Inconclusive chromosomal assessment after blastocyst biopsy: prevalence, causative factors and outcomes after re-biopsy and re-vitrification. A multicenter experience

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STUDY QUESTION: Can a second round of biopsy, vitrification and chromosomal testing provide a valid diagnosis where the first attempt fails?

SUMMARY ANSWER: The risk of inconclusive chromosomal-assessment after trophectoderm biopsy was 2.5% but a further biopsy and vitrification-warming appeared not to impair the competence of euploid blastocysts.

WHAT IS KNOWN ALREADY: The increasing implementation of multicell trophectoderm biopsy has significantly reduced the risk of inconclusive diagnosis after preimplantation-genetic-testing (PGT). Yet, few reports have defined the variables that influence the risk of failure or described the technical and clinical outcomes after re-biopsy.

STUDY DESIGN, SIZE, DURATION: Retrospective multicenter study involving 8990 blastocyst biopsies conducted between April 2013 and September 2017 at six IVF centers but analyzed at a single genetic laboratory. A total of 206 blastocysts were successfully re-biopsied after warming and re-expansion, then re-vitrified. And 49 of these blastocysts were diagnosed euploid and used in single-embryo-transfers (SETs). Logistic regression analyses were conducted.

PARTICIPANTS/MATERIALS, SETTING, METHODS: A total of 3244 PGT-for-aneuploidies (PGT-A) cycles with a freeze-all approach, vitrification and qPCR-based analysis were performed by 2687 consenting couples. DNA amplification failure (AF) or non-concurrent data resulted in inconclusive diagnoses. In case of DNA amplification, the cellularity of the biopsy was estimated according to a previously validated method. Euploid SETs were performed. Clinical pregnancy, miscarriage, live birth rates (LBR) and perinatal outcomes were monitored.

MAIN RESULTS AND THE ROLE OF CHANCE: Overall, 2.5% of trophectoderm biopsies resulted in an inconclusive diagnosis (N = 228/8990). Specifically, 2% (N = 176/8990) resulted in AF and 0.5% (N = 52/8990) in non-concurrent results. The only parameters significantly associated with inconclusive diagnoses were the IVF center and the embryo age (days) at biopsy. Among samples with successful amplification, the number of cells in the biopsy and the day of biopsy were critical to limit non-concurrent results. In total, 213 blastocysts with an
inconclusive diagnosis were warmed for re-analysis and the survival rate was 96.7% (N = 206/213). The euploidy rate in blastocysts biopsied twice was 51.9% (N = 107/206) and the euploid embryos were re-vitrified. Overall, 49 euploid embryos were warmed for replacement and all survived. The LBR after SET was 38.8% (N = 19/49). No minor/major obstetrical/perinatal complication was reported.

LIMITATIONS, REASONS FOR CAUTION: A single aneuploidy-testing method was adopted in this retrospective analysis. A more powered report of the clinical and obstetrical/perinatal outcomes after re-biopsied and re-vitrified blastocyst euploid SET requires a larger sample size.

WIDER IMPLICATIONS OF THE FINDINGS: It is important to re-biopsy and re-vitrify undiagnosed blastocysts since healthy live births can result from them.

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Introduction

Since the initial implementation of preimplantation-genetic-testing (PGT) in IVF, the use of blastocyst biopsy has significantly increased. The ESHRE PGD Consortium reported that between 2011 and 2012 in Europe only ~4% of the biopsy procedures were conducted at this stage of embryo development [De Rycke et al., 2017], a rate that has subsequently increased to ~60% in 2016 as recently reported by De Rycke at the ESHRE annual meeting held in Barcelona [De Rycke, 2018]. Therefore, the number of trophectoderm biopsies in Europe now outnumber single blastomere biopsies, and the same is true worldwide [International Federation of Fertility Societies Surveillance, 2016]. This shift towards blastocyst biopsy is driven by accumulating evidence supporting its safety, reliability and clinical value [Scott et al., 2012, 2013a, 2013b; Capalbo et al., 2016; Cimadomo et al., 2016]. From a technical perspective, trophectoderm biopsy allows the retrieval of a multicellular fragment, which, by limiting the risk of DNA amplification failure (AF) and low-quality molecular data, reduces the need of an embryo re-biopsy. Nonetheless, very few reports exist that investigated this important issue from both a technical (i.e. rate and causes) and a clinical (i.e. survival rate to re-biopsy and re-vitrification, miscarriage, live birth rates (LBR) and perinatal outcomes) perspective. To our knowledge, only one paper investigated the incidence of inconclusive diagnoses after blastocyst biopsy [Zhang et al., 2014]; two papers instead investigated the clinical outcomes after re-biopsy and re-vitrification [Zhang et al., 2014; Bradley et al., 2017]. Therefore, the data related to this topic can be mostly gathered from few studies presented at the ASRM annual meetings (Brower et al., 2014; Kaing et al., 2015; Swain et al., 2015; Lee et al., 2016; Neal et al., 2017a, 2018).

In this retrospective multicenter observational study, we aimed to assess the prevalence of inconclusive diagnoses after trophectoderm biopsy conducted at six IVF centers during PGT-A cycles, and to define their causes. Furthermore, we reported the clinical and perinatal outcomes after single re-biopsied and re-vitrified euploid blastocyst transfers (single-embryo-transfer (SET)).

Material and Methods

Study design and outcome measures

The workflow of this retrospective multicenter study is shown in Fig. 1. 8990 blastocysts underwent trophectoderm biopsy at six IVF centers in Italy and were vitrified between April 2013 and September 2017. These blastocysts derived from 3244 PGT-A cycles conducted by 2687 consenting couples (mean maternal age: 38.5 ± 3.9, range: 23–45 years). The main indications were advanced maternal age (AMA, ≥35 years), recurrent implantation failure (RIF, ≥3 previous failed attempts), recurrent pregnancy loss (RPL, ≥3 previous miscarriages) or couple’s choice. The biopsies were stored at −20°C and shipped overnight to a single genetic lab. Comprehensive-chromosom-al-testing (CCT) was conducted by qPCR [Treff et al., 2012]. The plots were inspected by a certified geneticist and given either conclusive (aneuploid/euploid) or inconclusive diagnoses (AF/non-concurrent) as previously defined [Capalbo et al., 2016] (Fig. 2A–D). The main parameters putatively affecting the chance of obtaining a conclusive diagnosis were investigated using logistic regression (i.e. maternal age, PGT indication, IVF center, blastocyst morphological quality and day of biopsy). Blastocyst biopsy showing DNA amplification failure were investigated also for the number of trophectoderm cells retrieved, estimated through a method previously published [Capalbo et al., 2016; Neal et al., 2017b] and recapitulated in the next paragraph. These data were inspected to define how many cells are required to achieve conclusive diagnosis and its correlation with the other variables under investigation.

Lastly, 213 of the 228 undiagnosed blastocysts were warmed to be re-biopsied, re-vitrified and re-analyzed. The re-biopsy and re-analysis were free of charge for the couple. The 49 euploid blastocysts after re-biopsy were re-warmed and underwent SET. The mean gestational weeks and birthweight of the newborns were reported.

Technical procedures and clinical outcomes

The IVF-related protocols (i.e. ovarian stimulation, oocyte retrieval, ICSI, embryo culture, vitrification and warming, and transfer) have been described previously [Rienzi et al., 1998; Cobo et al., 2008; Ubaldi et al., 2015; Cimadomo et al., 2018]. The participating clinics use a trophectoderm biopsy approach that does not entail Day 3 zona-opening [Capalbo et al., 2014, 2016]. The blastocysts were biopsied when they reached full expansion on Day 5, 6 or 7. They were classified as excellent, good, average or poor according to Capalbo et al. (2014), a method adapted from Gardner and Schoolcraft (1999).

The samples were analyzed by qPCR, a CCT platform validated to detect full-chromosome meiotic aneuploidies, but not segmental or mitotic ones [Treff et al., 2012; Capalbo et al., 2015]. This method requires the targeted pre-amplification of four non-variable sequences per each chromosome. Then, during the proper qPCR step, the TaqMan assays generate specific curves representative of each of these sequences. The Ct data are then compared to a historical pool of results produced from euploid male blastocysts to calculate specific copy numbers (CNs). Four CNs from four technical replicates per each of these four sequences per chromosome are obtained in the analytical phase. Specific chromosome CNs were calculated from the mean of the replicates, while specific
concordances were derived from their SD. Finally, an overall inter-chromosome SD (i.e. the SD of all chromosome CNs) and an overall-concordance (i.e. the mean of all chromosome concordances) are also calculated. A plot resulting into an overall-concordance ≥0.4 is considered non-concurrent and cannot be reliably diagnosed (Fig. 2D).

A standard curve was built based on the qPCR mean Ct data obtained by analyzing samples composed of an increasing known number of easily countable and loadable cells (1, 2, 3, 4, 5, 10, 15 and 20, respectively) from cell lines (Capalbo et al., 2016; Neal et al., 2017b). Each mean Ct value obtained from the analysis of the trophectoderm biopsies resulting in a DNA amplification (i.e. conclusive/non-concurrent results) was interpolated with this standard curve to estimate the number of cells composing them.

Only euploid vitrified-warmed SETs were performed. A clinical pregnancy was defined as a gestational sac with fetal heartbeat. A miscarriage was defined as a loss before the 22nd gestational week.

**Statistical analyses**

Continuous variables are shown as mean ± SD and range. Shapiro–Wilk tests were conducted to investigate the normal distribution of the data. ANOVA or t-tests were performed to assess statistically significant differences. Categorical variables are shown as rate with 95% CI. Fisher’s exact or chi-squared tests were performed to assess statistically significant differences. Logistic regression analyses upon the risk of a trophectoderm biopsy to result in an inconclusive diagnosis and, among amplified samples, in non-concurrent results were performed for all the parameters under investigation. All statistical analyses were conducted with the software R. A P < 0.05 was considered significant.

**Results**

**Inconclusive diagnoses and investigation of their related causes**

In the study period, 8990 blastocysts were biopsied; 8762 resulted in conclusive (97.5%, 95% CI: 97.1–97.8) and 228 in inconclusive (2.5%, 95% CI: 2.2–2.9) diagnoses, respectively. Among the latter, 176 because of AF (2.0%) and 52 because of non-concurrent results (0.5%).
The undiagnosed blastocysts belonged to 172 PGT cycles (N = 172/3244, 5.3%) performed by 172 couples (N = 172/2687, 6.4%). In detail, 94.7% (N = 3072), 3.8% (N = 124), 1.2% (N = 40) and 0.3% (N = 8) PGT cycles showed 0, 1, 2 and 3 undiagnosed blastocysts, respectively. No patient received more than a single report with inconclusive diagnoses during the study period. Supplementary Table SI shows these data by center; centers 1, 2 and 4 had significantly higher rates of PGT cycles where a conclusive report (i.e. no inconclusive diagnosis) was produced at the first analysis (95.6, 96.5 and 94.5%, respectively). Conversely, these rates were lower at centers 3, 5 and 6 (89.5, 91.2 and 87.7%; P < 0.01). Similar significant trends resulted from a patient-based analysis (Supplementary Table SII). The proportion of blastocysts undiagnosed was variable among the six centers, with a minimum rate at center 2 (N = 19/1294, 1.5%) and a maximum rate at center 6 (N = 11/222, 5.0%) (P < 0.01; Fig. 3A). On the contrary, a worse blastocyst morphological quality did not result into higher rates of inconclusive diagnoses. Indeed, the data were rather homogeneous among excellent- (N = 104/3900, 2.7%), good- (N = 34/1295, 2.6%), average- (N = 51/1752, 2.9%) and poor-quality (N = 39/2043, 1.9%) blastocysts (P = NS; Fig. 3B). Finally, the day of biopsy was highly correlated with the chance of achieving a conclusive diagnosis. Specifically, the rate of inconclusive results was 3.7% (N = 116/3151) on Day 5, 2.1% (N = 104/5011) on Day 6 and 1.1% (N = 8/738) on Day 7 (P < 0.01; Fig. 3C). Logistic regression analyses were conducted and did not show any correlation between the maternal age, the PGT indication and the blastocyst morphological quality with the risk of not achieving conclusive diagnoses. The only significant predictors were the IVF center (P < 0.01) and the day of biopsy (P < 0.01) (Fig. 3D). Supplementary Fig. S1 presents the data clustered in three groups according to the outcome of the qPCR analysis (i.e. conclusive diagnosis, AF or non-concurrent results) per day of biopsy and IVF center. The figure outlines superimposable trends from the two categories of inconclusive diagnoses (i.e. AF and non-concurrent results) versus the day of biopsy (Supplementary Fig. S1B and C). Conversely, while the rate of AF showed an increasing trend from the most to the least experienced clinics, the rate of non-concurrent results was more stable across them (Supplementary Fig. S1B and C).

Among the samples resulting in a successful DNA amplification, conclusive diagnoses originated from biopsies composed of a significantly higher number of trophectoderm cells (8.0 ± 3.0, 2–15) than biopsies resulting in non-concurrent results (6.6 ± 3.0, 2–13; P < 0.01). In general, the mean number of cells retrieved increased according to both the IVF center (i.e. their expertise) and the day of biopsy (P < 0.01; Supplementary Fig. S2A and B). We performed a logistic regression analysis aimed at assessing whether the cellularity of the biopsy could predict, even when corrected for both these variables, the risk of

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**Figure 2** Examples of qPCR plots: aneuploid (A), euploid (B), DNA amplification failure (AF) (C) and non-concurrent result (D). The graph represents each chromosome-specific copy number (Chr CN) as a black bar with standard error (SE) between the chromosome-specific thresholds (light blue bars) for trisomy (first from the top), monosomy (second) and nullisomy (last). The red and blue lines represent the average cut-off CNs for trisomy (2.69) and monosomy (1.3), respectively.
producing non-concurrent results. Interestingly, the IVF center did not show any significant correlation, while the estimated number of cells and the day of biopsy did (Supplementary Fig. S3A). Specifically, biopsies retrieved on Day 5 and resulting in conclusive diagnoses were characterized by a higher estimated number of trophectoderm cells (7.6 ± 2.9, 2–15) than those resulting in non-concurrent data (5.6 ± 2.3, 2–13) (Supplementary Fig. S3B). Similarly, biopsies retrieved on Day 6/7 and resulting in conclusive diagnoses presented a higher number of cells (8.1 ± 3.0, 2–15) than those resulting in non-concurrent data (7.8 ± 3.3, 4–13) (Supplementary Fig. S3B). Of note, the biopsies resulting in a successful diagnosis at the first round of analysis of euploid (N = 4000) and aneuploid (N = 4762) consisted of a similar number of cells (8.1 ± 3.0, 2–15 and 7.9 ± 3.0, 2–15, respectively), even if the data were further clustered according to the different days of biopsy or IVF centers (Supplementary Table SIII). Variability was instead observed between the aneuploidy rates reported by the IVF centers, however, this is imputable to different mean maternal ages (P < 0.01) across them rather than to their experience (P = NS) (Supplementary Table SIV).

Blastocyst re-biopsy and re-vitrification and related outcomes

Overall, 213 of the 228 undiagnosed blastocysts were warmed; 206 survived (96.7%), were re-biopsied and re-vitrified (Fig. 1). No blastocysts were undiagnosed after re-analysis and the euploidy rate was 51.9% (N = 107/206, 95% CI: 44.9–58.9). Among euploid re-biopsied blastocysts, 49 were warmed to undergo SET. All of them survived (100%) and were transferred (Fig. 1). The clinical pregnancy (N = 21/49, 42.9%, 95% CI: 29.1–57.7), miscarriage (N = 2/21, 9.5%, 95% CI: 1.7–31.8) and live birth (N = 19/49, 38.8%, 95% CI: 25.5–53.8) rates of re-biopsied euploid blastocysts were not different from those reported for euploid blastocysts biopsied and warmed only once (48.8, 12.2 and 42.9%, respectively) (Supplementary Table SIV). Overall, 19 babies were born after re-biopsy and re-vitrification. The mean gestational age and birthweights were 38.8 ± 1.3, 37–41 weeks and 3428.8 ± 462.7, 2650–4200 g, respectively (Supplementary Table SV). No minor/major obstetrical or perinatal complication was reported.

Discussion

Blastocyst biopsy involved a low risk of inconclusive chromosomal assessment (2.5%), in line with previous reports (2–6%) (Brower et al., 2014; Zhang et al., 2014; Kaing et al., 2015; Swain et al., 2015; Lee et al., 2016; Neal et al., 2017a). This confirms this approach as technically solid.

The day of trophectoderm biopsy represents the main variable affecting both the presence and quality of the DNA analyzed. Specifically, as the days to reach full-blastulation increased from 5 to 7, the rate of inconclusive diagnoses decreased from 3.5 to 1%. Possibly, longer culture in-vitro and larger blastocyst expansion is associated with the trophectoderm cells being smaller. Therefore, a fragment retrieved from a Day 5 blastocyst might contain fewer cells (i.e. lower DNA
content and quality) than a fragment of similar size retrieved on Day 6/7. Notably, by contrast, blastocyst morphological quality did not correlate with the risk of inconclusive diagnosis, suggesting that trophectoderm cells retrieved from poorer-quality embryos contain good quality genomic-DNA, justifying their biopsy. Even if characterized by a lower euploidy rate (Capalbo et al., 2014), at least poor-quality blastocysts do not seem to involve higher risks of inconclusive diagnosis.

The variability between the IVF centers is mainly ascribable to their expertise in biopsy and tubing procedures. In general, the differences are more pronounced when comparing AF ratios (i.e. absence/degradation of genomic-DNA) rather than the quality of the qPCR data. This might rely upon a trend towards the collection of bigger fragments in less-experienced clinics, possibly because of the initial fear of retrieving insufficient cells to achieve reliable results. Indeed, only the cellularity and the day of biopsy, but not the IVF center, significantly correlated with the risk of non-concurrent results among amplified samples after biopsy, tubing and shipment.

This study demonstrates that ideally eight trophectoderm cells should be collected to limit the risk of inconclusive diagnosis. The ideal timing is Day 6. Nevertheless, although blastocyst biopsy has been reported as a safe approach (Scott et al., 2013b; Cimadomo et al., 2016), the number of cells removed should be limited according to the biomass of the blastocyst at the time of biopsy. Recently, Neal et al. (2017b), using qPCR and the same estimation of cellularity used here, observed that the DNA content in a biopsy sample is associated with a reduction in the implantation of euploid blastocysts only in the fourth (upper quartile overall range: 1–20 cells). In the light of this, eight cells retrieved from a fully expanded blastocyst on Day 6 might represent the ideal balance between obtaining good quality molecular data and a minimal biopsy-dependent reduction of embryo competence.

The aneuploidy rates among successfully diagnosed blastocysts were different between the centers, but these data were biased by statistically different mean maternal ages. Indeed, the ‘IVF center’ variable corrected for the ‘maternal age’, did not show any correlation with blastocyst chromosomal constitution. Conversely, the risk of inconclusive diagnoses is mainly imputable to technical aspects rather than to intrinsic patient- (i.e. maternal age, PGT indication) or embryo-related (i.e. blastocyst quality) features. Indeed, no patient received a genetic report with inconclusive diagnoses more than once during the study period, while in 1.5% of the PGT cycles ≥2 blastocysts from the same cohort showed inconclusive diagnoses, perhaps suggesting a reiterated technical issue. In fact, as the overall rate of undiagnosed blastocysts was variable among the centers, also the rate of PGT cycles characterized by ≥2 undiagnosed blastocysts was significantly higher from less-experienced clinics. These evidences support that proper training and specific key-performance-indicators are all required to limit the risk of inconclusive diagnoses after blastocyst biopsy. In fact, if we deeply inspect our data by comparing the worst-performing center (#6) to the best-performing one (#2), this risk decreased from 5 to 1.5%, consequently affecting 12.3 and 3.4% of the PGT cycles, and 13.6 and 3.9% of the couples, respectively. Methodological studies like ours are therefore important to clarify the consequences of the techniques adopted in the daily activity of an IVF clinic. These evidences are pivotal to propose hints, corrective measures and solid data for counseling.

The CCT platform adopted here could not discriminate plots compatible with mosaicism. Therefore, future methodological studies adopting more sensitive platforms need to clarify whether variability exists in the rate of allegedly mosaic blastocysts reported from different centers/operators. Indeed, it is crucial to define if and to what extent a poor-quality biopsy, rather than a pure biological issue, may result in a plot suggestive of ‘mosaicism’.

From a clinical perspective, the survival rate after warming and re-biopsy (97%) was similar to the previous reports (91–98%) (Brower et al., 2014; Zhang et al., 2014; Kaing et al., 2015; Swain et al., 2015; Lee et al., 2016; Neal et al., 2017b). The euploidy rate (52%) did not differ from blastocysts biopsied only once. The survival rate to the second vitrification-warming cycle of euploid re-biopsied blastocysts was 100%, as for previous reports except for Zhang et al.’s study (95%) (Zhang et al., 2014). This evidence, beyond supporting the reliability and safety of vitrification (Rienzi et al., 2017), further suggests that human blastocysts are resistant to several sources of stress (e.g. IVF-required manipulations). The implantation rate of survived blastocysts from the previous reports ranged 38–57% (Brower et al., 2014; Zhang et al., 2014; Kaing et al., 2015; Swain et al., 2015; Lee et al., 2016; Neal et al., 2018); here we reported a 39% LBR, similar to standard euploid blastocyst SETs at our clinics (43%). To date, only Bradley et al. (2017) reported a reduced LBR after trophectoderm re-biopsy and two vitrification-warming cycles: 27% (N = 6/22) versus 50% (N = 734/1468) for blastocysts biopsied and vitrified only once. However, the morphological quality of the blastocysts in the former group (poor: 28%) was significantly lower than the control (poor: 9%) and the clinical outcomes were not corrected through a logistic regression analysis. Therefore, it cannot be discriminated whether the lower LBR reported in their study is ascribable to an additional biopsy and vitrification or simply to a lower-quality of the blastocysts transferred. Future larger studies should address this issue. Lastly, Bradley et al. (2017) did not show any worse result in the neonatal outcomes after re-biopsy and re-vitrification; yet, the report involved just six newborns. Only another study (Neal et al., 2018) assessed the obstetrical and perinatal outcomes of re-biopsied euploid blastocysts (N = 64) versus control ones (N = 4846); again no differences in the gestational age and birthweight were reported. Here, we also monitored the same parameters from 19 newborns in the study group: the mean birthweight was 3428.8 ± 462.7 g, 2650–4200 g, not statistically different from blastocysts biopsied and vitrified-warmed only once, and within the range of normality (2500–4500 g). Clearly, the sample size is still limited to make any solid statement, therefore, more data must be gathered to investigate this issue.

To conclude, this retrospective study applies to a clinical policy based on trophectoderm biopsy without zona-opening on Day 3, vitrification and qPCR. Furthermore, the number of blastocysts transferred after re-biopsy and re-vitrification is still limited to draw clear conclusions upon the clinical and neonatal outcomes. Still, the data are consistent with the previous reports based on different blastocyst biopsy strategies and CCT platforms. All these evidences encourage to re-biopsy and re-vitrify undiagnosed blastocysts and rescue them for the clinical use. Clearly, this is crucial for patients not producing any euploid blastocyst from the same cohort, but might be important for any couple experiencing this issue after PGT. Moreover, if an absent/limited clinical impact would be confirmed from future studies, any blastocyst requiring a confirmatory diagnosis might benefit from a re-biopsy and re-vitrification. For instance, re-biopsy might be advised to confirm plots generated via different platforms (e.g. NGS) and
Outcomes of re-biopsied blastocysts

suggestive of mosaicism/segmental aneuploidies, or to further test an euploid blastocyst for novel conditions (i.e. monogenic diseases) that might be identified in the parental genotype after PGT has been already conducted.

Lastly, we could not investigate cost-effectiveness (i.e. re-biopsy and re-analysis were free of charge for the couple), which is therefore advisable from future studies and, ideally, should include also time-to-pregnancy among the outcomes.

**Conclusion**

Trophectoderm biopsy is confirmed to be technically solid and safe. The increasing expertise of the clinic, thecellularity of the biopsy (ideally eight cells) and the day of biopsy (ideally Day 6) are all crucial to keep a rate of inconclusive diagnoses at ≤2.5%. Nevertheless, accumulating evidence suggests it is worthwhile to rescue undiagnosed blastocysts for clinical use through a second biopsy, analysis and vitrification-warming cycle.

**Supplementary data**

Supplementary data are available at Human Reproduction online.

**Author’s roles**

D.C. and A.C. designed the study. D.C., A.C. and L.R. drafted the article. All authors contributed to data collection, analysis and discussion.

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**Conflict of interest**

The authors have no conflict of interest to declare related to this study.

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