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ARTICLE

Consistent results of non-invasive PGT-A of human embryos using two different techniques for chromosomal analysis

BIOGRAPHY

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KEY MESSAGE

Diagnostic concordance between PGT-A and non-invasive PGT-A (niPGT-A) seems independent of the genetic analysis technique. niPGTA may be influenced by factors such as DNA contamination and embryo mosaicism. Culture conditions and medium retrieval may be potential targets to improve niPGT-A reliability. This study provides new data on improving the accuracy of niPGT-A.

ABSTRACT

Research question: Are discordances in non-invasive preimplantation genetic testing for aneuploidies (niPGT-A) results attributable to the technique used for chromosomal analysis?

Design: A prospective blinded study was performed (September 2018 to December 2019). In total 302 chromosomal analyses were performed: 92 trophectoderm PGT-A biopsies and their corresponding spent embryo culture medium (SCM) evaluated by two methods (n = 184), negative controls (n = 8), and trophectoderm and inner cell mass biopsies from trophectoderm-aneuploid embryos (n = 18). Trophectoderm analyses were carried out using Veriseq (Illumina), and SCM was analysed using Veriseq and NICS (Yikon).

Results: Genetic results were obtained for 96.8% of trophectoderm samples versus 92.4% for both SCM techniques. The mosaicism rate was higher for SCM regardless of the technique used: 30.4% for SCM-NICS and 28.3% for SCM-Veriseq versus 14.1% for trophectoderm biopsies (P = 0.013, P = 0.031, respectively). No significant differences in diagnostic concordance were seen between the two SCM techniques (74.6% for SCM-NICS versus 72.3% for SCM-Veriseq; P = 0.861). For embryos biopsied on day 6, these rates reached 92.0% and 86.5%, respectively. On reanalysing trophectoderm-aneuploid embryos, the discrepancies were shown to be due to maternal DNA contamination (55.6%; 5/9), embryo mosaicism (22.2%; 2/9) and low resolution in SCM-NICS (11.1%; 1/9) and in both SCM techniques (11.1%; 1/9).

Conclusions: This is the first study evaluating the consistency of different chromosomal analysis techniques for niPGT-A. In conclusion, the diagnostic concordance between PGT-A and niPGT-A seems independent of the technique used. Optimization of culture conditions and medium retrieval provides a potential target to improve the reliability of niPGT-A.

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KEY WORDS

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INTRODUCTION

Chromosomal abnormalities are very frequent in human embryos and can be identified during IVF by performing preimplantation genetic testing for aneuploidies (PGT-A). Blastocyst-stage biopsy represented an important improvement in the PGT-A technique and therefore its outcome. Several preclinical and clinical studies recognized its importance soon after it was introduced (*Scott et al., 2013*), so it has gradually replaced cleavage-stage and polar body biopsy approaches. Recent studies have reported that there is no effect of trophectoderm biopsy on the implantation potential of the embryo (*Cimadomo et al., 2018*). The power of trophectoderm biopsy resides in its higher technical and biological robustness (*Cimadomo et al., 2016*). However, some factors related to trophectoderm biopsy are technically challenging. The number of biopsied cells is likely to reduce the embryo implantation rate (*Zhang et al., 2016*) as are the number of laser pulse lengths used for the embryo biopsy procedure (Montag and Morbeck, *2017*) and the technique used for cell retrieval (*Herrero et al., 2019*). Therefore, it seems that there could be an impact on clinical outcomes related to increasing manipulation of the embryo.

The trophectoderm biopsy technique is costly as it requires specialized equipment (i.e. laser) and a team of experienced embryologists. It is also time-consuming, which, overall, is additionally reflected in the elevated cost. Indeed, some clinics may be unable to set up a PGT-A programme or have to outsource trophectoderm biopsy. Finally, in some countries, embryo biopsy is not allowed, legal reasons thus representing the major barrier to performing the technique. Moreover, more research is needed to assess the effects of trophectoderm biopsy on the future development of babies born after PGT-A, and other changes at the molecular level have also not been studied (*Zacchini et al., 2017*).

For all these reasons, an ideal aim for PGT-A is to avoid embryo biopsy and report euploidy using non-invasive methods. Non-invasive PGT-A (niPGT-A) may provide a simpler, safer and less costly approach to PGT-A. In addition, such a method could potentially allow the analysis of poor-quality embryos, which are often considered inappropriate for biopsy and are discarded.

DNA has been found in human embryonic fluid (*Tobler et al., 2015*) and culture medium (*Xu et al., 2016*), and its extracellular origin is still under investigation. Cell-free DNA in the spent embryo culture medium (SCM) is likely to be present in very low quantities and/or degraded, and the latter may, for instance, be due to cell death processes (*Galluzzi et al., 2015*). A low concentration and poor integrity of DNA seem to be more successfully overcome by some amplification strategies and genetic analysis techniques. Therefore, the particular amplification technology and chromosomal genetic test adopted could be key for the reliability of niPGT-A. The variable concordance rates between niPGT-A using SCM and trophectoderm biopsy reported in different studies (*Leaver and Wells 2020*) may thus be explained by the genetic test used. However, whether the accuracy of niPGT-A could be improved by different genetic analysis techniques has not yet been explored.

To the authors' knowledge, there are no published studies to evaluate the accuracy of niPGT-A when comparing different chromosomal analysis techniques. The aim of this study was to compare the niPGT-A results obtained from the same SCM analysed using two different chromosomal genetic analysis techniques and from trophectoderm biopsy. Furthermore, diagnostic discordances were analysed in order to detect the possible cause of the diagnostic error using two approaches: (i) trophectoderm-aneuploid embryos donated for research purposes and found to be euploid on SCM analysis were thawed and an analysis of the trophectoderm and inner cell mass (ICM) was undertaken; and (ii) the outcomes of IVF using trophectoderm-euploid embryos shown to be aneuploid on SCM analysis were investigated.

MATERIALS AND METHODS

Study design

A prospective blinded validation study was undertaken from September 2018 to December 2019. A total of 92 trophectoderm biopsies were included from 29 couples who attended the authors' clinic for PGT-A (maternal age 41.3 ± 3.4 years). Indications for PGT-A were as follows: advanced maternal age (over 38 years), recurrent implantation failure (failure to achieve a clinical pregnancy after the transfer of at least four good-quality embryos), repeated pregnancy loss (three consecutive pregnancy losses prior to 20 weeks' gestation) and severe male factor infertility. The corresponding SCM were divided into two aliquots and evaluated using two different chromosomal genetic analyses (n = 184). In order to assess DNA contamination, negative controls were included in each batch of analyses (n = 8). Finally, to investigate the diagnostic discrepancies, trophectoderm-aneuploid embryos that had been donated for research purposes were warmed and re-analysed using trophectoderm and ICM biopsies (n = 18). In total 302 chromosomal analyses were performed. The trophectoderm biopsy results were compared in a blind manner with the SCM results from the same embryo.

This study was approved by the University Hospital of San Juan (Alicante) Ethics Committee Review Board (18/342 Tut/20/12/2018; trial registration number NCT03879265). All included patients signed written informed consent forms.

IVF cycle and laboratory protocols

Protocols for ovarian stimulation were personalized in line with each patient's medical history and gynaecologist's recommendations. In brief, multifollicular development was achieved using either urinary or recombinant FSH/LH (150-300 IU/day) under a long gonadotrophin-releasing hormone (GnRH) agonist protocol or a GnRH antagonist protocol. The protocol was selected according to the patient's age, the predicted ovarian reserve assessed by serum anti-Müllerian hormone concentrations and the patient's body mass index. Oocyte maturation using 6500 IU recombinant human chorionic gonadotrophin and/or a bolus of GnRH agonist depending on the type of protocol was employed when the dominant follicle reached a diameter of more than 17 mm as measured by transvaginal ultrasonography. Oocyte retrieval was performed according European Society for Human Reproduction and Embryology recommendations for good clinical practice (*D'Angelo et al., 2019*).

TABLE 1 COMPARISON OF ACCURACY OF NIPGT-A USING NICS (YIKON) OR VERISEQ (ILLUMINA) VERSUS TROPHECTODERM BIOPSY FOR PGT-A

Parameter	niPGT-A NICS (*n* = 92)	niPGT-A Veriseq (*n* = 92)	*P*-value
Informativeness rate, % (*n*/*N*)	92.4 (85/92)	92.4 (85/92)	1.0
Mosaicism rate, % (*n*/*N*)	30.4 (28/92)	28.3 (26/92)	0.872
Positive predictive value	79.6	75.5	0.796
Negative predictive value	67.7	66.7	1.0
Sensitivity	78.0	80.0	1.0
Specificity	69.7	60.6	0.608
Diagnostic concordance, % (*n*/*N*)[a]	74.7 (62/83)	72.3 (60/83)	0.861
Full concordance, % (*n*/*N*)	45.2 (28/62)	41.7 (25/60)	0.837
Partial concordance, % (*n*/*N*) (%)	22.6 (14/62)	23.3 (14/60)	1.0
Complementary concordance, % (*n*/*N*)	4.8 (3/62)	3.3 (2/60)	1.0
Diagnostic concordance, maternal contamination excluded, % (*n*/*N*)	79.5 (62/78)	76.9 (60/78)	0.847
Full concordance, mosaicism excluded, % (*n*/*N*)	66.1 (41/62)	65.0 (39/60)	1.0

Diagnostic discordance: the result from the trophectoderm biopsy and the SCM were euploid–aneuploid; full concordance: the result from the trophectoderm and the SCM were exactly the same; partial concordance: the result from the trophectoderm biopsy and the SCM were aneuploid and matched for some aneuploidies; complementary concordance: the result from the trophectoderm biopsy and the SCM were aneuploid and the aneuploidies were complementary.

niPGT-A, non-invasive preimplantation genetic testing for aneuploidies; SCM, spent embryo culture medium.

[a] Only informative results from trophectoderm biopsy and SCM were included in the calculations.

All oocytes were fertilized by intracytoplasmic sperm injection (ICSI) using standard IVF protocols. Embryos were cultured in continuous media (Global Total LP, CooperSurgical, USA) in 50 µl microdrops until day 3. Zona pellucida opening by assisted hatching was carried out on day 3. Embryos were then washed three times in Global Total LP medium and moved to fresh 20 µl drops of Global Total LP medium until embryo biopsy. Trophectoderm biopsy was performed on day 5 (*n* = 53) or day 6 (*n* = 39) on good-quality expanded blastocysts with trophectoderm grades A or B and a visible ICM, using a 200 mW diode laser (Saturn; Research Instruments, UK and Hamilton Thorne, USA). The biopsied cells were transferred to polymerase chain reaction (PCR) tubes with 1 µl of phosphate-buffered saline (PBS). PGT-A of the trophectoderm biopsies was performed using Veriseq (Illumina, USA). Embryos were transferred to individual droplets in different dishes until vitrification (Vitrification Freeze Kit (Vit Kit – Freeze; Irvine Scientific, USA). Embryos identified as euploid by the trophectoderm biopsy were warmed and transferred in the subsequent cycle.

SCM collection and chromosomal analyses

SCM collection was carried out before trophectoderm biopsy, once the embryo had been moved to a new plate for the biopsy. Volumes of 15 µl of blastocyst medium from each embryo were transferred into RNAase-DNAse-free PCR tubes. Two aliquots were separated out, one for each technique used for chromosomal analysis. As a negative control, unconditioned culture medium was collected. All collected samples were immediately frozen and stored at –80°C until used for the niPGT-A assay.

Two methods were used for the chromosomal analysis of genetic material from the culture medium: NICS (Yikon Genomics, China) and Veriseq) using the multiple annealing and looping-based amplification cycle (MALBAC) and SurePlex (PicoPlex) methods of single-cell whole genome amplification (WGA), respectively, following the manufacturer's protocols. Sequencing was performed using a MiSeq (Illumina) sequencer. ChromoGo (Yikon Genomics, China) and BlueFuse Multi software (Illumina) were used for chromosomal analyses. Embryos were reported as mosaic if the analysed sample contained over 25% but less than 50% of aneuploid cells.

Donated blastocysts

Directly after warming, donated blastocysts were placed in 20 µl microdrops of Global Total LP supplemented with human serum albumin until full expansion of the blastocyst. Trophectoderm and ICM were separated using laser pulses and the flicking technique, in line with the trophectoderm biopsy technique. Trophectoderm and ICM samples were transferred into RNAase-DNAse free PCR tubes containing 1 µl of PBS. All samples were frozen immediately after collection and stored at –80°C until analysed.

Statistical analysis

Diagnostic rates were estimated by considering the successfully amplified samples in relation to the total number of analysed samples for trophectoderm biopsies and SCM. When assessing the concordance between SCM and trophectoderm, the results were independently compared at the chromosomal level, looking at autosomal and sex chromosomes. Full chromosome concordance was defined when the results for trophectoderm and SCM were exactly the same. Diagnostic concordance was identified when the result from the trophectoderm biopsy and the SCM were euploid–euploid or aneuploid–aneuploid from the two sample types. Results between samples were classified as discordant where no concordance with regard to chromosomal composition was identified. Positive predictive value (PPV), negative predictive value (NPV), sensitivity and specificity were calculated for euploidy versus aneuploidy. Categorical variables were presented as percentages.

Diagnostic concordance without maternal contamination was calculated by excluding samples in which maternal

TABLE 2 COMPARISON OF ACCURACY OF NIPGT-A USING NICS (YIKON) OR VERISEQ (ILLUMINA) VERSUS TROPHECTODERM BIOPSY FOR PGT-A ACCORDING TO THE DAY OF EMBRYO BIOPSY

Parameter	niPGT-A NICS			niPGT-A Veriseq		
	Day 5 (*n* = 46)	Day 6 (*n* = 37)	*P*-value	Day 5 (*n* = 46)	Day 6 (*n* = 37)	*P*-value
PPV	68.2	88.9	0.156	66.7	82.8	0.305
NPV	54.2	100	0.030*	54.5	100	0.062
Sensitivity	57.7	100	0.001*	61.5	100	0.003*
Specificity (%)	65.0	77.0	0.737	60.0	61.5	1.000
Diagnostic concordance (%)	60.9	92.0	0.003*	60.9	86.5	0.020*
Full concordance (%)	53.6	38.2	0.345	39.3	43.7	0.931
Partial concordance (%)	3.6	35.3	0.007*	3.5	37.5	0.005*
Complementary concordance (%)	10.7	0	0.177	7.1	0	0.418
Diagnostic concordance, maternal contamination excluded (%)	68.3	92.0	0.023*	68.3	86.5	0.104
Full concordance, mosaicism excluded (%)	75.0	58.8	0.198	71.4	59.4	0.484

Only 83 SCM analyses instead of 92 (the total) were included because the non-informative results were discarded.

Diagnostic discordance: the result from the trophectoderm biopsy and the SCM were euploid–aneuploid; full concordance: the result from the trophectoderm and the SCM were exactly the same; partial concordance: the result from the trophectoderm biopsy and the SCM were aneuploid and matched for some aneuploidies; complementary concordance: the result from the trophectoderm biopsy and the SCM were aneuploid and the aneuploidies were complementary.

niPGT-A, non-invasive preimplantation genetic testing for aneuploidies; SCM, spent embryo culture medium.

contamination was identified. Pearson's chi-squared test was used to determine significant differences between groups, with $P < 0.05$ representing significance. All statistical analyses were performed using Statistical Package for the Social Sciences software (IBM SPSS Statistics for Windows, version 20.0; IBM, USA).

RESULTS

From the SCM analysis, information on 24-chromosome ploidy (rate of informativeness) was successfully gathered from 92.4% (85/92) of samples regardless of the method used, in comparison to 96.8% of trophectoderm biopsies. One out of three non-informative (i.e. no result was obtained) trophectoderm biopsies remained so after SCM analysis. Full results are reported in Supplementary Table 1. Comparisons between the two SCM techniques showed 95.2% consistency in the diagnosis between NICS and Veriseq for niPGT-A analyses. No DNA was measurable in any of the amplified blank samples cultured under identical conditions in the two SCM techniques used. Mosaicism was detected in 14.1% of trophectoderm biopsies but in a greater proportion of SCM samples regardless of the technique used: 30.4% in SCM using NICS ($P = 0.013$) and 28.3% in SCM using Veriseq ($P = 0.031$) (TABLE 1).

Regarding diagnostic concordance (euploid trophectoderm/euploid SCM versus aneuploid trophectoderm/aneuploid SCM) between each SCM technique and trophectoderm biopsy, values of 74.7% for SCM using NICS versus 72.3% for SCM using Veriseq were obtained. Using trophectoderm biopsy, 37 embryos were identified as euploid and 33 results were informative on both SCM techniques; 23 of these showed concordance with SCM when analysed using NICS and 20 showed concordance when analysed using Veriseq. The remaining 10 and 13 samples showed chromosomal abnormalities (false-positive results), giving a specificity of 69.7% and 60.6%, respectively (TABLE 1). Out of the 50 embryos that were identified by trophectoderm biopsy as having chromosomal abnormalities, 39 presented chromosomal abnormalities with niPGT-A by NICS and 40 by Veriseq as well, but the remaining (11 by NICS and 10 by Veriseq) were identified as having a normal karyotype with niPGT-A (false-negative results), resulting in a sensitivity of 78.0% and 80.0% respectively (TABLE 1). The PPV and NPV of niPGT-A for identifying chromosomal abnormalities were 79.6 and 67.7 for SCM analysed using NICS, and 75.5 and 66.7 for SCM analysed using Veriseq (TABLE 1).

Overall, analysing concordance for the full chromosome set, the cytogenetic results were exactly the same as for the trophectoderm biopsy in 45.2% of SCM samples using NICS and 41.7% of SCM samples using Veriseq (TABLE 1). The overall concordance for autosomal chromosomes between the trophectoderm biopsy and the SCM samples was similar for the two techniques: 54.2% for SCM using NICS, and 53.0% for SCM using Veriseq. As for the sex chromosomes, the concordance rates were 86.6% for SCM using NICS, and 79.5% for SCM using Veriseq. Moreover, in 20.1% of SCM samples analysed using NICS and 23.3% SCM using Veriseq, the results were discordant only in the mosaicism diagnosis (data not shown). The rest of discordances were: (i) partial, in which at least one abnormal chromosome was diagnosed in both samples, but other unshared aneuploidies were also detected (22.6% for SCM using NICS versus 23.3% for SCM using Veriseq); and (ii) complementary in terms of loss versus gain of chromosomes (4.8% for SCM using NICS versus 3.3% for SCM using Veriseq) (TABLE 1).

When the day of the embryo biopsy was considered, the diagnostic concordance rates for SCM reached 92.0% for NICS and 86.5% for Veriseq when embryos were biopsied on day 6, versus 60.9% on day 5 biopsied embryos for the two techniques ($P = 0.003$, $P = 0.020$). The PPV and NPV for niPGT-A for embryos biopsied on day 6 were 88.9 and 100 for NICS, and 82.8 and 100 for Veriseq. The difference in NPV according to the day of embryo biopsy was significant for the SCM samples analysed by NICS ($P = 0.030$) and close to significance

TABLE 3 DISCORDANT RESULTS BETWEEN TROPHECTODERM BIOPSY AND SCM ANALYSES

Embryo ID	Trophectoderm biopsy	niPGT-A NICS (Yikon)	niPGT-A Veriseq (Illumina)	Trophectoderm re-biopsy	ICM re-biopsy	Cause of niPGT-A discrepancy
2	48,XX,+16,+20,+21,–22	46,XX	46,XX	48,XX,+16,+20,+21,–22	48,XX,+16,+20, +21,–22	Maternal contamination
4	47,XX,+20	46,XX, mos –20 [40%]	46,XX, mos –20 [40%]	47,XX,+20	47,XX,+20	Mosaic embryo
24	46,XX,+4p(pter→16.3 ~46M, × 3),–4q(35.2→ qter~139M, × 1)	46,XX	46,XX	46,XX,–4q(35.2→ qter~139M, × 1)	46,XX	Mosaic embryo
32	46,XY,–10q(26.3→ qter~39M, × 1)	46,XX	46,XX	46,XY	46,XY	Maternal contamination
33	47,XX,+15	46,XX	46,XX	47,XX,+15	47,XX,+15	Maternal contamination
41	44,XX,–4,–10	46,XX	46,XX	44,XX,–4,–10	44,XX,–4,–10	Maternal contamination
45	47,XX,+21	46,XX,mos+21[40%]	47,XX,+21	47,XX,+21	47,XX,+21	NICS low resolution
101	47,XX,+9	46,XX,mos+9q[40%]	46,XX,mos+9q[40%]	47,XX,+9	47,XX,+9	NICS and Veriseq low resolution and/or maternal contamination
134	47,XY+16	46,XX	46,XX	47,XY+16	47,XY+16	Maternal contamination

Embryo ID number is not correlative because some SCM were excluded as these patients did not sign the informed consent form.

ICM, inner cell mass; niPGT-A, non-invasive preimplantation genetic testing for aneuploidies; SCM, spent embryo culture medium.

for SCM analysed using Veriseq (P = 0.062). However, no significant differences in PPV were reported for the two SCM techniques and the day of embryo biopsy. The sensitivity for the SCM analyses performed using the two techniques was 100% for embryos biopsied on day 6 (TABLE 2).

To identify the cause of the discrepancies, trophectoderm-aneuploid donated embryos were reanalysed. The whole embryo was re-biopsied, separating the ICM and trophectoderm. Comparisons were performed between SCM analysis and the trophectoderm and ICM. The results are summarized in TABLE 3, showing that 55.6% (5/9) of the discrepancies were due to DNA contamination (maternal origin), 22.2% (2/9) to embryo mosaicism, 11.1% (1/9) to low resolution in SCM-NICS and 11.1% to low resolution in both techniques used for genetic analysis of the SCM.

In terms of IVF outcome, based on the trophectoderm results there were 37 euploid embryos among the 92 embryos that had PGT-A results. Fifteen of these were warmed and transferred, resulting in three live births, three ongoing pregnancies (pregnancies having completed ≥20 weeks of gestation) and one miscarriage (spontaneous loss of pregnancy before the 20th week). Thirteen of the euploid embryos had a result from the niPGT-A, and IVF outcome was analysed according to the chromosomal genetic technique. The clinical pregnancy rate was 55.5% for trophectoderm-euploid/NICS-euploid versus 57.1% for trophectoderm-euploid/Veriseq-euploid embryos (TABLE 4). Moreover, no significant differences were reported when the pregnancy rate was compared between the two techniques for discordant trophectoderm and SCM results (TABLE 4). The miscarriage rate was 0% for trophectoderm-euploid/NICS-aneuploid versus 33.3% for trophectoderm-euploid/Veriseq-aneuploid embryos, and the ongoing pregnancy rate was 44.4% for trophectoderm-euploid/NICS-aneuploid versus 33.3% for trophectoderm-euploid/Veriseq-aneuploid embryos. Comparing the two methods of SCM chromosomal analysis, Veriseq seems to be more predictive of IVF outcome, but, because of the low sample size, the differences were not significant.

DISCUSSION

This study reported for the first time the consistency between two different chromosomal analyses techniques for niPGT-A, which differ mainly in

TABLE 4 CLINICAL OUTCOME AFTER TROPHECTODERM-EUPLOID SINGLE-EMBRYO TRANSFER (N = 13)

Parameter	Trophectoderm- euploid/ NICS-euploid	Trophectoderm-euploid/ Veriseq-euploid	P-value	Trophectoderm-euploid/ NICS-aneuploid	Trophectoderm-euploid/ Veriseq-aneuploid	P-value
No. of of transfers	9	7	–	4	6	–
Female age, years (mean + SD)	32 ± 7.8	31.9 ± 8.8	0.973	35.0 ± 5.2	34.2 ± 4.8	0.799
Positive β-HCG rate (%)	55.5	57.1	0.951	50.0	50.0	1.0
Clinical pregnancy rate (%)[a]	55.5	57.1	0.951	50.0	50.0	1.0
Clinical miscarriage rate(%)[b]	20	0	0.371	0	33.3	0.414
Ongoing pregnancy rate (%)[c]	44.4	57.1	0.626	50.0	33.3	0.617

HCG, human chorionic gonadotrophin.

[a] Clinical pregnancy was defined when the pregnancy was confirmed by ultrasound visualization of a gestational sac with a heartbeat.

[b] Clinical miscarriage was defined as the spontaneous loss of a pregnancy before the 20th week.

[c] Ongoing pregnancy was defined when the pregnancy had completed ≥20 weeks of gestation.

the WGA technique used. The two techniques showed similar results in terms of PPV, NPV, sensitivity and specificity when compared with PGT-A using trophectoderm biopsy. In addition, diagnostic, full, partial and complementary concordances rates were equivalent. Moreover, this study agrees with previous work showing higher concordance rates in embryos biopsied on day 6 versus day 5. The analysis of discrepancies reported that maternal DNA contamination and embryo mosaicism are the main factors limiting the accuracy of niPGT-A, rather than this being because of the chromosomal analysis technique. This study advances another step towards optimizing and improving the accuracy of niPGT-A.

In clinical IVF, PGT-A using trophectoderm biopsy is, due to its relatively low invasiveness, currently the most widely used genetic test for identifying aneuploidies in embryos. However, the potential damage, which might compromise implantation potential, as well as potential issues relating to long-term effects on the offspring are very difficult to measure. In addition, trophectoderm biopsy requires experienced embryologists to perform the embryo manipulation, as well as specialized equipment, increasing the costs of performing PGT-A. The presence of DNA in the embryo culture media (*Stigliani et al., 2014*), and also in the blastocoel fluid (*Palini et al., 2013*), has opened up the possibility of niPGT-A. niPGT-A of embryo culture medium is less invasive, simpler and safer than collection of blastocoel fluid. It also does not require special expertise and can therefore potentially be used routinely in clinical practice. Another advantage of using SCM instead of trophectoderm biopsy is its usefulness in evaluating blastocysts of poor quality. When performing trophectoderm biopsy, it is not unusual to discard a poor-quality blastocyst because, when these blastocysts are biopsied, their PGT-A results often report aneuploidy or no result due to the suboptimal quantity and quality of the cells biopsied. Therefore, SCM could theoretically offer genetic information that clinicians might otherwise be unable to obtain.

The concordance between PGT-A and niPGT-A has already been reported and has shown variable results (*Ho et al., 2018*; *Vera-Rodriguez et al., 2018*; *Xu et al., 2016*). Moreover, a recent multicentre study showed a concordance rate ranging from 72.5% to 86.3% between centres, without statistical significance (*Rubio et al., 2020*). The cell-free DNA in the SCM is likely to be very low in amount and/or degraded (*Galluzzi et al., 2015*). A low concentration and poor integrity of DNA are associated with a lack of sensitivity of molecular methods and seem to be more successfully overcome by some amplification and genetic analysis techniques than others. The overall requirement is to distinguish real genetic changes from noise that is introduced by the technology, especially during the WGA step.

In the current study, a comparison was made between the SurePlex DNA amplification method, included in the Veriseq kit, and the MALBAC kit, used in the NICS kit. The two methods are considered quasi-linear methods that include a 'linear' phase followed by a limited number of PCR-based cycles, allowing a robust approach to detect copy-number variation. In a previous study comparing the two methods, the MALBAC system showed a higher false-positive rate than the SurePlex technique. Losses, gains and unbalanced chromosomal structural alterations larger than 10 Mb are better detected by SurePlex because the genome amplification achieved using this method is more uniform (*Deleye et al., 2015*). However, MALBAC provides higher sensitivity to background contamination thanks to a smaller proportion of reads that map to the target genome (*de Bourcy et al., 2014*). Previous studies for developing niPGT-A have used these techniques independently. Xu and colleagues validated an niPGT-A method based on SCM by using MALBAC for WGA and obtained ploidy information for all 24 chromosomes (*Xu et al., 2016)*. Recently, niPGT-A on SCM used for the first time SurePlex with no modification to the amplification cycles in WGA (*Yeung et al., 2019*). So far, no study has compared the superiority of one method over another. The results reported here show that both techniques demonstrated similar rates for informativeness and consistency when comparing SCM with the trophectoderm biopsy result for the same embryo. For both techniques the rates of informativeness were high (92%.4 for both SCM-NICS and SCM-Veriseq) and clinically acceptable, as fewer than 10% of embryos remained undiagnosed. The current study included freshly cultured embryos, avoiding the limitation of previous studies, which used frozen-thawed embryos reporting similar results: the rate of apoptosis is higher in frozen and thawed embryos than fresh embryos, which could potentially increase the quantity of cell-free DNA in the SCM and allow for higher rates of informativeness.

Up until now, trophectoderm results have been considered to be the gold standard for genetic diagnosis in embryos. Thus, all the niPGT-A performance indices were calculated with this assumption in mind. In terms of the accuracy of niPGT-A, the sensitivity of the SCM analyses for the two techniques (78.0 for NICS and 80.0 for Veriseq) were higher than the specificity (69.7 for NICS and 60.6 for Veriseq). The high sensitivity suggests that the method is more effective in identifying embryos with chromosomal abnormalities than in selecting normal and transferrable embryos. Therefore, the transfer of euploid embryos identified by niPGT-A has a low risk of an aneuploid pregnancy. However, discarding aneuploid embryos as a result of niPGT-A might reduce the number of embryos available for transfer. NiPGT-A using SCM might be improved to reach similar efficiency to PGT-A in classifying euploid embryos and potentially increasing live birth rates. Interestingly, this study found that SCM analyses of embryos biopsied on day 6 carried a higher sensitivity than those biopsied on day 5 regardless of the technique used for the genetic analysis. This might be directly associated with the longer time during which the embryo could release DNA into the medium. Moreover, the diagnostic concordance between SCM analysis and trophectoderm biopsy was higher in embryos biopsied on day 6 than day 5. A previous study showed that it could increase the concentration of embryonic DNA in the media, reduce the contamination from maternal sources, presumably cumulus cells, and reduce the likelihood of detecting polar body DNA (*Lane et al., 2017*). The findings of the current study agree with those of previous studies reporting improved results when analysing SCM from later stages of embryo development (*Babariya et al., 2019*; *Lane et al., 2017*; *Rubio et al., 2019*).

Some studies have reported different strategies to improve the results of niPGT-A. Collapsing the embryo prior to collection of the medium would increase

the diagnostic concordance between niPGT-A and trophectoderm biopsy (*Kuznyetsov et al., 2018*). Collapsing the embryo could thus be considered as a tool to improve the accuracy of niPGT-A. However, niPGT-A, if clinically applicable, would not require extra work in addition to the current laboratory routine other than collecting spent medium. Another approach to improving the accuracy of niPGT-A has been described by Jiao and colleagues (*Jiao et al., 2019*). This study proposed the new possibility of using BCM blastocoel fluid and spent blastocyst medium, which raises the possibility that the detection rate could be improved by mixing blastocoel fluid and spent medium. The concordance rate between BCM blastocoel fluid and spent blastocyst medium and embryo biopsies was 90%. However, as previously mentioned, this approach requires embryo manipulation and additional intervention on top of current laboratory protocols.

WGA techniques are more susceptible to contamination with exogenous DNA, giving false-negative results. In order to detect DNA contamination, negative controls were also collected (blank media from the same dish under the same culture conditions). The lack of DNA amplification in negative controls provided a strong suggestion that the contamination did not originate from the laboratory environment, the staff or the manufacturing of the media. In the current study, no evidence of DNA contamination was detected for either technique spent culture medium.

In order to identify the cause of diagnostic discordances, next-generation sequencing was performed on nine donated trophectoderm-aneuploid embryos to confirm the discordant results between trophectoderm and SCM analysis. By using the whole embryo to take one biopsy from the trophectoderm alone and one biopsy from the ICM, it was found that only 1 of the 9 embryos (37.5%) had inconsistent result between biopsy sites within the same embryo. The trophectoderm biopsy diagnosis was a segmental aneuploidy, and it has been reported that the concordance rate between ICM and trophectoderm in embryos diagnosed as having segmental aneuploidies is lower than in those with complete aneuploidies (*Victor et al., 2019*). From these data, it could be suggested that the false-negative results might have resulted from maternal contamination from the cumulus cells and embryo mosaicism. Therefore, the false-negative rate could be further reduced by carefully removing all cumulus cells before embryo culture. Moreover, culture medium replacement and extra embryo washes may decrease the probability of contamination from remaining cumulus cells**.** This limitation has been the major difficulty for most studies trying to use niPGT-A as an embryo selection tool (*Ho et al., 2018*; *Vera-Rodriguez et al., 2018*; *Xu et al., 2016*).

Despite all measures taken to avoid contamination (ICSI for fertilization, additional refreshes of medium and sterile tips), some cumulus cells might remain in the after oocyte denudation, and slow degeneration of the two polar bodies confined inside the zona pellucida might release maternal DNA (*Hammond et al., 2016*). Therefore, maternal DNA contamination would be associated with decreased sensitivity values. On the other hand, the false-positive results are most probably due to embryo mosaicism. Previous studies have suggested that, during embryo development, embryos can eliminate aneuploid cells to outside the embryo (*Taylor et al., 2014*). It is therefore possible that the cells eliminated from the embryo into the culture medium will produce false-positive results. Moreover, inadequate amplification of degraded DNA, as is expected to be present in the SCM, has the potential to produce noisy next-generation sequencing results and subsequent diagnostic errors.

To distinguish true embryo aneuploidy from the noise associated with WGA, a threshold for mosaicism has to be carefully selected. One recent piece of research reported that setting a threshold of mosaicism of 60% allows improved efficacy of niPGT-A compared with previous reports (*Huang et al., 2019*). The current study use the same mosaicism cut-off for the trophectoderm and SCM analyses, reporting a higher mosaicism rate in SCM than in trophectoderm biopsies. This result is in line with the previous work, and modifying the threshold could increase the accuracy. However, in order to avoid any bias and for a valid comparison of the different chromosomal techniques, it was decided that the same protocol would be used and the same threshold maintained for the SCM and trophectoderm analyses.

Clinical outcomes were compared retrospectively according to the SCM results on a group of patients after single-embryo transfer performed according to the trophectoderm biopsy results. Interestingly, ongoing pregnancy rates were higher when both trophectoderm and SCM results were given as euploid using Veriseq as the SCM technique, suggesting that Veriseq could have a higher prognostic value for IVF outcome than NICS. The low sample size here means that confirmatory research is needed, but this observation is nevertheless worthy of further investigation.

Current knowledge on the origin of DNA in embryo culture medium is limited. There are four sources: (i) maternal DNA from the polar bodies is very unlikely as these undergo apoptosis within 24 h (*Schmerler and Wessel, 2011*); (ii) the presence of maternal DNA originating from the cumulus cells could be minimized by removing all these cells in preparation for ICSI; and embryonic DNA could originate from either (iii) euploid or (iv) aneuploid apoptotic cells. Certain cells undergo apoptosis throughout the developing embryo *in vitro* (*Gahan and Swaminathan, 2008*) and therefore release DNA into the medium. As cells in both the ICM and the trophectoderm experience apoptosis during preimplantation embryo development, the DNA in SCM is expected to originate from both of these cell lines. Moreover, these apoptotic events increase exponentially in line with the total cell number (*Hardy et al., 1989*).

A recent study showed that a high proportion of cells showing aneuploidy undergo apoptosis and are removed from the embryo (*Zhu et al., 2018*). However, euploid cells could similarly undergo apoptosis and be excluded into the medium. Therefore, niPGT-A would be less accurate for diagnosing euploid embryos. Moreover, Bolton and colleagues showed in an animal model that cells from the ICM and the trophectoderm could be affected by this process (*Bolton et al., 2016*). They also reported that the percentage of chromosomically normal and abnormal cells that became apoptotic differed between the ICM and trophectoderm depending on their chromosomal constitution: aneuploid (41.4% versus 3.3%) or euploid (19.5% versus 0.6%).

For all of the above reasons, the most plausible source of cell-free DNA in spent medium might be apoptosis. Over the last decade, non-invasive prenatal testing for fetal aneuploidy has been developed and globally adopted in routine clinical protocols (*Shaw et al., 2020*) based on a similar origin of DNA from the apoptosis and release of cell-free DNA from the placenta into the maternal circulation (*Heitzer et al., 2020*). Therefore, SCM analyses might be useful for the analogous development of niPGT-A and might achieve better results in reflecting the ploidy status of the embryo.

The current study has some limitations, and larger prospective studies are warranted to draw definite conclusions on the accuracy of niPGT-A in representing the genetic constitution of the whole embryo. In addition, randomized clinical trials are needed to report the potential of niPGT-A to improve pregnancy outcomes. Moreover, to exactly determine the effect of maternal contamination on the concordance results, genotyping analysis is needed to reveal the real origin of SCM cell-free DNA, because for a 46,XX karyotype it would not be possible to differentiate between full concordance or maternal contamination.

In conclusion, the diagnostic and concordance rates are similar between the two genetic techniques used for niPGTA, suggesting that discordances may be due to embryo mosaicism or DNA contamination rather than technical limitations. Optimization of culture conditions and medium retrieval could improve the reliability of niPGTA. More studies are needed with new approaches to minimize maternal DNA contamination and to understand embryo mosaicism.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.rbmo.2020.10.021.

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