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Embryonic Cell-free DNA in Spent Culture Medium: A Non-invasive Tool for Aneuploidy Screening of the Corresponding Embryos

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Abstract. *Background/Aim: Preimplantation genetic testing (PGT) for chromosomal screening, based on embryo biopsy, has significant limitations. Cell-free DNA (cf-DNA) has been detected in spent culture medium (SCM), opening new horizons for the development of non-invasive PGT (ni-PGT). In this study, we evaluated the diagnostic performance of ni-PGT for aneuploidy (niPGT-A), comparing the results of trophectoderm biopsies (TE) and respective SCM from individually cultured embryos via Next Generation Sequencing (NGS). Materials and Methods: Forty fresh embryos were analyzed. TE and SCM from blastocysts were collected and analyzed. Results: We detected cfDNA in 100% of samples tested. The overall concordance rate between the ni-PGT-A and PGT-A was 27/33 (81.8%). The full concordance rate was 21/33 (63.6%). The aneuploidy agreement was 91.66%, and the euploidy agreement was 76.19%. Conclusion: We found a good accordance between TE and SCM analysis, suggesting that niPGT-A could be a reliable alternative for chromosomal abnormalities assessment of in vitro cultured embryos.*

The development of *in vitro* fertilization (IVF) over the last four decades has reached significant milestones in infertility treatment. Nowadays, IVF is the most successful and efficient approach to help infertile couples (1, 2). A key aspect in embryology, known since its introduction, is that different gametes have different potentials for successful growth, even in the most favorable environment. In the early days of IVF application, this knowledge led to the transfer of many embryos in each attempt, chosen by only morphological criteria (3, 4).

Multiple embryo transfers have an increased pregnancy rate, but this comes at a price of complications such as perinatal mortality, premature birth, *etc*. Across the world, various national legal frameworks have been established, stating that according to age, there is a restriction to how many embryos can be transferred to prevent multiple pregnancies and their complications. The international consensus is the selection and single transfer of the best possible healthy and viable embryo, which will lead to the birth of a living and healthy baby. One of the most critical dilemmas that all clinical embryologists have had is choosing the appropriate embryos for transfer.

Scientists are pursuing the goal of finding reliable embryo selection techniques and procedures. The different approaches to the best and most effective embryo selection technique or strategy can be divided into two broad categories, the non-invasive and the invasive. Invasive methods require the removal of specific cells from the embryo through biopsy to analyze its genetic composition.

Chromosomal makeup analysis of the embryo can serve as a prognostic factor of its implantation potential. Embryos with aneuploidy exhibit diminished potential of implanting and a lower pregnancy rate (5-8). The assessment of chromosomal status in each embryo can allow the selection of only euploid ones, significantly improving the results of *in vitro* fertilization (9). While this assumption has always been valid, the first attempts to examine fetal aneuploidy

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were insufficient (10, 11). At first, the molecular techniques that were applied were not accurate enough to be clinically relevant. More recently, molecular technology has constantly progressed in clinical sensitivity and specificity (12-15).

Preimplantation genetic testing (PGT) is an invasive procedure to acquire embryo DNA by removing some cells from the embryo through biopsy, but this has significant limitations. Embryo biopsy provides a "snapshot" in time of embryo DNA composition as embryo development occurs. Cells removal from day three embryos can dramatically affect their development (16, 17). Similarly, there is an inverse association between the number of cells derived from embryo biopsies and the likelihood of embryo implantation, especially in blastocysts with poor trophectoderm quality (18). Biopsies cannot be successfully performed in early blastocysts since there is a danger of accidental aspiration of the inner cell mass, as ICM lies very close to trophectoderm cells (16, 17). As a result, some healthy embryos with slower development are falsely deemed unsuitable candidates, thus resulting in the loss of potentially viable embryos.

Moreover, invasive procedures undermine the reproductive potential of embryos. Biopsy at the cleavage stage leads to impaired development of the fetus (19-22), with potential long-term adverse effects (23-25). Animal studies showed that offspring from biopsied embryos demonstrate epigenetic alterations and disorders in neurodegenerative tissues, adrenal glands, and ovarian deficiencies (26-30). Still, there are safety concerns about the prolonged culture of embryos up to the blastocyst stage to be accessible for trophectoderm biopsy (31, 32). Extensive embryo culture beyond the embryo's genome activation stage has been associated with an increased risk of monozygotism, perinatal mortality, genetic anomalies, premature birth, and infants (33, 34). Finally, invasive procedures cost more, are laborious, require more time, and demand highly skilled embryologists (17), so an alternative robust PGT method is needed.

Another aspect of embryo biopsies is that we can collect only a small portion of cells from specific points from the embryo, a limiting factor of genetic diagnosis of the embryo DNA integrity. As a result, we cannot identify mosaic embryos accurately because they show DNA heterogenicity, leading to a false genetic diagnosis (35-38). However, mosaic embryos can give birth to healthy babies (39, 40), but the dynamic implantation decreases compared to euploid embryos (41, 42). In addition, researchers have found a discrepancy between the chromosomal states of cells in trophectoderm (TE) biopsies, so these cells do not necessarily represent the whole embryo (40, 43, 44).

Recent studies have shown detection of cf-DNA in biological fluids, blastocyst fluid, and spent culture medium of *in vitro* cultured embryos (39, 40, 45-50), opening new roads for the implementation of non-invasive procedures in assisted reproductive technology. The cf-DNA that can be detected by the embryonic developmental culture material, SCM, seems to be the best choice for non-invasive PGT (ni-PGT). Many research groups have detected cfDNA and are under evaluation as a potential candidate method of assessing *in vitro* cultured embryo's chromosomal status (26, 39, 40, 45, 48, 51). A recent review of 15 published studies (46) showed that spent culture medium DNA detection is a safe and effective method for the chromosomal status of the developing embryos. However, the different methodologies followed in various studies undermine the validity of the findings as it is impossible to correlate the results directly.

Another problem is that several studies have detected extra-embryonic DNA in spend culture medium (SCM), a major limiting factor of the diagnostic and clinical performance of niPGT-A. Therefore, to implement niPGT-A procedures, we need to have concrete data derived from large-scale studies with comparable methodologies. Furthermore, provision must be taken to accurately determine the origin of the DNA detected (embryonic or non-embryonic).

In the present study, we compared the results of NGS aneuploidy control from TE biopsies and the SCM of the respective embryos in PGTA cycles. In addition, we tried to implement all the proposed measures to avoid contamination, to investigate the reliability of ni-PGT with optimized procedures. These results can offer helpful insight in research of reproductive biology.

Patients and Methods

Patients. We collected forty embryos from 13 infertile patients undergoing PGT-A in 2019-2020 at the IVF Unit of Mitera General Hospital in Athens.

Outcome assessments. The primary evaluation of this study was the embryos chromosomal status; for this reason, embryos were cultured to the blastocyst stage (d5/d6). NGS analyzed trophectoderm biopsies and respective SCMs from individually cultured embryos, and we compared the results. The average number of the collected eggs was 1.7+0.8, from women with mean age 35.3+4.2. Before being biopsied and frozen, we assessed the blastocysts according to the Gardner system of classification (52).

Embryo culture - Embryo biopsy - Collection and storage of SCM. The oocyte's fertilization was performed by intracytoplasmic sperm injection (ICSI) in all cases, using only mature (metaphase II) oocytes. After ICSI, the fertilized oocytes were cultured in groups, up to 3 embryos per 25 μl microdroplets, from day1 (D1) to day3 (D3), in Sage 1-Step culture material (Origio, Denmark) under oil (Origio).

We removed all remaining cumulus cells on day 3 of embryo culture and rinsed them thoroughly at least three times. Then we transferred to separate 10μl fresh microdroplets with Sage 1-Step culture material (Origio) under oil (Origio) for individual culture up to the blastocyst stage (day 5 or 6). Once the embryo has reached a fully expanded blastocyst, we collect and release the blastocyst fluid (BF) into the culture medium (SCM), then the blastocyst collapsed,

and each blastocyst was hatched by laser in order some cells (3-5) to come out of the zona, those cells were collected for preimplantation genetic analysis. When the embryo was suitable for the biopsy procedure, we transferred them to a biopsy plate, taking the cells for genetic analysis. Then the embryo was cryopreserved by vitrification according to standard procedures (53). Finally, the combined SCM and BF samples, intended for non-invasive PGT-A, were collected and frozen at –80˚C until analyzed. Then, we performed total genome amplification (WGA) and analysis of these samples for niPGT-A.

Whole genomic amplification (WGA) and chromosome analysis. All trophectoderm samples after biopsies and cell-free DNA from blastocyst culture material were amplified using the SurePlex (BlueGnome Ltd., Mill Court, Great Shelford, Cambridge, UK) kit according to the manufacturing instructions and quantified by the Qubit 3.0 Fluorimeter (Thermo Fisher Scientific, Waltham, MA, USA). In addition, the amplified DNA was evaluated for complete and partial chromosome aneuploidy testing with a previously validated VeriSeq™ PGS kit in ThermoFisher ion Reproseq PGS 520 kit, for cell-free DNA and in Illumina VeriSeq PGS Kit with automatic analyzer Bluefuse multi-data analysis, which controls 24 chromosome aneuploidies for trophectoderm biopsies.

We set a reliability threshold for the results of Illumina's VeriSeq PGS Kit analysis system; when the DLR was <0.4, the read after filtering was >150,000, the average quality score was >30, and the average alignment score was >30. Regarding the ThermoFisher ion R proseq PGS 520 kit analysis system, we considered the results reliable when the MAPD was <0.3 the reads after filtering was >100,000.

We used pure culture material as a negative control to check if there was cell-free DNA in material with no embryo development and to ensure the methodology of the technique.

Evaluation of chromosomal content. Both cf-DNA samples and TE biopsies were analyzed using the protocol as mentioned earlier. In addition, their chromosomal status (euploid *vs*. aneuploid) and the type and size of the chromosomal disorder in aneuploidy were evaluated using BlueFuse Multi software (Illumina, San Diego, CA, USA) and the ThermoFisher software.

We considered samples as full concordant when they were identical concerning the extent of polyploidy and mosaicism. Samples that agreed on the state of ploidy (euploid *versus* aneuploid) were considered as overall concordant, even if they differed in the type of aneuploidy.

We performed a chromosomal analysis of the whole embryo in specific samples deemed aneuploidy by TE biopsy and SCM. The patients permitted the destruction of the embryos. These samples are considered the gold standard in the process of evaluating the reliability of the niPGT-A method.

Statistical analysis. The PGT-A results by TE biopsies and niPGT-A results by SCM were correlated and analyzed using the McNemar test; also, we performed ROC analysis. In all statistical tests, we set the level of statistical significance to 95% (p<0,05). In addition, we calculated 95% confidence intervals (95%CI).

Ethical approval and consent to participants. The present study has permission from the Ethics committee of the Democritus University of Thrace and by the Council of Science of the Mitera Hospital in Athens. Furthermore, all couples in this study have signed a consent agreement stating that free nucleic acids will be tested in the remaining culture material, otherwise rejected, by their *in vitro* cultured embryos as part of their assisted reproduction program. It is also explicitly stated that their decision to participate or not in this research would not affect the provision of services by the Assisted Reproduction Unit of Mother Hospital.

Results

In this study, we examined embryos with an unknown chromosomal profile. We performed trophectoderm biopsy on blastocysts of the fifth or sixth day of growth, *i.e*., PGT-A samples (n=40). At the same time, the spent culture material (SCM) of the respective embryos was collected for the analysis of cell-free DNA, *i.e*., niPGT-A samples (n=40) (Figure 1). A total of 40/40 samples were amplified after WGA and analyzed by NGS. We collected the SCM samples from embryos cultured for 48-72 h after day3 to amplify cfDNA. After whole-genome amplification (WGA), the DNA concentration in each sample ranged between 2,500-30,000 ng/ml for TE biopsies and 2,000-20,400 ng/ml for SCM. The negative controls (new and unused culture medium) showed overall no amplification. Although the culture time was different between the day 5 and day 6 blastocysts, the DNA concentration of the culture medium collected for niPGT-A was not statistically different concerning the day of collection (day5 embryo group: 20.0 ng/μl *vs*. day6 embryo group 20.8 ng/μl; p=0.807).

We categorized the samples as euploid, aneuploid, or mosaic, according to the results. Table I presents the analysis of the results. We excluded seven samples from the SCM due to the low quality of the DNA in the NGS analysis, so we included 33 samples in the PGT-A and niPGT-A analysis (Table II).

The concordance rate between autosomal and sex chromosomes of PGT-A and niPGT-A samples for each embryo was estimated (Table III). The full concordance rate between the ni-PGT-A and PGT-A from samples taken from the same embryo was 21/33 (63.6%). The overall concordance rate between the ni-PGT-A and PGT-A, from samples taken from the same embryo, was 27/33, 81.8% (95% CI=68-96%). McNemar test: p=0.687 (non-statistically significant). The sex chromosomes concordance rate was 100%.

To estimate the diagnostic accuracy of niPGT-A, we calculated sensitivity (*i.e*., true positive) and specificity (*i.e*., true negative) in the PGT-A and the niPGT-A (Table IV). The sensitivity (aneuploidy agreement) was 91.66%, and the specificity (euploidy agreement) was 76.19%. In ROC analysis, AUC was 82.3% (95% CI=66.9-97.8). Of the 33 samples, 7 were male (XY), according to TE biopsy and SCM analysis, which confirms the safety of the method, as it shows no contamination by maternal DNA. In 5 samples, ni-PGTA from SCM detected euploidy, while TE biopsy showed embryomonosomes, possibly due to mosaicism.

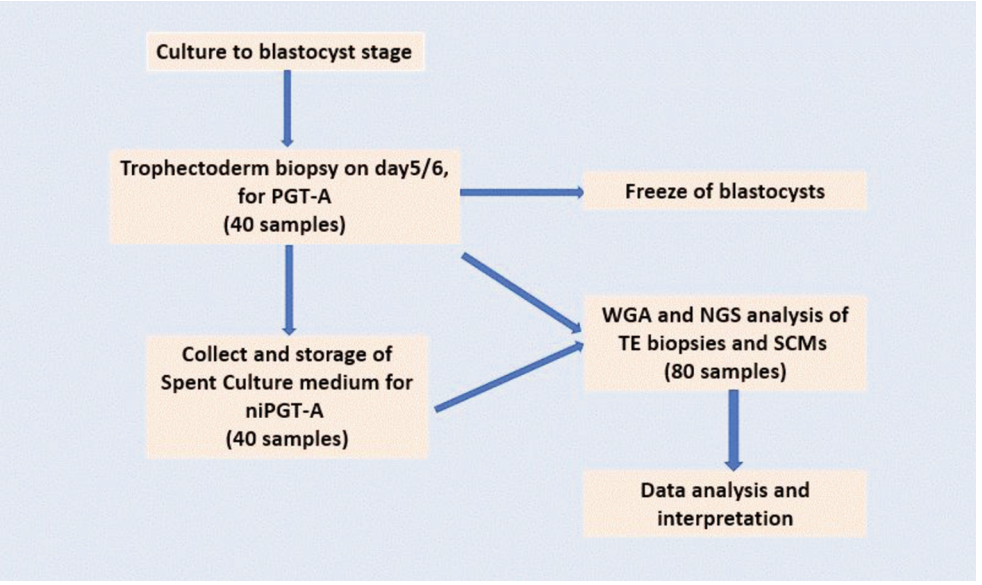

Figure 1. *The study flow chart.*

In 4 samples that have been detected aneuploid both by TE and SCM analysis, and we had permission from the patients, we performed a chromosomal analysis of the entire embryo, and we found aneuploidy as well (Table II; samples 14, 28, 33, 34). In sample 33, the results of the genetic analysis of the whole blastocyst were identical with the SCM analysis. Of particular interest is one sample, where trophectoderm biopsy showed euploidy, while niPGT showed a 6-fold increase in a small portion of chromosome 2.

Table I. *Ploidy analysis of trophectoderm (TE) biopsied embryos and spend culture medium (SCM) of the corresponding embryos.*

	PGT-A	niPGT-A
Number of analyzed embryos	40	40
Failed NGS analysis	0 (0%)	7 (17,5%)
Euploid	16 (40%)	16 (48,48%)
Aneuploid	24 (60%)	17 (51,5%)
Mosaic	4 (10%)	0 (0%)

NGS: Next generation sequencing; PGT-A: preimplantation genetic testing for aneuploidy; niPGT-A: non-invasive preimplantation genetic testing for aneuploidy.

Discussion

Due to its limitations, PGT-A by TE biopsy cannot accurately depict the whole genome of the developing embryos. Several studies have documented PGT-A results through TE biopsy. The degree of congruity between the biopsies of trophectoderm and inner cell mass (ICM) karyotyping ranged between 62.1-86.2% (39, 43, 44, 54). A recent study calculated that the sensitivity (probability of diagnosing an abnormality in ICM by TE biopsy) was 90.9%, while the specificity (the percentage of embryos with normal ICM diagnosed as clinically appropriate), according to the TE biopsy, was 66.7% (44). Based on these findings, they estimated the overall diagnostic accuracy at 75%. Furthermore, the overall rate of chromosomal mosaicism was determined in 37.9% of the examined embryos, suggesting that blastocysts are more chromosomaly diverse at a far more significant rate than reported before (44). These findings have shed doubts regarding the use of NGS, the PGT-A diagnostic performance, and the predictive power of TE biopsy. In a recent study (40) in IVF cases where infertile couples had only blastocysts with aneuploidy or mosaicism after TE biopsy check, the patients decided to transfer the blastocyst with mosaicism with a clinical pregnancy rate of 50%. Therefore, if a percentage of blastocyst mosaics can lead to healthy babies, the results of this procedure are undoubtedly at risk of their exclusion. However, the potential for embryo implantation and development with euploid ICM and mosaic or abnormal TE still needs to be elucidated. Currently, the whole blastocyst's DNA makeup is tough to precisely assess by TE biopsies (39, 40, 44, 54).

Biopsies of embryos have a documented negative impact on embryo dynamic and maybe undermine the implantation

Table II. *Chromosomal status of trophectoderm (TE) biopsies, spend culture media (SCM) and whole blastocysts. Data were obtained from 40 embryos from 13 patients.*

No	Day of biopsy/ freeze	Age	Grade	PGT-A	niPGT-A	Whole blastocyst
1	5	36	2BB	46XX	46XX	
2	5	36	4AA	46XX	N/A	
3	5	36	3AA	46XX	N/A	
4	5	38	3AA	46XY	N/A	
5	5	38	3AB	46XX	46XX	
6	5	38	3AB	47XX, +16,-14	46XX	
7	5	33	3AA	46XY	N/A	
8	5	33	3AA	46XX	46XX	
9	5	33	3AA	43XX, -8,-10,-22	N/A	
10	5	36	4AA	46XX	46XX	
11	5	36	4AA	46XX	46XX	
12	5	39	4AA	44XX, -15,-22	45XX,-15	
13	5	39	3AA	46XY,+3,-14 (p21.2;q32.13)	46XY,+3,-14 (p21.2;q32.13)	
14	6	40	5AA	48XX,+16,+22	48XX,+16,+22	48XX,+16,+22
15	6	40	5AB	45XY,-16	N/A	
16	5	36	3AB	46XX	46XX	
17	6	36	4AA	45XX,-22	45XX,-22	
18	5	36	4AA	47XX,+15	N/A	
19	5	36	3AA	46XX	46XX	
20	5	44	3AA	45XY,+13,-19,-22	4XY,+3,-6,-8,+13,-22	
21	5	44	3AA	48XY,+18,+20,+22,-21	46XY, +16,-21	
22	5	44	3BB	Chaotic, XX	Chaotic, XX	
23	5	44	4AA	47XY,+21, mosaic	46XX	
24	5	44	3AB	43XX,-10,-18,-20	47XX,+18	
25	5	44	4AA	47XX,+22,mosaic	46XX	
26	5	39	3BB	46XX	46XX	
27	5	39	4AA	46XX	46XX	
28	5	39	3AA	47XX,+18	47XX,+18	47XX,+18
29	5	31	3AA	46XX	46XX	
30	5	31	3AA	46XX	46XX	
31	5	31	4AA	47XX,+13,mosaic?	46XX	
32	5	31	3AA	47XX,+13,mosaic?	46XX	
33	5	45	3AB	45XY,-4,+7,-12,+20,-21	47XY,-4,+7,+20	47XY,-4,+7,+20
34	5	45	3BB	46XY,+2,-13	49XY,+2,-13	49XY,+2,-13
35	6	41	4AA	45XX,-22	45XX,-22	
36	6	41	4AA	48XX,+9,+19	47XX,+9,+19	
37	6	41	5AA	46XX	46XX, 6 times increase in a small fragment of 2 chromosome	
38	6	41	5AA	45XX,-15	45XX,-15	
39	6	41	5AA	48XY,+9,+18	47XY,+9	
40	6	41	5AA	46XY,-18,+19	46XY,-18,+19	

PGT-A: Preimplantation genetic testing for aneuploidy; niPGT-A: non-invasive preimplantation genetic testing for aneuploidy.

potential (21, 39). Moreover, studies in animals have shown that embryo biopsy can cause neurodevelopment and adrenal disorders, but the impact in humans remains unclear (26, 29). A more reliable and efficient non-invasive method to produce clinically relevant results without the hazards of invasive methods would be a valuable tool for genetic screening in IVF clinics.

The cf-DNA detected by the embryonic developmental culture material is probably an excellent alternative to traditional invasive PGT. The niPGT-A methodology is easier than invasive methods and does not require expensive hardware, *e.g.*, lasers. However, the diagnostic accuracy of niPGT-A is, for the moment, unclarified and under scrutiny. Previous researchers have reported a low, 20.8%, concordance rate between TE and SCM for monosomy testing (39). In another study, the SCM/entire D3 embryo concordance rate was 56.3%, increasing to 65% at d5 of the development (39). Jiao *et al*., using a modified MALABAC

Table III. *Chromosomal concordance rates of preimplantation genetic testing for aneuploidy (PGT-A) vs. non-invasive preimplantation genetic testing for aneuploidy (ni-PGT-A).*

	Overall concordance	Full concordance	Sex chromosomes concordance
PGT-A *vs.* niPGT-A	27/33 (81.8%)	21/33 (63.6%)	33/33 (100%)

Table IV. *Aneuploidy/Euploidy agreement between preimplantation genetic testing for aneuploidy (PGT-A) vs. non-invasive preimplantation genetic testing for aneuploidy (ni-PGT-A) groups.*

	Sensitivity (aneuploidy agreement)	Specificity (euploidy agreement)
PGT-A *vs.* niPGT-A	91.66%	76.19%

method increased the agreement on chromosomal DNA between spent culture medium and blastocysts to 90% (39). In the present study, we estimated the agreement at the state of ploidy (overall concordance rate) between niPGT-A samples and PGT-A samples at 81.8%.

We chose to collect the SCM two to three days after single embryo culture because in that time frame, as other studies have shown (46, 48), there is the highest amount possible of cfDNA with the lowest degradation rate. The quality and the quantity of cfDNA have a direct impact on the niPGT-A diagnostic accuracy. The rationale behind the blastocyst fluid and SCM mix is the qualitative and quantitative enhancement of cell-free DNA. Other research groups have also used this technique with good results (48). We can also confirm that this technique gives reasonable amounts of high-quality cfDNA. In our study, we found NGS applicable cfDNA in 33/40 samples. Although we had an amplification rate in all samples, the cfDNA has low quality in seven samples and thus did not give reliable results, probably due to degradation. We collect the SCM 48-72 h after culture, and maybe this time is extended for some DNA molecules, resulting in degradation. The secretory mechanism of cell-free DNA is unknown. A potential source would be from cells entering the apoptotic pathways inside the growing embryo, increasing the probability of degraded DNA (39, 48). The clinical performance of genetic testing is of paramount importance for preimplantation diagnosis; for that reason, we selected NGS, as it is a widely recognized methodology capable of providing timely and accurate results on chromosomal disorders and mosaic embryos (41).

We obtained a good correlation of PGT-A *vs.* niPGT-A results (overall concordance 81.8%, p=0.687, non-statistically significant). Maternal contamination DNA is a major limiting factor that can affect the diagnostic performance of the methodology. Previous studies have documented this effect (48, 51). In our study, we tried to optimize the whole procedure to avoid maternal or other contamination. We used Intracytoplasmic Sperm Injection (ICSI) as a fertilization procedure to avoid paternal contamination. We carefully denuded the oocytes from cumulus cells and rinsed them thoroughly carefully at least three times in fresh medium microdroplets. The embryos after day3 were cultured in single microdroplets up to the blastocyst stage. We had seven male samples (XY) and twenty-six females (XX); both TE biopsy and SCM analysis confirm these results, suggesting that this methodology is robust and safe. A further step to ensure the diagnostic accuracy of this methodology could be the analysis of the XX samples for single nucleotide polymorphisms (SNPs) to establish the origin of DNA, maternal or embryonic.

We found a discrepancy in euploidy status in 4 samples (Table II, samples 23, 25, 31, 32). TE biopsy showed aneuploidy, but SCM showed euploidy. We classified those embryos as mosaics. Other studies have also found various degrees of discrepancies (48). Li *et al.* (40) reported that when mosaic embryos were re-cultured and re-tested the whole blastocyst, they found euploidy at 85.4%. They also noted that setting the threshold of mosaicism at 50%, the niPGT-A concordance rate between the SCM and Whole Blastocyst analysis was 87.2%, more significant than the concordance rate with TE biopsy, suggesting that niPGT-A can more accurately diagnose the ploidy status of mosaic embryos. We collected DNA from both the blastocyst fluid and the spent culture medium, and we believe that this approach gives more representative results of the ICM chromosome status (48).

Interestingly, in sample 13, a translocation [t(3:14)(p21.2;q32.13)] of paternal origin was detected, both TE biopsy and SCM, showing the diagnostic power of niPGT-A in detecting various types of chromosomal abnormalities. Of particular interest is 1 sample, where trophectoderm biopsy showed euploidy, while niPGT-A showed a 6-fold increase in a small portion of chromosome 2; it is possible that this increase in such a small part of the chromosome cannot be detected in trophectoderm cells, which may not be representative of the whole embryo, while cfDNA may be more representative.

Whether the cfDNA in the SCM represents the full chromosomal status of the embryo remains to be clarified. However, a blastocyst expansion study using DNA-specific fluorochromes revealed that the source of apoptotic cells is mainly from the inner cell mass (54). Indeed, if the origin of this DNA is predominantly from the inner cell mass, niPGTA may give a more reliable picture of the future fetus (47).

Of the four samples we analyzed of the entire embryo, we found complete agreement between the TE biopsy, SCM, and Whole Blastocyst (WB) analysis in 3 out of the 4. In sample 33, we found a 100% match with the study of the cfDNA from the SCM. Thus, the WB analysis is a robust diagnostic performance index of the niPGT-A methodology, even if the samples are few.

Time-dependent degradation of DNA and contamination of maternal DNA are risks that must be accounted for (48). However, the careful removal of cumulus cells and the meticulous washing of the embryos in single culture microdroplets can significantly reduce the risk of maternal DNA contamination. At the same time, using ICSI as a fertilization method can help avoid the risk of paternal DNA contamination.

Conclusion

Genetic analysis in IVF settings is widely used to select viable embryos, leading to healthy offsprings; however, there is a need to replace the invasive procedure with safer and non invasive techniques. We used niPGT-A to assess the chromosomal status of embryos. Our results showed that cfDNA from SCM could be detected and amplified at 100%, but at a rate of 82.5%, the cfDNA samples were appropriate for analysis. This rate can be even more increased through further refinement of the collection and amplification steps. In this study, the small number of samples is a limitation; nonetheless, we managed to fully determine the genetic makeup of specific blastocyst used for this research, a gold standard for the accurate determination of this methodology. Despite the limitations, we can confirm that niPGT-A, a non-invasive process with minimal risks to embryos, is reliable and precise and can be used along invasive PGT-A as an alternative, especially in cases of mosaicism. Large-scale randomized control studies will be able to validate this very promising methodology and establish niPGT-A as a valuable tool to assist infertile couples in having healthy babies.

Conflicts of Interest

There are no conflicts of interest.

Authors' Contributions

Afrodite Sialakouma conceived the study, recruited the patients, performed the biopsies, collected the samples, performed the experiments, analyzed the data, wrote and edited the manuscript. Ioannis Karakasiliotis performed the experiments, analyzed the data, revised the manuscript. Vaia Ntala analyzed the data and revised the manuscript. Nikolaos Nikolettos and Byron Asimakopoulos revised the manuscript.

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