Noninvasive preimplantation genetic testing using the embryo spent culture medium: an update

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Purpose of review
The presence of cell-free DNA (cf-DNA) in the embryo spent culture medium allows to develop a noninvasive PGT-A (niPGTA). Noninvasive PGT-A may provide a simpler, safer and less costly approach to preimplantation genetic testing of aneuploidy (PGT-A). Furthermore, niPGTA would provide wider access to embryo genetic analysis and circumvent many legal and ethical considerations. However, the concordance rate between the results obtained by PGT-A and niPGTA varies among studies and, their clinical utility has not been already demonstrated. This review evaluates the niPGTA reliability based on SCM and adds new knowledge about the clinical relevance of SCM for noninvasive PGT-A.

Recent findings
The most recent concordance studies evaluating the accuracy of niPGTA using SCM showed a high variation in the informativity rate of SCM and the diagnostic concordance. Also, sensitivity and specificity showed similar heterogeneous results. Therefore, these results do not support the clinical utility of niPGTA. Regarding clinical outcome, the data are initial and further research, including randomized and nonselection studies are needed.

Summary
Further research, including randomized and nonselection studies, as well as optimization of embryo culture conditions and medium retrieval, are needed to improve the reliability and clinical utility of niPGTA.

Keywords
cell-free DNA, concordance rates, noninvasive preimplantation genetic testing for aneuploidies

INTRODUCTION

Chromosomal abnormalities are very frequent in human embryos and can be identified during IVF by performing preimplantation genetic testing for aneuploidies (PGT-A). This requires invasive embryo biopsy techniques. Although recent studies have reported the biopsy does not have any effect on the embryo [1] implantation potential, some factors related to embryo biopsy are technically challenging. Cell-free DNA (cf-DNA) has been found in spent culture media (SCM) [2], which has led to develop noninvasive PGT-A (niPGTA). Variable success and concordance rates between niPGTA and trophectoderm biopsy have been reported [3]. Discordances seem related to the DNA's low quantity and quality, embryonic mosaicism, favoured elimination of aneuploid cells, DNA contamination and the method used for genetic analysis. Thus the niPGTA clinical efficacy remains unknown and is challenging. The aim of this review is to report recent findings regarding noninvasive chromosomal genetic assessment of preimplantation embryos.

ORIGIN OF DNA IN SPENT CULTURE MEDIA

Cell-free genomic DNA (gDNA) in SCM has been detected as early as days 2–3 [4], and the amount of DNA increases during embryo culture, suggesting an embryonic origin [5]. The embryonic DNA-release mechanisms remain unclear. There are different possible sources: maternal DNA originating from the cumulus cells and embryonic DNA from either euploid or aneuploid apoptotic cells. Certain cells undergo apoptosis throughout the developing embryo *in vitro* and, therefore, release DNA into the medium. These apoptotic events increase

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KEY POINTS

- Cell-free genomic DNA (gDNA) in SCM is of embryonic origin and allows the development of niPGTA.
- Mechanisms underlying release of embryonic DNA remain unclear.
- The most recent studies in niPGTA continue to show discrepancies in the main outcomes regarding test accuracy.
- Clinical studies showed good results in pregnancy outcome and healthy babies born; however, further research is needed.
- The current literature does not support the clinical utility of niPGTA.

exponentially in line with the total cell number [6]. Noninvasive prenatal testing for foetal aneuploidy has been adopted in routine clinical protocols [7] based on a similar origin of DNA from the apoptosis and release of the placental cell-free DNA into maternal circulation [8]. Therefore, SCM analyses might be useful for the analogous development of niPGTA.

CONCORDANCE STUDIES

The concordance studies evaluate the accuracy of niPGTA when comparing the results obtained between the trophoectoderm biopsy, using current techniques, or whole embryo analysis and niPGTA using SCM. The main outcomes in these studies were the informativity rate of SCM, defined as the percentage of SCM samples with successful diagnosis, and the diagnostic concordance rate or the overall agreement between the SCM and the reference sample considering them euploid or aneuploid. Full chromosome concordance was defined when the results for trophoectoderm biopsy and SCM were exactly the same. Partial concordance was defined when the chromosomal status for some chromosomes differed between trophoectoderm biopsy and SCM but they were both aneuploidy. Results between samples were classified as discordant where no concordance with regard to chromosomal composition was identified. Other relevant parameters were the sensitivity and specificity and the percentage of false-positives and false-negatives.

Table 1 shows the most recent concordance studies. The sample size ranges from 11 to 283 samples. Gamete fertilization was performed mainly by intracytoplasmic sperm injection (ICSI) except in the case of Tsai's and Xie's study where ICSI and IVF was used for fertilization. The characteristics of the embryos associated to each SCM sample also were heterogeneous. Specifically, 66.7% of studies evaluated cf-DNA released from fresh embryos and 33.3% (2/6) of studies assessed cf-DNA secreted from frozen/thawed embryos. The release of cf-DNA in SCM was evaluated throughout blastocyst stage. The SCM-embryo incubation time varied from 6 to 72 h. The main genetic technique used was NICSInst from Yikon company, and although a previous study showed consistent results of niPGTA using different techniques for chromosomal analysis [9], the information about what technique had been used should be considered. Trophectoderm biopsy was typically chosen as the reference sample against which to compare SCM. In these cases, the studies showed that the informativity rate of SCM and the diagnostic concordance rate varied, ranging from 62.7 to 96.6% and from 62.5 to 88.9%, respectively [10▪,11▪▪–14▪▪,15▪]. In some studies, the whole embryo was also considered as the reference for comparison, not only the trophectoderm, obtaining higher results for the informativity rate (from 78.7 to 96.6%) and diagnostic concordance rate (from 78.1 to 93.8%). The better results from the studies using the whole embryo as gold standard add knowledge insights into the origin of the DNA molecules in culture medium. The study performed by Chen *et al.* [12▪▪] showed the highest informativity rates for both trophoectoderm and whole embryo references, 96.6%; this NICS validation test included one of the largest set of embryos. The embryos were poor-quality embryos not eligible for transfer. This fact could explain why although the informativity rates were the highest, the diagnostic concordance was lower than other studies. Poor-quality embryos may have higher incidence of debris and DNA fragmentation compared with the embryos eligible for transfer, which may contribute to the increase in DNA release but low accurate result. On the other hand, Hanson and co-workers reported the lowest informativity rate (62.7%) with a discrete concordance diagnostic rate, 75%. The greater media volume may explain these results. As a larger volume was used, the DNA was less concentrate, therefore, the embryos required prolonged exposure to the culture medium for the DNA amplification to be successful.

The metrics of sensitivity, specificity and false-positive and false-negative rates are often considered measures of diagnostic accuracy because they provide information on dichotomous tests, distinguishing between euploidy and aneuploidy blastocysts. Sensitivity and specificity range from 36.8 to 100% and 57.9 to 88.9%, respectively, in studies using trophoectoderm as reference. As for studies

Table 1. Summary of studies comparing results between spent culture medium and trophoectoderm biopsy or whole embryo

Study	Number of samples	Reference	Informativity rate % (*n*/*N*)	Concordance % (n/N)			Sensitivity (%)	Specificity (%)	False-positives (%)	False-negatives (%)	Drop volume (μl)	Time in culture	WGA method	Genetic technique
				Diagnostic	Full	Partial								
Shitara *et al.* (2021)	20	TE	95 (19/20)	88.9 (16/18)	55.6 (10/18)	2.2 (4/18)	80.0	88.9	5.6	5.6	–	Cultured for 24 h for D5 3 h for D6 blastocysts after warming	SurePlex (Illumina)	Veriseq (Illumina)
	20	WE	95 (19/20)	93.8 (15/16)	56.3 (9/16)	31.2 (5/16)	100	87.5	12.5	22.2	–			
Hanson *et al.* (2021)	166	TE	62.7 (104/166)	75 (78/104)	59.6 (62/104)	15.4 (16/104)	49.5	62.5	19.2	5.7	30	D3 or D4 to D5/6/7	MALBAC (Yikon)	NICSInst (Yikon)
Chen *et al.* (2021)	265	TE	96.6 (256/265)	72.2 (185/256)	70.3 (180/256)	1.9 (5/256)	76.2	73.2	10.5	14.8	20–25	D3 to D5/6	MALBAC (Yikon)	NICSInst (Yikon)
	265	WE	96.6 (256/265)	78.1 (200/256)	75.4 (193/256)	2.4 (7/256)	86.5	73.1	16.8	5.1				
Yin *et al.* (2021)	75	WE	78.7 (59/75)	89.8 (53/59)	32.2 (19/59)	57.6 (34/59)	100	45.5	10.2	0	25	Cultured for 24 h after warming	MALBAC (Yikon)	NICSInst (Yikon)
Tsai *et al.* (2022)	29	TE	82.8 (24/29)	62.5 (15/24)	33.3 (8/24)	12.5 (3/24)	36.8	80.0	4.2	29.2	25-30	D3 to D5/D6	MALBAC (Yikon)	NICSInst (Yikon)
	11	WE	63.6 (7/11)	85.7 (6/7)	0 (0/7)	28.6 (2/7)	55.6	33.3	0	16.7				
Xie *et al.* (2022)	27	TE-preclinical	96.3% (26/27)	69.2 (18/26)	42.3 (11/26)	57.7 (15/26)	100	57.9	30.8	0	25	D4–D5/6	MALBAC (Yikon)	NICSInst (Yikon)
	283	TE-clinical	93.6 (265/283)	73.97 (196/265)	35.8 (95/265)	38.1 (101/265)	82.1	59.4	19.6	5.2				

using whole embryo as gold standard for comparison, similar results were obtained from 55.6 to 100% and 45.5 to 87.5% for sensitivity and specificity, respectively. These values do not support the clinical utility of niPGTA. Regarding false-positive and false-negative rates, discrepancies were found among different studies. False-negative rate is thought to be partially attributable to maternal origin such as cumulus cells, which have a balanced chromosomal content and could lead to pregnancy with a chromosomal abnormality. This limitation has been the major difficulty for most studies trying to use niPGTA as an embryo selection tool. Otherwise, the false-positive results are most probably because of embryo mosaicism. 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. False-positive results might lead to discarding of many embryos with an entirely normal pregnancy potential.

Overall, the most recent studies in the niPGTA field continue to show discrepancies in the main outcomes regarding test accuracy. Contamination, DNA degradation, embryo mosaicism and different methodologies as embryo culture conditions (drop volume and time exposure), embryo manipulation or DNA genetic analysis could explain it. Consequently, a standardized protocol diminishing the contamination, decreasing the drop volume, prolonging embryo culturing and optimizing the genetic diagnosis are needed before including the noninvasive approach in the IVF routine.

CLINICAL RESULTS

PGT-A is widely used to select embryos having normal ploidy for transfer. Multiple clinical trials have demonstrated improved clinical outcomes with PGT-A [16]. In this respect, several groups have evaluated niPGTA with the transfer of euploid embryos, based only on SCM diagnosis, because the ultimate goal of the application of niPGTA may be to improve IVF outcome. Previously, pilot studies showed good results in pregnancy outcome and healthy babies born at least with rates comparable to those of PGT-A [2,17,18]. Most recently, new studies with higher sample size and different study design have been performed (Table 2).

The study published by Chen *et al.* [19▪▪] is the first large-scale retrospective study analysing the relationship between NICS results and clinical outcomes in frozen–thawed single blastocyst transfer. The study analysed retrospectively the data from 212 frozen–thawed single blastocyst transfers. The embryos were thawed and placed in incubation for

Table 2. Summary of studies comparing clinical outcomes

Reference	Study	Number of samples	Embryo transfer	Type of study	Study groups	Results
19	Chen *et al.* (2022)	212	Frozen embryo transfer	Nonselection	Aneuploid versus Euploid	The euploid group had a statistically significantly higher CPR (56.2 versus 29.4%), OPR (47.2 versus 22.1%,), and LBR (46.1 versus 22.1%, 6)
20	Gombs *et al.* (2021)	40	Fresh embryo transfer	Retrospective	Miscarriage versus healthy babies	Low cf-DNA in healthy babies
21	Chen *et al.* (2022)	345		Prospective observational blinded	Artificial intelligence algorithm classification: A: Euploid or low mosaic embryo B: Low-rate mosaic embryo C: High mosaic embryo or aneuploid	Higher LBR was observed in A versus C (50.4 versus 27.1%) and B versus C (45.3 versus 27.1%) and lower miscarriage rate in A versus C (15.9 versus 33.3%) and B versus C (14.3 versus 33.3).

CPR, clinical pregnancy rate; LBR, live birth rate; OPR, ongoing pregnancy rate.

6 h after warming. The transferred embryos were selected based on morphological criteria. Then SCM were collected for analysis by NICS. Ultimately, SCM of two embryos were not obtained, then 210 patients were included in the final analysis. The NICS informativity rate was 74.8%. Overall, the aneuploidy rate was 43.3%. The results showed that compared with the aneuploidy group, the euploid group had a statistically significantly higher clinical pregnancy rate [56.2 versus 29.4%, odds ratio (OR) 0.33, CI 0.15–0.72], ongoing pregnancy rate (47.2 versus 22.1%, OR 0.34, 95% CI 0.15– 0.77) and live birth rate (46.1 versus 22.1%, OR 0.39, 95% CI 0.18–0.86) but were nonsignificantly different from those in the chaotic abnormal embryos group. As a conclusion from this study, NICS combined with morphological evaluation can be clinically used to select blastocysts for transfer in frozen–thawed cycles. However, the main limitation of this approach is the need to refreeze embryos because of the time need for thawing and testing. Gombos *et al.* [20▪] published a retrospective study in 2021 in which they sampled SCM on day 3 to set up a comprehensive workflow, supporting a clinically applicable strategy for niPGTA that can be carried out within 48 h, which is critical for the same cycle blastocyst transfer and avoiding freezing. Only good-quality embryos were included in the study; the number of total transferred embryos was 514. The pregnancy rate was 34% leading to the live births of 83 healthy babies and 20 miscarriages. All SCM samples were used for the niPGTA analysis from the miscarriage group (group 0, $n = 20$) and matched with 20 SCM randomly selected from the transferred embryos that developed to healthy babies (group 1, $n = 20$). The cf-DNA in the SCM of the aborted embryos was found in higher copy numbers, whereas the low embryonic cf-DNA in SCM was consistent with a healthy pregnancy and live birth. Also, they found clinically significant autosomal ploidy alterations only among the aborted embryos (this affected 75% of them).

A recent study used random forest machine learning algorithm to predict blastocyst ploidy using 345 paired SCM and whole blastocyst samples [21▪▪]. The system was validated using a blinded prospective observational study in 266 patients. The authors investigated clinical outcomes between machine learning-guided and traditional niPGTA. The embryos were cultured to the blastocyst stage, previously medium was changed on day 4. Embryos were graded as A, B or C according to their predicted euploidy probability levels. Higher live birth rate was observed in A-grade versus C-grade embryos (50.4 versus 27.1%, $P = 0.006$) and B-grade versus C-grade embryos (45.3 versus 27.1%, $P = 0.022$) and lower miscarriage rate in A-grade versus C-grade embryos (15.9 versus 33.3%, $P = 0.026$) and B-grade versus C-grade embryos (14.3 versus 33.3%, $P = 0.021$). According to chromosomal composition, A-grade embryos were euploid, low mosaicism or small segmental aneuploidy; B-grade embryos showed a low rate of mosaicism; C-grade embryos had a high mosaicism proportion or a 100% aneuploid pattern. Therefore, embryos in group A have an euploid probability above 90% and should have the highest priority transfer.

Finally, an interesting, well designed study is ongoing by Huang and co-workers [22]. This is the first double-blinded multicentre randomized controlled trial comparing the ongoing pregnancy rate after embryo transfer selected by niPGTA versus conventional morphological evaluation, including 1148 couples in 13 different centres. Recruitment and randomization were performed when the couples had at least two blastocysts. The controlled ovarian hyperstimulation and IVF laboratory procedures performed through standard routine procedures according to each centre. On day 4, the embryos are individually transferred into a new 25 μl droplet. The blastocysts vitrified, and for the niPGTA group, 20 μl culture medium will be collected and tested. Blastocysts will be classified according to the euploid probabilities of at least 0.94 (a), 0.7–0.94 (b) and ≤0.7 (c) from a previous study [21▪▪]. The grading system had an area under the curve value of 0.92 and a negative-predictive value of 0.93. The last recruitment is estimated in December 2022. The results will add important clinical data for the niPGTA utility.

CONCLUSION

Conventional PGT-A is the gold standard for aneuploidy detection promoting IVF outcome improvement. However, this approach has some limitations that can be solved by niPGTA. The greatest advantage of noninvasive genetic testing is cost-effectiveness, avoiding embryo biopsy damage and provide wider access to embryo genetic analysis and circumvent many legal and ethical considerations. However, in the majority of cases, rates of concordance are relatively low and would likely be considered unacceptable for most clinical applications. Current literature indicates that discordance of genetic results obtained between SCM and embryo biopsy or whole embryos may be because of embryo mosaicism, the preferential elimination of aneuploid cells and/or DNA contamination. Regarding clinical data, the results are very preliminary. Further research, including randomized and nonselection studies, is needed to assess the clinical benefits of niPGTA.

In conclusion, currently, niPGTA should not be considered a substitute for trophectoderm biopsy, as it has not yet reached the accuracy needed for a diagnostic test. Optimization of culture conditions and medium retrieval with new approaches to minimize maternal DNA contamination could improve the reliability of niPGTA. Also, studies assessing the potential niPGTA of clinical utility of using SCM are needed.

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Conflicts of interest

There are no conflicts of interest.

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- ■ of special interest
- ■■ of outstanding interest

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