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The use of copy number loads to designate mosaicism in blastocyst stage PGT-A cycles: fewer is better

Laura Girardi [1,*,†], Matteo Figliuzzi[1,†], Maurizio Poli[1], Munevver Serdarogullari [2], Cristina Patassini[1], Silvia Caroselli[1], Ilaria Pergher[1], Francesco Cogo[1], Onder Coban[3], Fazilet Kubra Boynukalin[4], Mustafa Bahceci[4], Roser Navarro[5], Carmen Rubio[5], Necati Findikli[6], Carlos Simón[7,8,9], and Antonio Capalbo [1,*]

[1]Igenomix, Reproductive Genetics, Marostica, Italy [2]Faculty of Medicine, Cyprus International University, Northern Cyprus, Turkey [3]British Cyprus IVF Hospital, Nicosia, Cyprus [4]Bahceci Fulya IVF Centre, Infertility Clinic, Istanbul, Turkey [5]Igenomix Spain, Valencia, Spain [6]Bahceci Fulya IVF Centre, Embryology Laboratory, Istanbul, Turkey [7]Department of Obstetrics and Gynecology, Valencia University; and INCLIVA, Valencia, Spain [8]Department of Obstetrics and Gynecology, Harvard School of Medicine, Harvard University, Cambridge, MA, USA [9]Carlos Simón Foundation, Valencia, Spain

*Correspondence address. Igenomix, Reproductive Genetics, Marostica 36063, Italy. E-mail: laura.girardi@igenomix.com (L.G.) https://orcid.org/0000-0002-2252-6682; Igenomix, Reproductive Genetics, Marostica 36063, Italy. E-mail: antcapalbo@gmail.com (A.C.) https://orcid.org/0000-0003-0109-323X

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STUDY QUESTION: : How well can whole chromosome copy number analysis from a single trophectoderm (TE) biopsy predict true mosaicism configurations in human blastocysts?

SUMMARY ANSWER: : When a single TE biopsy is tested, wide mosaicism thresholds (i.e. 20–80% of aneuploid cells) increase false positive calls compared to more stringent ones (i.e. 30–70% of aneuploid cells) without improving true detection rate, while binary classification (aneuploid/euploid) provides the highest diagnostic accuracy.

WHAT IS KNOWN ALREADY: : Next-generation sequencing-based technologies for preimplantation genetic testing for aneuploidies (PGT-A) allow the identification of intermediate chromosome copy number alterations potentially associated with chromosomal mosaicism in TE biopsies. Most validation studies are based on models mimicking mosaicism, e.g. mixtures of cell lines, and cannot be applied to the clinical interpretation of TE biopsy specimens.

STUDY DESIGN, SIZE, DURATION: : The accuracy of different mosaicism diagnostic thresholds was assessed by comparing chromosome copy numbers in multiple samples from each blastocyst. Enrolled embryos were donated for research between June 2019 and September 2020. The Institutional Review Board at the Near East University approved the study (project: YDU/2019/70-849). Embryos showing euploid/aneuploid mosaicism (n = 53), uniform chromosomal alterations (single or multiple) (n = 25), or uniform euploidy (n = 39) in their clinical TE biopsy were disaggregated into five portions: the inner cell mass (ICM) and four TE segments. Collectively, 585 samples from 117 embryos were analysed.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Donated blastocysts were warmed, allowed to re-expand, and disaggregated in TE portions and ICM. PGT-A analysis was performed using Ion ReproSeq PGS kit and Ion S5 sequencer (ThermoFisher). Sequencing data were blindly analysed with Ion Reporter software to estimate raw chromosome copy numbers. Intra-blastocyst comparison of copy number data was performed employing different thresholds commonly used for mosaicism detection. From copy number data, different case scenarios were created using more stringent (30–70%) or less stringent criteria (20–80%). Categorical variables were compared using the two-sample *z* test for proportions.

MAIN RESULTS AND THE ROLE OF CHANCE: : When all the five biopsies from the same embryo were analysed with 30–70% thresholds, only 8.4% (n = 14/166) of patterns abnormal in the original analysis revealed a true mosaic configuration, displaying evidence of reciprocal events (3.6%, n = 6/166) or confirmation in additional biopsies (4.8%, n = 8/166), while most mosaic results (87.3% of total

[†]The authors consider that the first two authors should be regarded as joint first authors.

predicted mosaic patterns) remained confined to a single TE specimen. Conversely, uniform whole chromosome aneuploidies (28.3% of total patterns, n = 47/166) were confirmed in all subsequent biopsies in 97.9% of cases (n = 46/47). When 20–80% thresholds were employed (instead of 30–70%), the overall mosaicism rate per biopsy increased from 20.2% (n = 114/565) to 40.2% (n = 227/565). However, the use of a wider threshold range did not contribute to the detection of additional true mosaic patterns, while significantly increasing false positive mosaic patterns from 57.8% to 79.5% (n = 96/166; 95% CI = 49.9–65.4 vs n = 271/341; 95% CI = 74.8–83.6, respectively) ($P < 0.00001$). Moreover, the shift of the aneuploid cut-off from 70% to 80% of aneuploid cells resulted in mosaicism overcalling in the high range (50–80% of aneuploid cells), impacting the accuracy of uniform aneuploid classification. Parametric analysis of thresholds, based on multifocal analysis, revealed that a binary classification scheme with a single cut-off at a 50% level provided the highest sensitivity and specificity rates. Further analysis on technical noise distribution at the chromosome level revealed a greater impact on smaller chromosomes.

LIMITATIONS, REASONS FOR CAUTION: While enrolment of a population enriched in embryos showing intermediate chromosome copy numbers enhanced the evaluation of the mosaicism category compared with random sampling such study population selection is likely to lead to an overall underestimation of PGT-A accuracy compared to a general assessment of unselected clinical samples. This approach involved the analysis of aneuploidy chromosome copy number thresholds at the embryo level; future studies will need to evaluate these criteria in relation to clinical predictive values following embryo transfers for different PGT-A assays. Moreover, the study lacked genotyping-based confirmation analysis. Finally, aneuploid embryos with known meiotic partial deletion/duplication were not included.

WIDER IMPLICATIONS OF THE FINDINGS: Current technologies can detect low-intermediate chromosome copy numbers in preimplantation embryos but their identification is poorly correlated with consistent propagation of the anomaly throughout the embryo or with negative clinical consequences when transferred. Therefore, when a single TE biopsy is analysed, diagnosis of chromosomal mosaicism should be evaluated carefully. Indeed, the use of wider mosaicism thresholds (i.e. 20–80%) should be avoided as it reduces the overall PGT-A diagnostic accuracy by increasing the risk of false positive mosaic classification and false negative aneuploid classification. From a clinical perspective, this approach has negative consequences for patients as it leads to the potential deselection of normal embryos for transfer. Moreover, a proportion of uniform aneuploid embryos may be inaccurately categorized as high-level mosaic, with a consequent negative outcome (i.e. miscarriage) when inadvertently selected for transfer. Clinical outcomes following PGT-A are maximized when a 50% threshold is employed as it offers the most accurate diagnostic approach.

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Key words: mosaicism / thresholds / PGT-A / NGS / trophectoderm / inner cell mass / re-biopsies / binary classification

Introduction

Using preimplantation genetic testing (PGT) for aneuploidy, uniform whole chromosome aneuploidies affecting the whole embryo can be accurately predicted through next-generation sequencing (NGS)-based analysis of a trophectoderm (TE) biopsy (Victor *et al.*, 2019; Girardi *et al.*, 2020; Sachdev *et al.*, 2020; Capalbo *et al.*, 2021; Marin *et al.*, 2021). As previously shown by multifocal analysis, whole chromosome aneuploidies of meiotic origin are consistently detected across all blastocyst sections including the inner cell mass (ICM), confirming both the high reliability and accuracy of the methodology employed and the persistence and inherence of the abnormality throughout the whole blastocyst stage embryo (Girardi *et al.*, 2020; Kim *et al.*, 2022). The increased analytical sensitivity of current NGS-based platforms allows the identification of intermediate chromosome copy numbers, often interpreted as evidence of chromosomal mosaicism (Goodrich *et al.*, 2016; Vera-Rodriguez and Rubio, 2017; Rodrigo *et al.*, 2020). From a biological standpoint, a mosaic embryo is characterized by the presence of multiple cell lines with normal and abnormal karyotypes (diploid/aneuploid mosaic). Unlike meiotic whole chromosome alterations, mosaicism derives from mitotic errors occurring in the early stages of embryo development, thus it is not expected to be evenly distributed throughout the preimplantation embryo (Capalbo *et al.*, 2017). Currently, the interpretation of a mosaic diagnosis remains a topic of ongoing debate in the PGT field, with increasing uncertainty on the ability of intermediate chromosome copy numbers profiles to predict a real mosaic state in the blastocyst (Treff and Marin, 2021). To provide evidence on this topic, the chromosomal constitution of multiple portions of the same embryo has been recently evaluated in several multifocal biopsy/embryo disaggregation studies (Capalbo *et al.*, 2013, 2021; Popovic *et al.*, 2018; Lawrenz *et al.*, 2019; Sachdev *et al.*, 2020; Marin *et al.*, 2021; Wu *et al.*, 2021; Kim *et al.*, 2022). Multifocal biopsies analysis represents the most comprehensive investigative approach for evaluating embryo mosaicism status, providing true evidence of chromosomal mosaic patterns with high diagnostic reliability (Capalbo *et al.*, 2017; Popovic *et al.*, 2020; Treff and Marin, 2021). In a previous study, we demonstrated that the simultaneous analysis of five different specimens from the same embryo (i.e. the ICM and four consecutive TE biopsies) allows discrimination between different true aneuploid/mosaic segmental configurations from the presence of technical artefacts introduced by NGS procedures (Girardi *et al.*, 2020). Interestingly, previous re-biopsy studies showed low reproducibility of whole chromosome mosaic events (Kim *et al.*, 2022), where more than half of diagnoses of mosaicism in clinical TE (cTE) biopsies were not confirmed in subsequent biopsies (Marin *et al.*, 2021), and low levels of mosaicism were mostly associated with lower confirmation levels (Capalbo *et al.*, 2021; Wu *et al.*, 2021). From a strictly technical-analytical viewpoint, the combination of minor inconsistencies in the

NGS procedures employed, differences in technical validation procedures, and detection thresholds applied for diagnostic calls can result in subtle variations in analytical results, preventing a consistent discrimination between real biological signals and technical noise (Treff and Marin, 2021). Validation procedures for mosaicism detection usually employ mixtures of cell lines, where different ratios of abnormal and normal cells are present to calculate the accuracy in identifying the correspondent copy numbers for chromosomes tested (Goodrich et al., 2016; García-Pascual et al., 2020). In this experimental setting, cell lines represent very stable specimens with a known and defined number of cells included, where the biological variability in cellular quality and quantity is absent. Thus, mosaicism thresholds are extrapolated from a very stable setting which is likely to differ from the generally heterogeneous TE biopsy scenario. Hence, to avoid the potential introduction of classification errors or misdiagnoses, the validation of thresholds more suitable to be applied in the 'real-life' context would include not only cell line-based models but also multifocal biopsies experiments and/or non-selection studies. In this context, the stringency of the thresholds employed for diagnostic calls remains a key point that requires further considerations before an appropriate level of standardization in categorising and reporting mosaicism can be reached (Popovic et al., 2020). Indeed, the application of less stringent thresholds (e.g. 20–80%) (Leigh et al., 2022) can ultimately lead to incorrect mosaic classification of uniformly euploid and aneuploid embryos, resulting in increased mosaicism prevalence rates and higher false positive mosaic rates (Capalbo et al., 2017; Marin et al., 2021; Wu et al., 2021). The overestimation of mosaicism in TE biopsies could lower preimplantation genetic testing for aneuploidies (PGT-A) accuracy, ultimately affecting patient's treatment outcome from both a clinical and a psychological standpoint.

In this study, we applied NGS-based PGT-A analysis on human disaggregated embryos to compare the diagnostic predictivity of two mosaicism classification criteria, 30–70% and 20–80% of aneuploid cells with respect to the binary classification scheme (euploid/aneuploid) with single cut-off value at 50%.

These results provide evidence on the most accurate approach to detect real biological events and limit technical artefacts. Moreover, a concordance analysis showing the impact of mosaic findings on the reproducibility of PGT-A results across blastocyst re-biopsies was also performed.

Materials and methods

Ethical approval

Surplus cryopreserved blastocysts were donated for research at the British Cyprus IVF hospital. Approved informed consent forms were signed by all the individuals donating their embryos to this study. Ethical committee approval for the study was obtained from the Institutional Review Board (IRB) at Near East University (project number: YDU/2019/70-849).

Objective, study design, size, and duration

A cohort study blinded to the geneticist was carried out to evaluate the accuracy of different diagnostic classification thresholds for chromosomal mosaicism and aneuploidy detection in TE biopsies. Chromosome copy numbers comparison was performed in an intra-blastocyst, multifocal biopsies setting. To do so, three groups of embryos were included based on the result obtained from their first, cTE biopsy: embryos showing euploid/aneuploid mosaicism (n = 53), embryos diagnosed with uniform chromosomal alterations (single or multiple) (n = 25), and uniformly euploid embryos (n = 39) (see the 'Bioinformatic analysis and interpretation of sequencing data' section for the criteria employed for categorization). All embryos were disaggregated into four additional portions, the ICM, and three TE portions, allowing to test five specimens from each embryo. Collectively, 585 biopsies from 117 embryos were collected between June 2019 and September 2020. Individual samples were blindly analysed using the NGS Ion Torrent S5 platform. To determine how mosaicism detection rates vary according to the classification thresholds employed (i.e. 30–70% and 20–80% criteria), raw NGS data from all intra-embryonic specimens were compared irrespective of their biopsy order. Finally, cTE and ICM results were compared with subsequent portions' results to obtain diagnostic concordance rates with the remaining embryo (Fig. 1).

PGT-A analysis

ICM isolation and multiple TE biopsies were performed using a previously described and validated methodology (Capalbo et al., 2013). The PGT-A protocol employed in this study was previously validated for the detection of whole chromosome aneuploidies, segmental aneuploidies, and mosaicism using commercial cell lines/genomic DNA for 14 different affected chromosomes (García-Pascual et al., 2020). In brief, individual biopsies were blindly analysed using the semiautomated protocol which employs the Ion Chef™ equipment and the Ion S5 sequencer (ThermoFisher Scientific, MA, USA). All blastocyst biopsies were subjected to DNA extraction and whole-genome amplification (WGA) using Ion Reproseq PGS kit (ThermoFisher). Template preparation and chip loading were performed using Ion Chef system according to manufacturer's instructions. Chip was then loaded and sequenced on Ion S5™ XL Sequencer™. Acceptance values for quality parameters (QC), for both the entire run and individual samples, were employed as previously reported (García-Pascual et al., 2020).

Bioinformatic analysis and interpretation of sequencing data

Sequencing data obtained by the S5™ XL Sequencer were processed and sent to the Ion Reporter software for analysis. Aneuploidies and copy number variations were analysed with the Ion Reporter™ Software version 5.4 (ThermoFisher Scientific). This software uses the bioinformatic tool ReproSeq PGS workflow v1.1 (default parameters, 'medium' mode) and ReproSeq Low-Coverage Whole-Genome Baseline (5.4) to detect 24 chromosomes aneuploidies from a single whole-genome sample with low coverage (minimum 0.01×). Mosaicism values for each chromosome were established using the computed difference value (DV) parameter, which is applied on normalized segment obtained from Ion Reporter algorithm at bin level. This value is defined as DV = SNMC − CNMP × EP, where SMNC (Sample Normalized Mean Coverage) is the observed ratio of reads in the sample; CNMP1 (Control Normalized Mean Coverage for 1

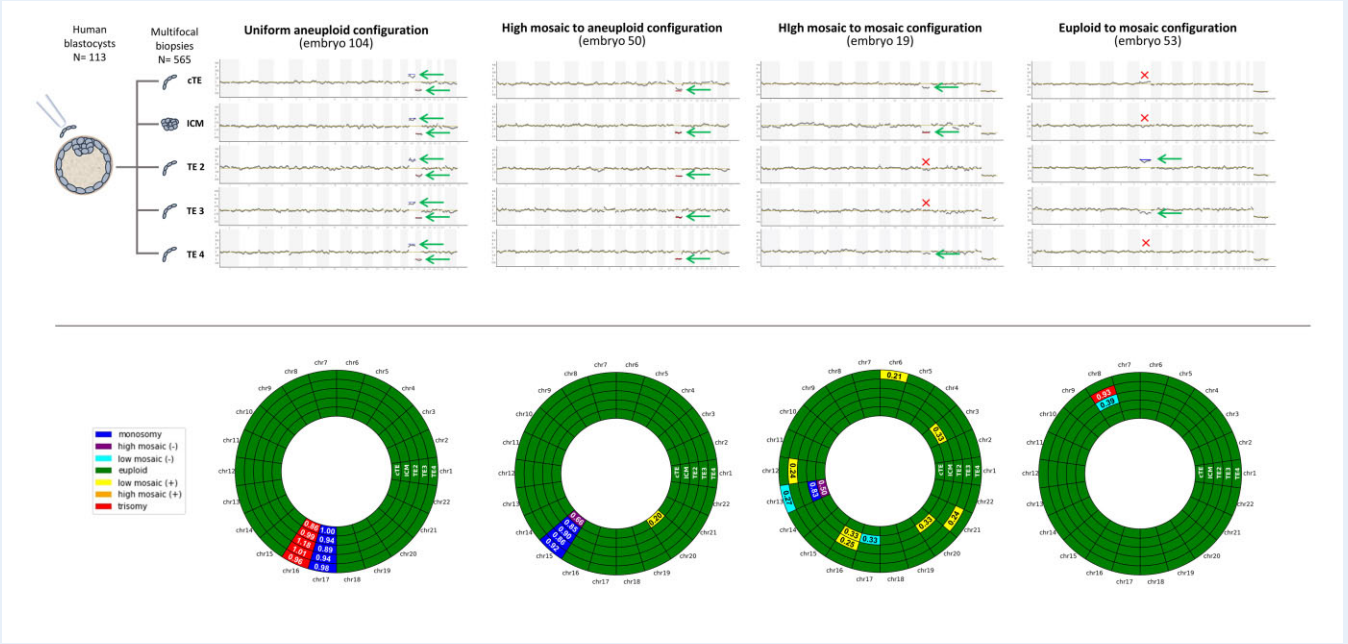


Figure 1. Study design and sample size. Multifocal analysis of 113 human embryos (565 biopsies), selected according to the clinical trophectoderm (cTE) preimplantation genetic testing for aneuploidy (PGT-A) result. Each blastocyst was disaggregated into five portions: four trophectoderm (TE) portions (cTE, TE2, TE3, and TE4) and the inner cell mass. Top: examples of next-generation sequencing plots for uniform aneuploid, high mosaic to aneuploid and false positive mosaic, high mosaic to mosaic and multiple false positive mosaic and a euploid to mosaic configuration detected in the study. Bottom: circle diagrams showing the corresponding difference value parameter for each chromosome.

copy) is the expected ratio of reads for 1 copy if the sample is normal; and EP (Expected Ploidy) is the expected number of copies (García-Pascual *et al.*, 2020). Intra-blastocyst comparison of DV data obtained from the Ion Reporter files for each embryo was performed employing different thresholds commonly used for mosaicism detection. When 30–70% threshold was applied, chromosome copy numbers <30% were considered euploid results and >70% were classified as aneuploid. The mosaic range was experimentally placed between 30% and 70%, where 50% threshold discriminated between low-grade mosaicism (<50%) and high-grade mosaicism (>50%). When the 20–80% criteria were considered, the euploid/aneuploid categories were extended below 20% and above 80% values, while the mosaic range was experimentally placed between 20% and 80% values.

Definition of confirmed mosaic and aneuploid patterns

To minimize the impact of technical over biological variation, intermediate chromosome copy numbers were classified in a confirmed mosaic pattern according to the following criteria: (i) the detection of the same mosaic alteration in at least three portions from the same embryo or in one additional portion over the 50% threshold or (ii) the detection of a reciprocal mosaic pattern involving the same chromosome in one additional portion. On the other hand, if the same alteration was uniformly detected in all biopsies above 50% threshold, the pattern was classified as uniform aneuploid. Single mosaic or aneuploid calls found only in one or two biopsies were interpreted as false positive or unconfirmed. The analysis presented here includes data from all the embryos enrolled in the study with informative results for all samples and considers both the reproducibility of the alteration identified in the first cTE biopsy and the detection of *de novo* aberrations in the subsequent embryo portions. Finally, based on the confirmed chromosome configuration results from all five specimens, each embryo was classified as mosaic, aneuploid or euploid (Supplementary Fig. S1A). In this study, single partial deletion/duplication of known meiotic origin were not considered.

Statistical analysis

Categorical variables were compared using the two-sample *z* test for proportion. The result is significant at $P < 0.05$. Mean was reported ±SD and percentages were reported with 95% CI. In the concordance analysis towards the ICM, to define the sensitivity and specificity of the binary classification approach, ICM sample was considered the reference and all biopsies were classified in euploid (DV < 0.5) and aneuploid (DV ≥ 0.5). TE results were considered true positive (aneuploid TE and aneuploid ICM), true negative (euploid TE and euploid ICM), false positive (aneuploid TE and euploid ICM), and false negative (euploid TE and aneuploid ICM). In the estimation of PGT-A accuracy in a realistic clinical scenario, the potential sampling bias derived from the particularly mosaics-enriched population enrolled in the study was corrected through 100 random resampling of embryos at fixed euploid/mosaic/aneuploid proportions. Chromosome-level specificity of the binary classification approach was estimated considering all biopsies from confirmed euploid configurations and dividing the number of

single biopsies with DV < 50% by the total number of euploid biopsies. The association between PGT-A noise and chromosome length was assessed by calculating the Pearson correlation coefficient and the *P*-value for the absence of linear correlation.

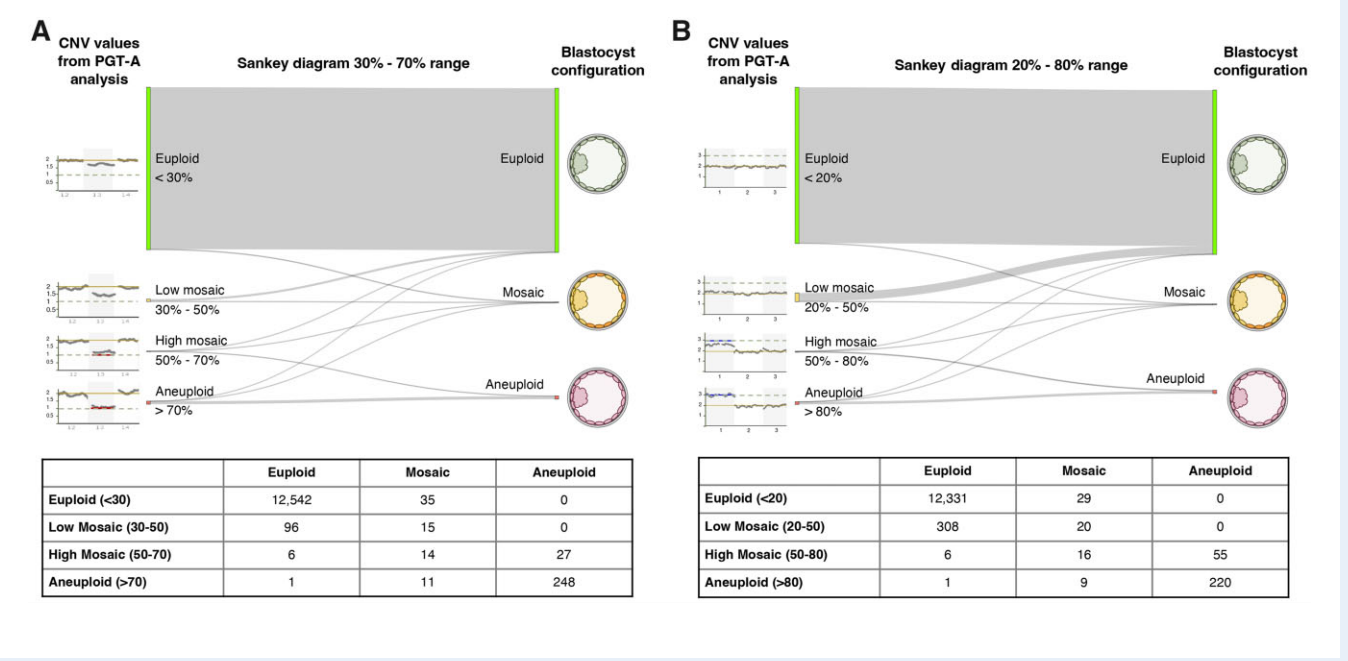
Results

Detection of aneuploid and mosaic configurations using 30–70% thresholds

A total of 585 samples from 117 embryos were analysed. Non-informative rate per sample, due to high mean absolute percent deviation values, was 0.7% (n = 4/585). Considering all the embryos with 5 informative results (n = 113) and showing all the alterations detected across all the portions from the same blastocyst (Supplementary Fig. S1A), when the 30–70% threshold was applied, a total of 418 single mosaic or aneuploid calls, corresponding to 166 different patterns, were identified (Supplementary Table SI).

Of these, only 8.4% (n = 14/166; 95% CI = 4.7–13.7) true mosaic patterns were confirmed ('Low and High mosaic to mosaic', Fig. 2A). In detail, 2.4% (n = 4/166; 95% CI = 0.7–6.0) of confirmed patterns involved at least three portions from the same embryo showing the same mosaic chromosome, 3.6% (n = 6/166; 95% CI = 1.3–7.7) involved a reciprocal alteration (2/4 with reciprocal whole chromosome and 4/6 patterns showing at least one segmental chromosome) in the subsequent portions, and 2.4% (n = 4/166; 95% CI = 0.7–6.0) showed at least one additional portion carrying the same alteration above 50% threshold. The remaining 5.4% (n = 9/166; 95% CI = 2.5–10.0) of patterns revealed a uniform aneuploid configuration for the same chromosome detected in the original mosaic call (Fig. 2A, 'Mosaic to aneuploid'). Conversely, 57.8% (n = 96/166; 95% CI = 49.93–65.44) of total findings (86.5% of mosaic patterns, n = 96/111; 95% CI = 78.7–92.2) identified in the cTE or detected *de novo* in the subsequent portions were not confirmed in other specimens from the same embryo, which all resulted as euploid (Fig. 2A, 'Mosaic to euploid'). These data suggest the presence of a technical artefact, or a very low grade of mosaicism, confined to a small portion of the embryo.

Intermediate chromosome copy numbers were also evaluated according to the possible level of mosaicism, as often categorized as low range (30–50%) and high range (50–70%). In detail, when only the high range was considered mosaic findings detected in the first biopsy were confirmed in 36.4% of cases (n = 7/19; 95% CI = 16.3–61.6). Moreover, 47.4% of high mosaic calls in the cTE biopsy (n = 9/19; 95% CI = 24.4–71.1) revealed a uniform aneuploid pattern, showing the same chromosome copy numbers above the 50% threshold in the subsequent specimens. Collectively, only 2.2% (n = 7/307; 95% CI = 0.9–4.4) of all single chromosome copy numbers above 50% detected in this study were not confirmed in other parts of the embryo (Fig. 2A, 'High mosaic to euploid' and 'Aneuploid to euploid'). In contrast, for the cases where the low mosaicism category (i.e. 30–50%) was applied, the overall confirmation rate across all 30–50% calls reported in this study was 13.5%, (n = 15/111; 95% CI = 7.8–21.3) (Fig. 2A, 'Low mosaic to mosaic').



	Euploid	Mosaic	Aneuploid
Euploid (<30)	12,542	35	0
Low Mosaic (30-50)	96	15	0
High Mosaic (50-70)	6	14	27
Aneuploid (>70)	1	11	248

	Euploid	Mosaic	Aneuploid
Euploid (<20)	12,331	29	0
Low Mosaic (20-50)	308	20	0
High Mosaic (50-80)	6	16	55
Aneuploid (>80)	1	9	220

Figure 2. Concordance analysis results for different mosaicism classification criteria. Sankey diagrams depicting concordance between single biopsy classification and multifocal classification, according to 30–70% (**A**) and 20–80% criteria (**B**). In each diagram, left nodes (euploid, low mosaic, high mosaic, aneuploid) represent the chromosome classification based on the copy number variation analysis from a single biopsy, right nodes (confirmed euploid, confirmed mosaic, confirmed aneuploid) represent the multifocal classification of the embryo, and the width of each flow is proportional to the number of events. Per chromosome, data are reported in tables below each diagram.

In addition, considering the initial biopsy, a total of 47/166 (28.3%; 95% CI = 21.6–35.8) uniform aneuploid patterns were explored in this study. When the uniform aneuploid range was considered (i.e. >70%), 97.9% (n = 46/47; 95% CI = 88.7–99.9) of single aneuploid findings detected in the cTE specimen were present in all the subsequent portions from the same embryo, confirming the high diagnostic reproducibility in uniform meiotic aneuploidies detection. Only for one blastocyst, the aneuploidy present also in the ICM was not present in two sequential TE biopsies, suggesting the presence of a true mosaic pattern (Fig. 2A 'aneuploid to mosaic' and Supplementary Table SI). Collectively, considering all specimens analysed in this study (n = 565), the only false positive alteration detected in the uniform aneuploid range was a trisomy of chromosome 14 in a single TE sample (Fig. 2A 'Aneuploid to euploid' and Supplementary Table SI). Common embryonic origin of all re-biopsied specimens from this sample was confirmed with fingerprinting analysis.

Detection of aneuploid and mosaic configurations with less stringent thresholds (20–80%)

Considering less stringent thresholds for mosaic classification (i.e. 20–80% aneuploid cells) (Supplementary Fig. S1A), a total of 635 single mosaic or aneuploid calls, corresponding to 341 different patterns, were detected (Supplementary Table SII). The overall mosaicism rate with this threshold increases from 20.2% (n = 114/565; 95% CI = 27.0–34.8) per biopsy with 30–70% to 40.2% (n = 227/565; 95% CI = 36.1–44.3) with 20–80%, just by applying different reporting criteria.

In detail, when 20–50% threshold was applied, the overall confirmation rate from all single mosaic calls detected in this range significantly decreased to 6.1% (n = 20/328; 95% CI = 3.8–9.3; $P = 0.01278$) (compared to 13.5% when 30–50% thresholds were applied) (Fig. 2B, 'Low mosaic to mosaic'). In particular, the inclusion of very-low mosaic (20–30%) results added a significantly higher number of unconfirmed calls to this range (97.7%; n = 212/217; 95% CI = 94.7–99.2) compared to the 30–50% (Fig. 2B, 'Low mosaic to euploid') ($P < 0.00001$). Moreover, when the upper cut-off was experimentally placed at 80% of abnormal cells, instead of 70%, and the uniform aneuploid calls in cTEs were considered, complete propagation of aneuploid findings in subsequent TE biopsies decreased to 87.2% (n = 164/188; 95% CI = 81.6–91.6) compared to 95.7% when 70% was employed as upper threshold (n = 180/188; 95% CI = 91.8–98.1) ($P = 0.00318$). Indeed, shifting the aneuploidy level cut-off above 80% resulted in mosaicism overcalling in the high range (50–80%), even if the same alteration was uniformly shown in all the biopsies from the same embryo above 50% threshold (Fig. 2B, 'High mosaic to aneuploid'). Overall, no additional true mosaic patterns were detected when 20–80% classification was employed (n = 14) respect to 30–70% classification (n = 14), while false positive mosaic patterns significantly increased from 57.8% to 79.5% (n = 96/166; 95% CI = 49.9–65.4 vs n = 271/341; 95% CI = 74.8–83.6, respectively) ($P < 0.00001$).

Concordance analysis towards the ICM

A comparison between all TE portions and the ICM was performed to assess how well a single TE biopsy result predicts the propagation of a mosaic or uniform aneuploidy throughout the embryo (Supplementary Fig. S1B). Considering the ICM as the reference sample and comparing PGT-A results from all TE specimens from the same blastocyst, when 30–70% thresholds were applied, the overall concordance rate per chromosome was 98.0% (n = 10 189/10 396; 95% CI = 97.7–98.3). The application of the 20–80% threshold range led to an overall reduction of the concordance rate with the ICM to 95.0% (n = 9879/10 396; 95% CI = 94.6–95.4) ($P < 0.00001$). Interestingly, chromosomal mosaicism, and in particular low-grade mosaicism, represents the main contribution to discordant rates towards the ICM. Concordance analysis using a binary classification with 50% as single threshold was also evaluated: sensitivity and specificity per chromosome were 93.33% (n = 224/240; 95% CI = 89.40–96.14) and 99.80% (n = 7846/7859; 95% CI = 99.67–99.88) respectively. It is worth noting that these metrics are affected by the biased sample under analysis (which is enriched in mosaic configurations): we expect even greater sensitivity and specificity testing this classification approach on an unbiased representative sample of chromosome configurations. Indeed, when only uniform euploid and uniform aneuploid cTE biopsies were considered, chromosome confirmation rates between TE and ICM significantly increased to 99.7% (n = 5595/5612; 95% CI = 99.5–99.8) and to 97.7% (n = 5483/5612; 95% CI = 97.3–98.1) for 30–70% and 20–80% classification criteria, respectively.

Binary classification and estimation of PGT-A accuracy in a realistic clinical scenario

As previously explained, the embryo population enrolled in this study was intentionally enriched in mosaic configurations and it is therefore not representative of the typical distribution of chromosomal anomalies found in a clinical sample. To evaluate PGT-A performance in a realistic clinical scenario, we first classified the embryos according to the multifocal definition of confirmed mosaic and uniform aneuploid patterns: embryos with unconfirmed mosaic or aneuploid pattern in any of the chromosomes were classified as true euploids, embryos with at least one confirmed mosaic pattern and unconfirmed uniform aneuploidies as true mosaics, and embryos with at least one confirmed uniform aneuploid pattern as true aneuploid. Then, to correct for the sampling bias, we randomly resampled euploid, aneuploid, and mosaic embryos 100 times at clinically realistic proportions (42% euploid, 55% aneuploid, 3% mosaic). These proportions were fixed considering different reference studies where the same NGS protocol or similar study design were employed (Girardi *et al.*, 2020; Rodrigo *et al.*, 2020; Capalbo *et al.*, 2021; Kim *et al.*, 2022). For each biopsy, the PGT-A test was considered positive (i.e. predicted genetically abnormal) if the highest copy number variation across all chromosomes was greater than a given DV threshold, negative otherwise. Then, we evaluated the performance of a dichotomous classification approach in identifying abnormal embryos (true aneuploid and true mosaic) by estimating the sensitivity and specificity at different DV thresholds (Fig. 3). The analysis clearly showed that a dichotomous classification approach based on a single DV = 50% threshold achieves an optimal accuracy trade-off in terms of specificity (98.2%) and sensitivity (94.0%). When mosaicism is reported, a decrease in specificity is observed for the 30–70% range (81.9%) which becomes crucial with the 20–80% range (54.5%), while only a minimal increase in sensitivity is observed (97.7% or 98.2%, respectively).

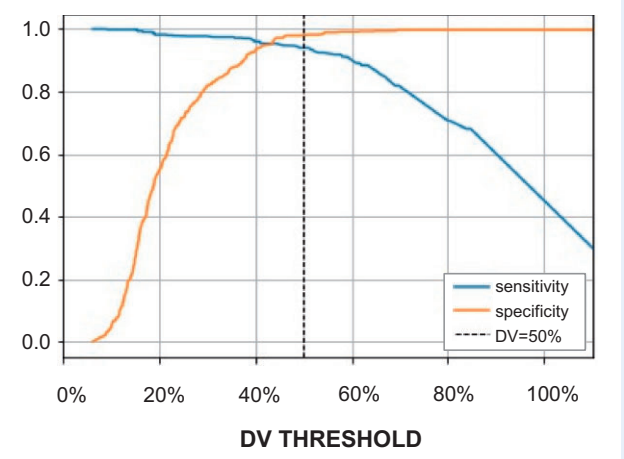


Figure 3. Binary classification performance in a clinically realistic scenario. The figure shows the sensitivity and specificity of the binary classification approach as a function of the difference value threshold. In the analysis, we considered positive cases of those embryos with at least one confirmed mosaic or one confirmed aneuploid configuration. Data have been randomly resampled to correct for sampling bias.

Analysis of chromosome copy number variation

To characterize the technical and biological noise affecting chromosome copy number analysis in PGT-A, we considered the distribution of chromosome copy numbers estimated from a single embryo biopsy stratified by chromosome configuration as assessed by multifocal analysis (disomic, aneuploid or mosaic configurations, Fig. 4A). We also estimated the fraction of disomic, confirmed mosaic, and confirmed aneuploid configurations falling above or below a given DV threshold (Fig. 4B). For disomic configurations, the deviation from the expected chromosome copy number DV is usually close to zero, with most values lying in the 0.0–0.3 range; seldom, absolute DV values can be >0.2, 0.3, or 0.5 in 5.0%, 1.2%, and 0.06% of cases, respectively. Such high values are not replicated by secondary biopsies: the variability observed across disomic configurations is entirely ascribable to technical noise (e.g. uneven DNA amplification, or inaccurate biopsy procedures). Focusing on confirmed aneuploid configurations, the absolute deviation from expected copy number for disomic chromosomes typically ranges between 0.6 and 1.4. Absolute DV can sometimes be lower than 0.8 or 0.7 (22.5% and 10.4% of cases, respectively) but never lower than 0.5. Again, the observed fluctuations are driven by technical noise since we detected a consistent propagation of the aneuploid finding across the whole embryo. The variability in estimated chromosome copy number is much larger for confirmed mosaic configurations, with absolute DV ranging from 0.0 to 1.0, partially overlapping both with the uniform disomic and uniform aneuploid distributions: in this case, chromosome copy number variations are caused both by technical noise but also by true biological variability (fraction of abnormal cells in the biopsied sample).

The number of mosaic and aneuploid configurations across the 113 embryos was insufficient for a systematic and statistically robust chromosome-level analysis of mosaic patterns and concordance rates: the average number of mosaic configurations per chromosome is small (0.61), and no mosaic anomaly was present for most chromosomes (Supplementary Fig. S2), nor aneuploidy for some specific chromosomes (e.g. chromosomes 7 and 10). Therefore, focusing on euploid configurations confirmed by multifocal analysis, the size of the technical noise across different chromosomes was evaluated. This analysis revealed that smaller chromosomes were typically affected by a higher level of technical noise (Pearson $R = -0.61$, $P = 0.0015$, Supplementary Fig. S3) as it could be expected considering that smaller chromosomes are likely to have lower number of amplicons and a smaller amount of genetic material amplified by WGA procedures. Moreover, the specificity of the binary classification approach (based on a DV = 50% threshold) at the single chromosome level was estimated to be >0.996 for any chromosome, including the smaller ones (Supplementary Fig. S4).

Discussion

This work represents the largest blastocyst disaggregation study where five independent specimens from each embryo are analysed, and we believe that it is the first to provide evidence of the most appropriate criteria for mosaicism classification through multifocal biopsy design. Unlike other studies, the report of intermediate chromosome copy numbers is based on raw NGS data analysis from a well-validated platform (García-Pascual *et al.*, 2020), without the application of any proprietary algorithm or chromosome-specific threshold. Furthermore, the evaluation of thresholds for mosaicism classification in TE samples instead of cell lines provides a better representation of outcomes, more transferable to the clinical setting. The multifocal analysis of 113 human donated embryos (n = 565 biopsies) showed that only a small proportion of all putative mosaic calls are confirmed in other parts of the embryo, so failing to confirm the presence of a true mosaic pattern. In detail, we first explored the reliability of mosaic and aneuploid findings when the more stringent threshold (i.e. 30–70%) was applied. Among all 14 putative mosaic calls identified in the cTE, only two were confirmed through the detection of a reciprocal whole chromosome alteration, which represents the strongest evidence of the presence of true mosaicism (Capalbo *et al.*, 2017). In the remaining patterns, the mosaic/aneuploid finding involved only one or two additional embryonic portions, suggesting a distribution of aneuploid cells confined to a small part of the embryo. Moreover, nine were initially detected in the high mosaic range (i.e. 50–70%) and revealed a uniform distribution among all the subsequent TE biopsies and the ICM. On the contrary, when the initial biopsy showed single or multiple alterations in the aneuploid range (>70%), almost 98% of them showed consistent results in all subsequent TE portions and ICM, confirming the high diagnostic reproducibility and complete propagation of uniform chromosomal alterations of meiotic origin. These data support our previous and other works (Victor *et al.*, 2019; Sachdev *et al.*, 2020; Wu *et al.*, 2021; Capalbo *et al.*, 2022) where meiotically derived aneuploidies are reliably detected from a single TE biopsy based on copy number analysis and highly predictive of negative reproductive outcome (Capalbo *et al.*, 2022). Similarly, when 37 embryos with a

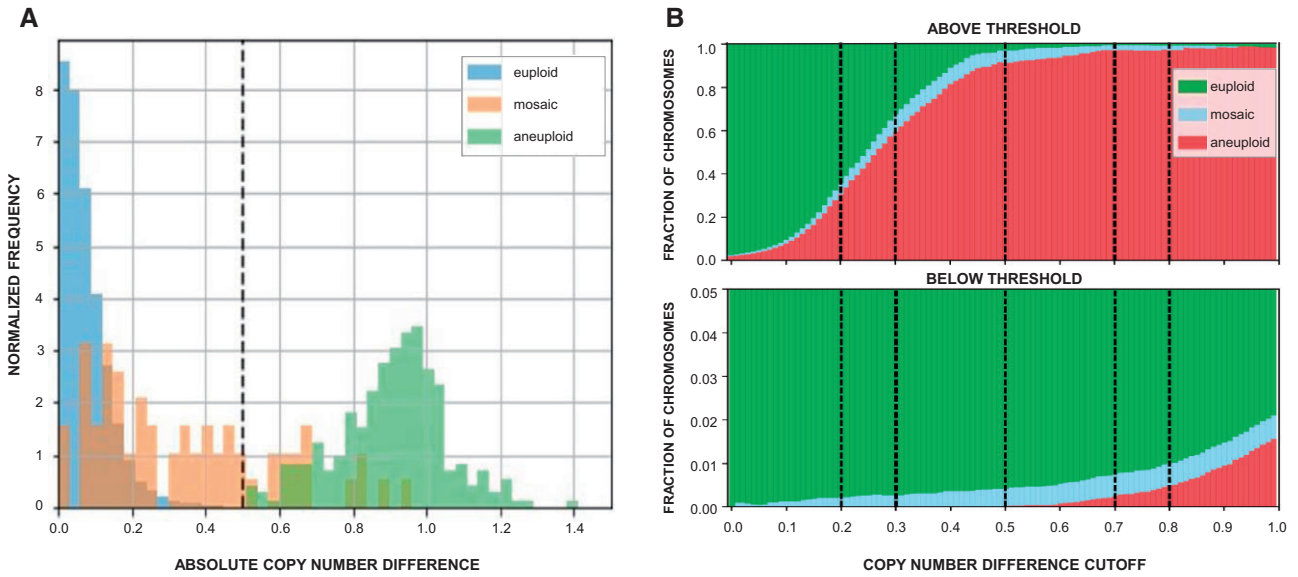


Figure 4. Parametric analysis of copy number variation (CNV) classification thresholds. (**A**) Distribution of the (modulus of the) difference value parameter, stratified by multifocal chromosomal classification. (**B**) At different CNV thresholds, we estimated the relative frequency of confirmed euploid, confirmed mosaic, or confirmed aneuploid chromosomes (based on multifocal classification) above (top panel) or below (bottom panel) the threshold.

euploid cTE result were dissected, only one evidence of true mosaicism was reported in two subsequent TE biopsies (reciprocal whole chromosome aneuploidy) out of 185 analysed (1.08%), while six embryos showed a single low mosaic profile, unconfirmed in the 4 remaining specimens. Taken together, these data confirm the high predictivity of euploid and aneuploid results from cTE biopsy while demonstrating the low ability of mosaic diagnosis in predicting the chromosomal conformation of the remaining embryo even when stringent criteria for mosaicism detection are employed.

As expected, when less stringent criteria (i.e. 20–80%) are applied, false positive mosaicism designations increased significantly. However, less stringency did not result in higher sensitivity, that is higher detection of true mosaic cases. In other words, no additional true mosaic configurations were discovered with the use of a wider threshold range. Excluding the 14 confirmed patterns previously identified with the more stringent range and the aneuploidies of confirmed meiotic origin, the remaining 80% of single putative mosaic calls detected with the 20–80% thresholds could not be confirmed (i.e. false positive), with the majority of them detected in the very low range 20–30%. Consequently, lowering the limit for mosaic classification to 20% can potentially reduce the number of euploid embryos available for transfer, as a large proportion of them could be inaccurately categorized into the mosaic group and, as a result, potentially excluded or deprioritized. Moreover, when the aneuploidy classification criteria were experimentally placed at the 80% threshold, a proportion of results previously categorized as aneuploid by the 70% cut-off, were shifted into the high mosaic group, even if the aneuploid finding was uniformly distributed throughout the embryo. In other words, when wider threshold ranges are applied, previous aneuploid results are now called as high mosaics. This re-classification is not driven by a different biological mechanism, since the abnormality is constitutive and still affecting all embryonic segments. Therefore, with wider ranges, there is an increased risk of misclassifying uniform aneuploid embryos in the high mosaic category. As a consequence, those patients that for some reason decide to transfer a high mosaic embryo, will have a higher chance of negative outcomes since they are probably transferring an aneuploid (Capalbo *et al.*, 2022). Although the presence of analytical noise, amplified by WGA methods, biopsy procedures, and/or other experimental variability, is typical of any platforms validated for chromosome copy numbers analysis, the use of more stringent thresholds (i.e. 30–70%) for mosaic assignment decreases error rate, reducing false positive mosaic and false negative aneuploid results. Another interesting point that emerged from this study is the correlation between the level of mosaicism detected in a single TE biopsy and diagnostic confirmation rate within the remaining embryo. When all intermediate CNVs were initially classified from the cTE biopsy as low or high mosaic according to the more stringent parameters (i.e. 30–70%), only 13.5%, of low mosaic results showed a coherent segregation in other parts of the embryo, while the majority remained unconfirmed (86.5%). The false positive rate further increased to 97.7% when the very low mosaic range (20–30%) was considered. In contrast, high mosaic findings were confirmed in 87.2% of cases detected in this study. It is interesting to note that 47.4% of high mosaic initially reported in cTE revealed a uniform aneuploid configuration in the following biopsies. These data are consistent with other recent works (Capalbo *et al.*, 2021; Marin *et al.*, 2021; Wu *et al.*, 2021) where increasing levels of mosaicism in TE biopsies associated with higher concordance rates with the ICM and uniform aneuploid configuration, while low levels mosaicism

mostly revealed false positives calls. From a clinical perspective, our data support the evidence of a relationship between the level of mosaicism identified in the TE biopsy and the transfer outcome of the corresponding mosaic embryo (Capalbo *et al.*, 2021; Leigh *et al.*, 2022). Indeed, embryos classified as low mosaic (i.e. 20–50% threshold) showed live birth and miscarriage rates similar to euploid embryos (i.e. <20% threshold). Conversely, the transfer of embryos with high mosaic results leads to clear reduced success rates, as the majority of these embryos shared a uniform aneuploid configuration (Viotti *et al.*, 2021). Finally, what emerged from this study is that a binary classification scheme (euploid/aneuploid) with a single cut-off at 50% level is the best strategy to increase overall PGT-A accuracy.

One of the main limitations of this study is that aneuploidy/mosaicism analysis was limited to chromosome copy number thresholds and lacked genotyping information. Adding genotyping data in both clinical and research settings provides a valuable strategy to confirm meiotic origin of some trisomies, reveal true mosaicism for apparently monosomic chromosome with heterozygosity, check for biopsy identity in multifocal experiments, and exclude intermediate copy number caused by ploidy alteration. Moreover, aneuploid embryos with known meiotic partial deletion/duplication were not included, even though this topic was previously addressed by our group. In this study, the sample under analysis was voluntarily enriched in mosaic configurations selected in cTE biopsies. An unbiased sample representative of all chromosome configurations would provide greater PGT-A accuracy levels. Furthermore, several assumptions were made to establish a true mosaicism pattern. Although reasonable and founded, slightly different outcomes might originate using different assumptions. Finally, this work was performed using a single PGT-A assay and other assays need to go through similar validations to establish their sensitivity to different levels of aneuploidy.

In conclusion, based on this large embryo disaggregation study, to maximize PGT-A accuracy, single TE biopsy results showing intermediate chromosome copy numbers should be reported according to euploid/aneuploid classification, based on a single cut-off at 50%. When mosaicism classification is employed, more stringent parameters (i.e. 30–70%) showed better performance and predictive values compared to wider ranges (i.e. 20–80%), which reported suboptimal values. Reporting inaccurate mosaic results in PGT-A cycles may have negative consequences for patients including anxiety for the choice of transfer or not the 'putative' mosaic embryo, additional session of genetic counselling, additional PGT cycles to obtain euploid embryos, and unnecessary higher costs associated to these procedures. One of the major clinical consequences of this method of reporting mosaicism is the potential reduction of the total number of normal embryos available for transfer per cycle because perceived as abnormal by patients. This strategy of reporting and deselecting 'putative' mosaic embryos can also impact established criteria for morphology-based embryo selection among euploid embryos, which are always transfer as the first option even of worst morphology. In addition, the use of wide thresholds for detecting intermediate chromosome copy numbers up to 80% reduces PGT-A ability to discriminate true mosaic from uniformly aneuploid embryos, lowering overall diagnostic accuracy. As a result, a large proportion of the embryos diagnosed as high-level mosaic (i.e. between 70% and 80% of aneuploid cells) may actually be uniformly aneuploid and inadvertently transferred. Considering that uniform aneuploid embryo transfer is almost never associated with the live birth of a healthy baby (Tiegs *et al.*, 2021; Capalbo *et al.*, 2022), the resulted overcalling of high mosaics above 70% will lead to negative outcome for patients when selected for transfer (i.e. miscarriage) with consequent reduced overall success rates. Finally, even if the rate of mosaic embryo transfer confirmed in pregnancies to date is extremely low (Kahraman *et al.*, 2020; Treff and Marin, 2021), transferring mosaic embryos after PGT-A can generate unjustified indication for invasive prenatal follow-up through amniocentesis, which is associated to a known risk of iatrogenic abortion. Taken together, our data provide useful information for patient counselling and may assist in the prioritization and clinical utilization of embryos presenting putative mosaic results.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

Data availability

The data underlying this article are available in the article and in its online supplementary material. Additional data will be shared on reasonable request to the corresponding author.

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Authors' roles

L.G. and M.F. performed, data curation, formal analysis, supervision, writing—original draft, writing—review editing. M.P. contributed to writing—original draft, writing—review editing. M.S., O.C., F.K.B., and M.B. have been involved in investigation and methodology. C.P., S.C., I.P., and F.C. worked on methodology. R.N. performed data curation. C.R., N.F., and C.S. have been involved in the supervision of the study and revision. A.C. performed conceptualization, supervision of the study, writing—review, and editing.

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Conflict of interest

The authors not employed by Igenomix have no conflicts of interest to declare.

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