a normal or low-mosaic TE biopsy, a normal or low-mosaic ICM was observed.

Conclusion: Despite the low cytogenetic concordance rate due to chromosomal mosaicism present in blastocysts, it was found that a single TE biopsy could correctly predict whether the ICM consists of mostly normal or abnormal cells in the majority of cases.

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KEYWORDS

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INTRODUCTION

Implantation failure, spontaneous miscarriage and congenital birth defects in humans are related to chromosomal imbalances (*Macklon et al., 2002*). The gain or the loss of a chromosome or chromosomal region is commonly found in human preimplantation embryos (*Baart et al., 2006*; *Daphnis et al., 2008*; *Delhanty et al., 1997*; *Munné et al., 1994a*; *Santos et al., 2010*; *Vanneste et al., 2009*). In young women, 10–20% of embryos are uniformly abnormal, as the result of a meiotic error in the oocyte (*Baart et al., 2006*; *McCoy et al., 2015*). This proportion of embryos increases dramatically with maternal age (*Dang et al., 2019*; *Demko et al., 2016*; *Gruhn et al., 2019*). Moreover, the first cell divisions in the early embryo are highly prone to mitotic chromosome segregation errors (*Baart et al., 2006*; *Daphnis et al., 2008*; *Delhanty et al., 1997*; *Munné et al., 1994a*; *Santos et al., 2010*; *Vanneste et al., 2009*), which are independent of maternal age (*Reich et al., 2020*). Mitotic errors result in the presence of two (or more) distinct cytogenetic populations of cells in an embryo, defined as chromosomal mosaicism. Mosaic embryos can consist of a mixture of normal and abnormal cells, but also of cells with different abnormalities. The underlying mechanisms in chromosome segregation in human preimplantation embryos are not fully understood, but so far appear to be multifactorial (*McCoy, 2017*; *Popovic et al., 2020*; *Tšuiko et al., 2020*). It is hypothesized that the molecular pathways that monitor accurate chromosome segregation in somatic cells operate at reduced stringency in early embryos, to allow the rapid cleavage divisions needed for the coordinated development of the embryo (*Akera et al., 2019*; *Vázquez-Diez et al., 2019*).

Preimplantation genetic testing for aneuploidies (PGT-A) was introduced (*Harper et al., 1995*; *Munné et al., 1994b*) in an attempt to identify chromosomally normal embryos before embryo transfer. Initially, one or two blastomeres from IVF embryos at the 8-cell stage were removed and they underwent subsequent cytogenetic analysis with fluorescence in-situ hybridization (FISH) or comprehensive chromosome screening (CCS) (*Imudia and Plosker, 2016*). Studies that analysed human IVF embryos at the 8-cell stage showed that chromosomal mosaicism affected up to 91% of these embryos (*Baart et al., 2004*, *2006*; *Bielanska et al., 2002*; *Chow et al., 2014*; *Mantzouratou et al., 2007*; *Mertzanidou et al., 2013*; *Vanneste et al., 2009*; *Voullaire et al., 2000*; *Wells and Delhanty, 2000*). These studies provided evidence that the biopsy and analysis of a single blastomere is not really representative of the chromosomal constitution of the entire embryo (*Baart et al., 2004*, *2006*). When a meta-analysis of randomized controlled trials on the effect of PGT-A demonstrated no added value, the use of PGT-A at the 8-cell stage as an embryo selection tool was largely abandoned (*Mastenbroek et al., 2011*).

After the morula stage, the incidence of chromosomal mosaicism appears to decrease as embryos reach the blastocyst stage (*Santos et al., 2010*). After compaction and cavitation, there seems to be a selection against embryos, or cells within the embryo, carrying complex abnormalities and monosomies (*Fragouli et al., 2019*; *McCoy et al., 2015*). However, cytogenetic analysis of 5–10 trophectoderm (TE) cells following biopsy still reveals chromosomal mosaicism in blastocysts (*Fragouli et al., 2019*; *Popovic et al., 2018*; *Starostik et al., 2020*), although establishing the prevalence remains a challenge (*Fragouli et al., 2019*; *Popovic et al., 2020*; *Starostik et al., 2020*). Therefore, there is an ongoing debate over whether mosaicism in a TE biopsy correctly reflects true mosaicism or whether it results from technical artifact (*Capalbo and Rienzi, 2017*; *Marin et al., 2020*; *Popovic et al., 2020*). Single TE biopsies detected mosaicism in 2–19% of blastocysts investigated (*Capalbo et al., 2021*; *Katz-Jaffe et al., 2017*; *Munné et al., 2019*; *Ruttanajit et al., 2016*; *Stankewicz et al., 2017*). However, if mosaicism is present in blastocysts, abnormal cells might be unevenly distributed within and over the two embryonic lineages, the inner cell mass (ICM) and the TE (*Gleicher et al., 2017*; *Popovic et al., 2018*; *Starostik et al., 2020*). A diagnosis based on a TE sample only may thus not represent the entire embryo and in particular the ICM that will form the fetus.

Recent studies analysed multiple embryo biopsies, including the ICM, of embryos donated for research and the reported chromosomal mosaicism rates were between 14% and 80% (*Chuang et al., 2018*; *Huang et al., 2017*; *Lawrenz et al., 2019*; *Lin et al., 2020*; *Orvieto et al., 2016*; *Popovic et al., 2018*; *Sachdev et al., 2020*; *Tšuiko et al., 2018*; *Victor et al., 2019*). In addition, the same studies showed that the concordance rate between the chromosomal content of ICM and TE varied between 18% and 86%. However, as this type of research into mosaicism relies on embryos donated for research, only small cohorts of embryos have been investigated. Moreover, the previous studies mainly used embryos that were selected based on an abnormal PGT-A result, introducing a major selection bias.

Despite insufficient knowledge about the true incidence of mosaicism and the concordance rates between the cytogenetic constitution of TE and ICM, PGT-A is offered to an ever-increasing number of patients worldwide (*Roche et al., 2021*). To determine the accuracy of PGT-A it is important to clarify to what extent cytogenetic analysis of a single TE sample is representative of the ICM. This study investigated good-quality blastocysts developed from cryopreserved embryos donated for research that were not cytogenetically pre-tested. Biopsies of the TE and corresponding ICM were performed and the chromosomal constitution was determined by next-generation sequencing (NGS). Concordance rates are discussed from a cytogenetic and a clinical perspective by taking into account the latest insights into the implantation potential of mosaic embryos.

MATERIALS AND METHODS

Ethical approval

Surplus cryopreserved human preimplantation embryos of unknown chromosomal constitution were donated with written consent from patients undergoing IVF treatment at the Erasmus MC, University Medical Center, Rotterdam. Use of these embryos for this study was approved by the Dutch Central Committee on Research Involving Human Subjects (CCMO, NL 38053.000.111, 13 March 2012) and the local institutional ethics committee (MEC-2011-372, 25 April 2012).

Embryo warming and culture

Ovarian stimulation, oocyte retrieval, IVF procedures and assessment of

embryo morphology were performed as described previously (*Hohmann et al., 2003*). Supernumerary good-quality embryos were cryopreserved at the morula stage between 2010 and 2012. Cryopreservation was performed in a controlled-rate freezer in straws in culture medium with 1.5 mol/l dimethylsulphoxide. Straws were cooled to –6°C before seeding and subsequently cooled to –40°C at 0.3°C/min. Finally, the straws were cooled rapidly at –25°C/min to –140°C, before immersion in liquid nitrogen and storage in nitrogen vapour. After donation, the embryos were thawed at room temperature. After release from the straw, the embryo was warmed using the RapidWarm™ Omni kit (Vitrolife, Göteborg, Sweden), according to the manufacturer's instructions. After thawing, each embryo was placed in a well of an EmbryoSlide™ (Vitrolife) culture dish containing 25 µl of SAGE 1-Step™ medium (Cooper Surgical, Trumbull, CT, USA) under 1.4 ml liquid paraffin oil (Cooper Surgical). The embryos were cultured for 24-48 h in an EmbryoScope™ time-lapse incubator (Vitrolife) at 36.8°C, 7% O_2 and 5% CO_2. After 24 h of culture, the embryos were morphologically evaluated according to the ESHRE consensus scoring system for blastocysts (*Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011*). Only embryos that managed to reach at least the full blastocyst stage and had at least several loosely grouped ICM cells were biopsied (TABLE 1). Early blastocysts were further cultured and re-evaluated after 24 h if they met the minimal biopsy requirements, as already described.

Embryo biopsy

The biopsy was performed on the heated stage of an inverted microscope (Leica Microsystems, Germany) equipped with a micromanipulation system (TransferMan®4m, CellTram®4m Air/Oil, Eppendorf, Hamburg, Germany). The biopsy took place in buffered human tubal fluid (Quinn's Advantage™ Medium with HEPES, Cooper Surgical) and the penetration of the biopsy pipette (type MBB-FP-SM-30, Cooper Surgical) in the blastocoel for aspiration of the ICM was achieved with the assistance of a holding pipette (type MPH-MED-30, Cooper Surgical) and an OCTAX NaviLase system (Vitrolife). It has previously been shown that ICM and TE cells can be reliably separated by biopsy (*Capalbo et al. 2013*). After collection of the ICM, a TE biopsy of 5–10 cells was performed on the opposite side of the ICM to avoid contamination of the TE sample. Both samples were separately washed in Dulbecco's phosphate-buffered saline (DPBS, Thermo Fisher Scientific, MA, USA) containing 0.1% polyvinyl alcohol (Sigma Aldrich, Missouri, USA) and were subsequently transferred in 0.5 ml Eppendorf microcentrifuge tubes containing 2.5 µl DPBS. Samples were kept on ice during the procedure and were then stored at –20°C for up to 7 days, until the cytogenetic analysis.

NGS analysis

Cytogenetic analysis of the samples was performed with an NGS-based approach. For the library preparation, the ReproSeq™ PGS Kit (Thermo Fisher Scientific) was used, according to the manufacturer's instructions. Briefly, the extracted genomic DNA of each sample was amplified and uniquely barcoded. This allowed the simultaneous pooling of 16 or 24 samples depending on the loading chip (Ion 510 or 520 chip, Thermo Fisher Scientific). Each library pool was diluted to the final concentration of 80 pmol/l. The templating and the chip loading were carried out with using the Ion Chef system (Thermo Fisher Scientific) and the loaded chips were sequenced on the Ion S5 XL Sequencer (Thermo Fisher Scientific). The sequencing data analysis and interpretation used Ion Reporter™ Software 5.10 (Thermo Fisher Scientific). Samples with an insufficient number of reads (<90,000) or with a median absolute pairwise difference (MAPD) higher than 0.3 were excluded from the analysis. The copy number of chromosomes for each sample was determined through the ReproSeq Mosaic PGS w1.1 workflow with low genome coverage (0.01 ×). According to the manufacturer's default setting, the software counts the sequences in 2 Mb bins and compares the number of reads for each bin to the reference baseline. A call is made when the copy number for a particular defined region deviates from the normal (2N) with a copy number variation confidence range set at 0.1.

Establishment of normal, fully abnormal and mosaic copy number ranges

Studies have shown that NGS can detect the presence of chromosomal mosaicism when 20–80% of the cells of a sample are abnormal (*Biricik et al., 2021*; *Chuang et al., 2018*; *Goodrich et al., 2016*; *Spinella et al., 2018*). The sensitivity of the ReproSeq Mosaic PGS kit (Thermo Fisher Scientific) to detect (mosaic) chromosomal abnormalities was validated by using cell lines with a known normal or abnormal karyotype. To test the thresholds, cell-mixing experiments of normal and abnormal cells in different ratios were performed (Supplementary Methods, Supplementary Table 1). Based on the results from these validation experiments, the copy number range for normal, fully abnormal and mosaic samples was determined (FIGURE 1A). For autosomal chromosomes and the X chromosome in XX samples, the normal copy number range was set between 1.75 and 2.25. A full-blown chromosomal loss or gain was considered when the copy number was <1.20 or >2.80, respectively. For the X and Y chromosomes in XY samples, the normal range was set between 0.75 and 1.25, whereas a call for a loss or gain was made when the copy number value was <0.75 or >1.25, respectively. The copy number range in between these ranges was considered mosaic (FIGURE 1A). Mosaic samples were also classified as low or high mosaic, based on the majority dominance of either normal or abnormal cells in the biopsied sample.

Cytogenetic interpretation of TE and ICM samples

First, according to the copy number values for each chromosome, the presence of a (partial) loss or gain was established. If a copy number value in the abnormal range was observed for a specific chromosome, a distinction was made between low-mosaic, high-mosaic and full-blown abnormality (FIGURE 1A, TABLE 1). Then, based on the copy number value of the combined abnormalities present, each sample (TE or ICM) was also classified in the normal, low-mosaic, high-mosaic or abnormal category. In low-mosaic samples the copy number value(s) of the aberration(s) was (were) in the low-mosaic range. A high-mosaic sample had at least one chromosomal abnormality with a copy number value in the high-mosaic range, while additional chromosome aberrations could show low-level mosaicism. Samples with at least one chromosome aberration with a copy number value in the full abnormal range were considered fully abnormal. Samples with more than five chromosome aberrations were

TABLE 1 **Morphology score and NGS results of TE and ICM per embryo. The CN value is presented next to each chromosomal abnormality. In partially concordant embryos, the common abnormality of TE and ICM is presented in bold. For chaotic samples, only the abnormalities present in both embryonic lineages are shown. Whole chromosomal abnormalities are presented only with the number of the involved chromosome; for segmental abnormalities the involved segment is shown; 2N normal. The letter (a–j) in the first column reflects the schematic categories from *FIGURE 3*.**

Concordant

Embryo	Morphology	TE	TE-CN value	ICM	ICM-CN value
5[a]	B421	2N	2N	2N	2N
7[a]	B411	2N	2N	2N	2N
8[a]	B411	2N	2N	2N	2N
11[a]	B411	2N	2N	2N	2N
14[a]	B411	2N	2N	2N	2N
23[a]	B412	2N	2N	2N	2N
29[a]	B411	2N	2N	2N	2N
26[a]	B413	2N	2N	2N	2N
35[a]	B411	2N	2N	2N	2N
40[a]	B412	2N	2N	2N	2N
41[a]	B412	2N	2N	2N	2N
44[a]	n/a	2N	2N	2N	2N
6[j]	B413	Loss 10 Loss 15 Loss 18	1.00 1.05 0.95	Loss 10 Loss 15 Loss 18	1.10 1.05 1.05
9[g]	B411	Gain 15	2.80	Gain 15	2.95
13[j]	B413	Loss 17	1.00	Loss 17	1.05
15[j]	B312	Gain 16	2.95	Gain 16	3.05
21[j]	B421	Gain 15	2.90	Gain 15	2.95
22[j]	B423	Gain 6 Gain 8 Gain 9 Gain 16 Gain 20	3.15 2.95 3.00 3.00 2.90	Gain 6 Gain 8 Gain 9 Gain 16 Gain 20	2.60 2.65 2.60 2.95 2.30
36[j]	B411	Gain 15	2.95	Gain 15	2.95
42[j]	B423	Gain 5	3.05	Gain 5	3.10
43[f]	B411	Gain 19	2.60	Gain 19	2.75
45[j]	B413	Loss 16	1.05	Loss 16	1.10

Partially concordant

Embryo	Morphology	TE	TE-CN value	ICM	ICM-CN value
2[j]	B421	Chaotic including **Loss 13**	 0.85	 **Loss 13**	 1.05
4[h]	B422	Chaotic including **Gain 4q32.1q35.2** **Loss 6p25.3p11.2**	 2.45 1.50	**Gain 4** **Gain 6** Gain 19 Loss 21	2.50 2.40 2.35 1.70
10[j]	B313	Loss 2p25.3p11.2 Loss 6p25.3p11.2 Loss 6q11.1q16.1 Loss 6q16.1q27 Gain 7 **Loss 22**	1.05 1.00 1.70 1.05 2.90 1.05	**Loss 22**	1.10
12[j]	B412	Chaotic including **Gain 6** **Loss 21** **Gain 22**	 2.90 0.70 3.65	 **Gain 6** **Loss 21** **Gain 22**	 2.90 1.00 2.85

(continued on next page)

TABLE 1 *(continued)*

Partially concordant

Embryo	Morphology	TE	TE-CN value	ICM	ICM-CN value
24[j]	B411	Gain 8 **Loss 13**	2.35 1.15	Gain 3 **Loss 13** Loss 19 Loss 22	2.30 0.90 1.70 1.70
28[j]	B411	Gain 9 **Loss 22**	2.40 1.05	**Loss 22**	1.10
37[i]	B412	**Loss 1q21.1q44** Gain 9q21.11q34.3 **Loss 16q11.2q24.3**	0.55 2.40 1.00	**Gain 1q21.1q44** **Gain 16**	2.65 2.35
39[j]	B322	**Loss 7** Loss 16	1.15 1.65	**Loss 7p22.3q31.2** **Gain 7q31.2q36.3**	1.05 3.95

Discordant

Embryo	Morphology	TE	TE-CN value	ICM	ICM-CN value
1[b]	B423	2N	2N	Loss 13	1.65
3[b]	B421	2N	2N	Gain X Loss Y	1.50 0.50
17[b]	B422	2N	2N	Loss 16p13.3p11.1	1.65
18[b]	B411	2N	2N	Gain 9 Gain 20	2.30 2.30
19[b]	B412	2N	2N	Gain 21	2.30
20[b]	B411	2N	2N	Gain 3 Gain 6 Gain 20 Gain 22	2.50 2.30 2.40 2.30
25[c]	B412	2N	2N	Chaotic	
30[b]	B321	2N	2N	Gain 14 Gain 22	2.30 2.35
31[c]	B422	2N	2N	Gain 1p36.33p36.12 Loss 2p25.3p13.1 Loss 2p13.1q37.3 Loss 4 Loss 13q21.31q34	3.40 1.25 1.65 1.70 1.25
34[c]	B422	2N	2N	Loss 5q15q35.3 Loss 9 Loss 13q11q21.33 Loss 13q21.33q34 Loss 17	1.20 1.35 1.50 0.80 1.45
32[d]	B411	Gain 22	2.35	2N	2N
38[e]	B311	Gain 10q11.22q26.3	2.55	2N	2N
46[d]	B312	Loss 9 Gain 15	1.70 2.40	2N	2N
27[d]	B421	Loss 6 Gain 18	1.70 2.40	2N	2N
16[h]	B412	Loss 16q11.2q24.3	1.00	Gain 5q11.1q35.3	2.45
33[g]	B322	Loss X (XX sample)	1.40	Chaotic	

CN = copy number; ICM = inner cell mass; NGS = next-generation sequencing; TE = trophectoderm.

considered chaotic and fully abnormal. It is important to note that with NGS on a sample of 5–10 cells, the chromosomal constitution of individual cells cannot be distinguished because the combined DNA of all cells is analysed and therefore only a net gain or loss can be detected (*Mamas et al., 2012*).

Embryo categorization

After evaluation of the chromosomal constitution of the TE and ICM samples based on the copy number values of the different abnormalities present, the embryos were categorized as cytogenetically concordant, partially concordant or discordant (FIGURE 1B). Concordant embryos were normal or

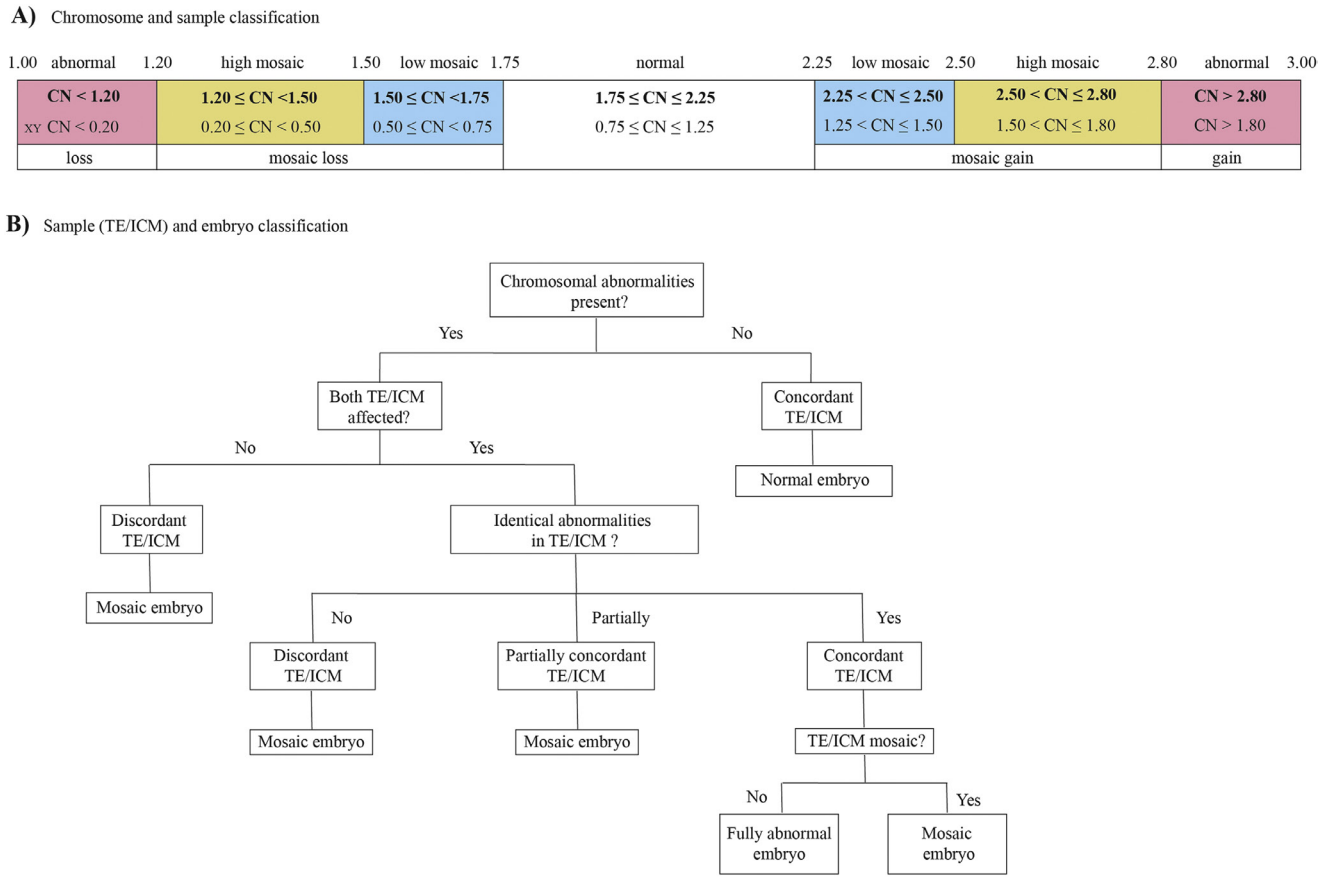

FIGURE 1 (A) CN value ranges to distinguish between normal chromosome copy number and low-mosaic, high-mosaic or fully abnormal chromosomal abnormalities. The same ranges were used to classify a TE or ICM sample as normal, low-mosaic, high mosaic or fully abnormal. The ranges are presented for both XX and XY samples. The ranges for autosomal chromosomes and the X chromosome in XX samples are presented in bold. (B) Flow chart presenting the classification process to the concordant, partially concordant or discordant embryo category. Embryos that are considered mosaic are also shown. CN = copy number; ICM = inner cell mass; TE = trophectoderm.

contained (an) identical chromosomal abnormality(ies) in both embryonic lineages, regardless of the copy number values for the involved chromosomal imbalance(s). In partially concordant embryos, at least one chromosomal abnormality was identical in both embryonic lineages, while additional chromosomal abnormalities could be present in one or both of them. In this category, the common abnormality could also concern the same chromosome or chromosomal segment showing a loss in one embryonic lineage and a gain in the other one, potentially originating from one mis-segregation event. Discordant embryos had either only one affected embryonic lineage or both, but with different chromosomal aberrations and none in common.

By definition, an embryo was considered to be mosaic when cytogenetically different cells were detected within the TE and/or ICM (FIGURE 1B). This includes embryos with (a) chromosome aberration(s) in either one or both embryonic lineages with (a) copy number value(s) in the mosaic range as well as all discordant embryos.

RESULTS

Cytogenetic findings of TE and ICM

In this study, a total of 152 good-quality frozen morula stage embryos of unknown chromosomal content and donated for research were thawed (FIGURE 2A). Seventy-one embryos that developed into a blastocyst with at least a visible ICM (morphology grade of at least B323 or higher) were biopsied. The TE and ICM samples were collected separately and were submitted for NGS analysis (FIGURE 2B). From 46 embryos, both biopsied samples could be successfully analysed with NGS data that passed the analysis thresholds. Successful analysis resulted in genome-wide copy number plots of ICM and TE, where chromosomal gains and losses are indicated (Supplementary Figure 1). Embryo morphology and the cytogenetic results of TE and ICM with copy number values per chromosome aberration are presented for each embryo (TABLE 1). In total, 33 embryos carried whole chromosome abnormalities. Segmental abnormalities ranging from 17 to 131 Mb were found in eight embryos (17%) (TABLE 1, Supplementary Table 2).

Concordance between cytogenetic results from TE and ICM

In order to determine whether a single TE biopsy accurately predicts the chromosomal constitution of the ICM, the concordance between the cytogenetic results of the ICM and the TE were evaluated for each embryo (TABLE 1, FIGURE 1B). Full concordance of the NGS results was observed in 48% (n = 22/46); 12 embryos were chromosomally normal in both TE and ICM, while in the other 10 embryos identical chromosomal aberrations were detected in both embryonic lineages. A subset of 17% (n = 8/46) was partially concordant, where

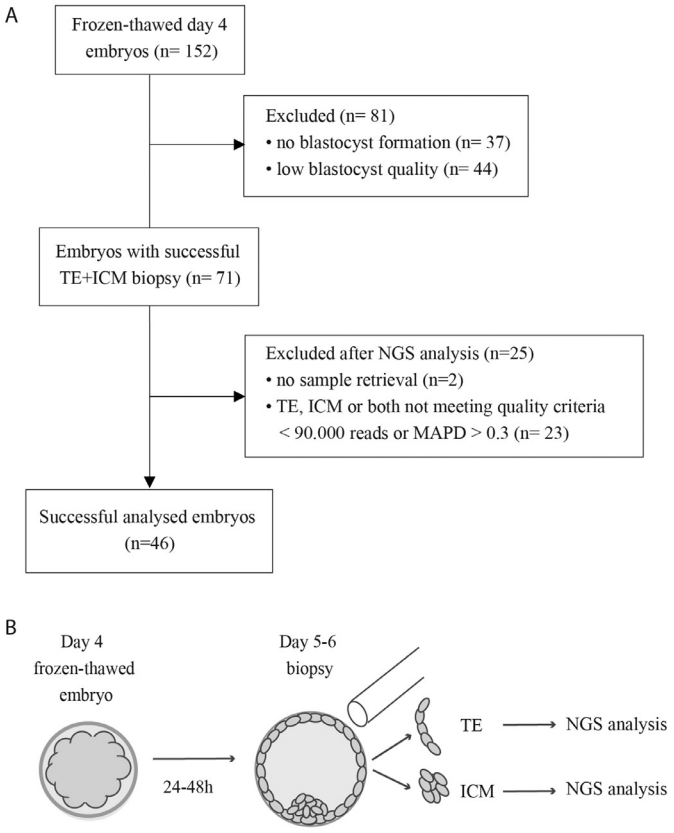

FIGURE 2 (A) Flow chart presenting the embryos that were thawed and biopsied. Embryos with a successful NGS analysis for both TE and ICM were included in this study. (B) Schematic representation of the biopsy approach. Human day 4 frozen–thawed embryos donated for research were cultured until day 5 or 6. Blastocysts that met the minimal morphological requirements were biopsied for both ICM and TE. The samples were analysed with NGS. ICM = inner cell mass; MAPD = median absolute performance difference; NGS = next-generation sequencing; TE = trophectoderm.

the two embryonic lineages shared at least one common chromosomal aberration and one or both lineages showed additional aberrations. In three of these embryos (embryos 4, 37, 39) the common abnormality concerned the reciprocal product (gain and loss) of the same chromosome segment. For the remaining 35% (n = 16/46) the cytogenetic results of ICM and TE were completely discordant: 10 embryos consisted of a normal TE with an affected ICM; four embryos had a normal ICM and an affected TE; two embryos had different chromosomal abnormalities in the two embryonic lineages. Thus, from a cytogenetic perspective, the current results show that in 48% (22 concordant embryos) of the embryos the chromosomal content of the TE sample and the corresponding ICM were fully concordant.

However, the concordance between TE and ICM was also investigated from a clinical point of view. TE and ICM samples were categorized as normal, low-mosaic, high-mosaic or abnormal, according to the copy number values of the involved chromosome aberration(s) (FIGURE 3). This categorization showed that when a TE biopsy was observed to be normal or low-mosaic, 88% (n = 22/25) of the embryos had a corresponding normal (n = 15) or low-mosaic (n = 7) ICM. In the remaining 12% (n = 3/25) of blastocysts with a normal TE biopsy, the ICM was abnormal. One embryo had a

TE \ ICM	normal	low mosaic	high mosaic	abnormal
normal	a) n=12 (55%)	b) n=7 (32%)	-	c) n=3 (13%)
low mosaic	d) n=3 (100%)	-	-	-
high mosaic	e) n=1 (25%)	-	f) n=1 (25%)	g) n=2 (50%)
abnormal	-	h) n=2 (12%)	i) n=1 (6%)	j) n=14 (82%)

FIGURE 3 Schematic comparison between TE biopsy and the corresponding ICM from a clinical perspective. Normal TE or ICM samples are presented in blue and affected samples in red. Samples with both colours represent mosaic samples with normal and abnormal cells. (–) = not observed. Differentiation between normal, high and low-mosaic or fully abnormal TE or ICM samples was made based on CN values (see *Figure 1A*). A sample was considered low-mosaic if the CN values of the involved chromosome aberrations were in the low-mosaic range. A high-mosaic sample had at least one chromosomal abnormality with a CN value in the high-mosaic range, with other aberrations showing low-level mosaicism. Samples with at least one chromosome aberration with a CN value in the full abnormal range were considered fully abnormal. Chaotic samples were also considered fully abnormal. Embryos were categorised into groups from a to j based on the chromosomal status (normal, low-mosaic, high-mosaic, abnormal) of TE and ICM, and this classification is also indicated for each individual embryo in *Table 1*. This figure shows that most embryos with a normal or low-mosaic TE also had a normal or low-mosaic ICM (blue background). Embryos with a high-mosaic or abnormal TE mostly had a high-mosaic or abnormal ICM as well (red background). CN = copy number; ICM = inner cell mass; TE = trophectoderm.

chaotic ICM and two embryos contained structural and/or whole chromosome abnormalities in the abnormal or high-mosaic copy number range. When a TE biopsy was observed to be high-mosaic or abnormal, 86% (n = 18/21) of blastocysts also showed a high-mosaic (n = 2) or abnormal (n = 16) ICM. The remaining 14% (n = 3/21) showed a normal or low-mosaic ICM.

Incidence of chromosomal mosaicism

By definition, an embryo was considered mosaic when cytogenetically different cells were detected within or between the TE and ICM (FIGURE 1B). Chromosomal mosaicism was found in 59% (n = 27/46) of the analysed embryos, of which 16 embryos showed discordant, eight partially concordant and three concordant results between TE and ICM (TABLE 1). In seven out of eight partially concordant embryos, at least one identical abnormality in ICM and TE presented a copy number value consistent with high-level mosaicism or a full-blown aberration, indicating the presence of a large proportion of affected cells. The same was observed in the three concordant embryos (embryos 9, 22 and 43). In contrast, the copy number values of the chromosomal abnormalities detected in 16 discordant embryos indicated low-level mosaicism in 10 out of 16 cases. Exceptions consisted of two embryos with a chaotic ICM and normal (embryo 25) or high-mosaic TE (embryo 33), three embryos with segmental abnormalities (embryos 31, 38, 16) and one embryo that contained both structural and whole chromosome abnormalities in the high-mosaic range (embryo 34).

DISCUSSION

This study explored chromosomal mosaicism and the potential impact on the diagnostic accuracy of PGT-A at the blastocyst stage by investigating how often the TE biopsy correctly reflects the chromosomal content of the ICM. Chromosomal mosaicism was observed (defined as the presence of cytogenetically different cells in one or both embryonic lineages) in 59% of the analysed embryos. This chromosomal mosaicism had a negative impact on the concordance rate, in terms of the type of aberration and the chromosome(s) involved. Cytogenetic findings were fully concordant between the TE biopsy and the ICM in 48% of the embryos. In an additional 17%, TE and ICM were partially concordant by sharing at least one chromosome aberration, whereas in 35% of the embryos TE and ICM were discordant. However, when TE and ICM samples were classified as normal or low-mosaic versus abnormal or high-mosaic, the TE classification corresponded to the ICM in most cases. So from a clinical perspective, concordance rates were found to be 86–88%.

Unfortunately, studies that investigate mosaicism involve small cohorts of embryos, as the number of untested donated embryos is limited. Therefore, every study using surplus embryos of good quality is of great value as it adds information about mosaicism to the already existing data. However, as long as there is no uniform way to define mosaicism in blastocysts (*Popovic et al., 2020*), it is important to extensively describe cytogenetic findings and sample or embryo classification processes in order to be able to compare results with other studies and to draw conclusions about the incidence of mosaicism. This study systematically describes the criteria for cytogenetic diagnosis and the classification of embryos, which may be useful for other researchers.

Four comparable studies also used NGS for the cytogenetic analysis of multiple biopsies of blastocysts with unknown chromosomal composition and reported 29–50% of blastocysts to be mosaic (*Chuang et al., 2018*; *Orvieto et al., 2016*; *Popovic et al., 2018*; *Tšuiko et al., 2018*). A somewhat higher rate of mosaicism was observed, which can be explained as follows. First, all studies dealt with small sample sizes, using two, 14, 29 and 34 embryos, respectively. Second, different NGS platforms were used for the cytogenetic analysis. Third, and most importantly, criteria to diagnose the biopsied sample or the whole embryo as normal, mosaic or abnormal were not always unambiguously reported. By definition in this study, embryos that involve cytogenetically different abnormal cells without normal cells were considered to be mosaic, whereas others may call these embryos abnormal. However, when only the TE results in the current data are considered, 48% of the embryos analysed would be diagnosed as normal, 37% abnormal and 15% as mosaic (8% high and 7% low-mosaic). In this case, the incidence of mosaicism observed is comparable to the range of 2–19% that has been previously reported for single TE biopsies (*Capalbo et al., 2021*; *Katz-Jaffe et al., 2017*; *Munné et al., 2019*; *Ruttanajit et al., 2016*; *Stankewicz et al., 2017*).

The concordance rate between the cytogenetic results of ICM and TE was lower in the current study (48%) than in the previous studies already mentioned. In these studies, the concordance rates varied between 50% and 86% (*Chuang et al., 2018*; *Lawrenz et al., 2019*; *Orvieto et al., 2016*; *Popovic et al., 2018*; *Tšuiko et al., 2018*). The reasons for these discrepancies may be the same as mentioned above to explain differences in incidence of mosaicism between studies. Notably, in (partially) concordant embryos it was found that the common chromosomal aberration(s) in TE and ICM mostly had copy number values consistent with high-level mosaicism or full-blown abnormality(ies). These abnormalities are most probably the result of either a meiotic error or a malsegregation event during the first cell divisions before TE and ICM lineage specification. The additional chromosomal aberrations that affected only one embryonic lineage in partially concordant embryos mostly displayed low-level mosaicism. The majority of discordant embryos had only one affected embryonic lineage with copy number values mostly indicating low-level mosaicism. The abnormal cells most likely arose after embryonic lineage differentiation, which also explains the restriction to one embryonic lineage.

From a strict cytogenetic perspective, the results from the TE biopsy showed a low concordance rate with the findings in the ICM. However, when mosaic findings in the TE biopsy and ICM were classified as high or low-mosaic, it was observed that 86% of the embryos with a high-mosaic or abnormal TE biopsy had a high-mosaic or abnormal ICM as well. In addition, 88% of the embryos with a normal or low-mosaic TE were cytogenetically normal or low-mosaic in the ICM. Thus, in most cases a high-mosaic or abnormal TE biopsy accurately predicted the ICM to be also affected with high-level mosaicism or full-blown abnormality(ies), while embryos with a low-mosaic TE biopsy also had a normal or low-mosaic ICM. A recent study disaggregating blastocysts into four TE portions and the ICM also observed that low-mosaic abnormalities in a TE biopsy are rarely confirmed in other parts of the embryo (*Capalbo et al., 2021*). The current results support recent clinical studies that classified transferred mosaic

embryos as low or high mosaic based on the TE biopsy (*Spinella et al., 2018*; *Viotti et al., 2021b*). They observed that embryos with high-mosaic TE biopsies had lower implantation and live birth rates than embryos with a low-mosaic or normal TE biopsy. Embryos with a low-mosaic TE had comparable clinical outcomes to those of normal embryos (*Spinella et al., 2018*). Similar observations were made by a double-blinded non-selection study that also transferred embryos diagnosed as low-mosaic (*Capalbo et al., 2021*). Together these studies gather evidence that the transfer of an embryo with low-level mosaicism in the TE biopsy can lead to a healthy live born in 36–42% of the cases (*Capalbo et al. 2021*; *Spinella et al. 2018*; *Viotti et al., 2021a*).

It has been argued that mosaicism in TE biopsies is the result of NGS technical artefacts (*Capalbo and Rienzi, 2017*; *Marin et al., 2020*; *Popovic et al., 2020*; *Treff and Marin, 2021*), and that mosaicism should not be considered in a PGT-A diagnosis (*Treff and Marin, 2021*). However, studies performed on the chromosomal constitution of human embryos over the last 30 years, using many different cytogenetic techniques, have firmly established that mosaicism is a common biological phenomenon (*Popovic et al., 2018*; *Starostik et al., 2020*; *Viotti et al., 2021a*). Single-cell analysis studies show the presence of low levels of abnormal cells in the majority of human blastocysts (*Baart et al., 2006*, *2007*; *Santos et al., 2010*), where it can be observed in both TE and ICM (*Starostik et al., 2020*). Later in development, in first-trimester chorionic villi, mosaicism can be observed as generalized or confined placental mosaicism in only 1–2% of pregnancies (*Pittalis et al., 1994*). There is increasing evidence that the decrease of mosaicism during embryonic development is the result of aneuploid cells being selectively eliminated and/or outcompeted by normal cells, as recently suggested by studies performed in mouse embryos (*Bolton et al., 2016*; *Singla et al., 2020*; *Zhou et al., 2019*). Consistent with this idea, human blastocysts that were diagnosed as mosaic by PGT-A were observed to have both higher levels of cell proliferation, as well as apoptosis, compared to normal embryos (*Victor et al., 2019*). Moreover, observations in in-vitro cultured mosaic human embryos show that the proportion of aneuploid cells decreases after extended culture through the peri-implantation stages (*Popovic et al., 2019*; *Santos et al., 2010*). Single-cell analysis of human embryos at different stages of development showed that after in-vitro culture to the post-implantation stage, aneuploidy decreases in the epiblast and is more frequently detected in the extra-embryonic trophoblast compartment (*Starostik et al., 2020*; *Zhou et al., 2019*). To further assess the outcome of mosaic embryos that implant, a recent study performed cytogenetic follow-up after mosaic embryo transfer in a few cases (*Capalbo et al., 2021*). They could not confirm the presence of chromosomal mosaicism in fetus or child. However, these studies were mainly restricted to postnatal saliva and a few amniotic fluid samples, whereas the tissues of most interest (first-trimester chorionic villi and/or placenta) were not investigated.

Although cytogenetically untested, good-quality blastocysts have been analysed to investigate whether the TE biopsy correctly reflects the ICM, there are some limitations to this study. Undoubtedly, the sample size is small and the findings should be confirmed with further studies. It is also possible that the entire ICM was not always removed from the embryo and contamination of the ICM sample with TE cells or cell-free DNA from the blastocoel also cannot be excluded (*Capalbo et al., 2013*; *Palini et al., 2013*). It should also be taken into account that, due to chromosomal mosaicism, the chromosomal content of the TE biopsy that was compared to the ICM might not reflect the entire TE (*Capalbo et al., 2013*; *Chuang et al., 2018*; *Gleicher et al., 2017*; *Popovic et al., 2018*). Furthermore, it remains unknown whether the findings also apply to fresh embryos, as all embryos were frozen–thawed and had been stored for an extended time. Another technical limitation is that shallow massively parallel sequencing on a sample of 5–10 cells will only reveal a net gain or loss and does not allow the determination of chromosome copy number in individual cells (*Fiorentino et al., 2014*; *Mamas et al., 2012*; *Spinella et al., 2018*). In future studies, this limitation can be overcome with a single-cell sequencing analysis approach, which will give more insight into chromosomal mosaicism at the individual cell level and the underlying mechanisms.

The current findings indicate that chromosomal mosaicism is still common at the blastocyst stage, negatively impacting the cytogenetic concordance rate between TE and ICM. However, despite this, it was found that in almost all cases a high-mosaic or abnormal TE biopsy accurately predicted the ICM to also be high-mosaic or abnormal. In addition, embryos with a normal or low-mosaic TE biopsy mostly contained a normal or low-mosaic ICM. Thus, the findings support the notion that diagnosing embryos as mosaic where appropriate after an NGS-based PGT-A result, and making the distinction between high and low mosaics, is clinically relevant (*Viotti et al., 2021a*). As the impact of abnormal cells in human embryo development is still being investigated, the interpretation of PGT-A results still warrants caution. Patients proceeding to PGT-A should be counselled about the technical and biological limitations, and in case of a finding of mosaicism, about the uncertain clinical outcomes after mosaic embryo transfers. In such cases, prenatal diagnosis or at least genome-wide non-invasive prenatal testing could be offered.

DATA AVAILABILITY STATEMENT

The data underlying the findings in this paper will be shared on reasonable request to the corresponding author.

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SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.rbmo.2022.06.004.

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