Embryonic cell-free DNA versus trophectoderm biopsy for aneuploidy testing: concordance rate and clinical implications

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Objective: To study whether embryonic cell-free DNA (cfDNA) in spent blastocyst media is representative of the chromosomal constitution of a blastocyst.
Design: Pilot prospective blinded study.
Setting: In vitro fertilization center and genetics laboratory.
Patient(s): A total of 115 trophectoderm (TE) biopsies and spent blastocyst media (SBM) from 46 patients with ages ranging from 32 to 46 years, whose indications for preimplantation genetic testing of aneuploidy (PGT-A) were advanced maternal age, recurrent miscarriage, or recurrent implantation failure.
Interventions(s): Spent blastocyst media collection and TE biopsy.
Main Outcome Measure(s): Concordance rates, sensitivity, and specificity between TE biopsies and SBM. Clinical outcomes in cases with euploid TE biopsies and euploid SBM compared with cases with euploid TE and aneuploid SBM.
Result(s): In general, the total concordance rate for ploidy and sex was 78.7%, and sensitivity and specificity were 94.5% and 71.7%, respectively. A significant increase for all parameters was observed for day 6/7 samples compared with day 5 samples, with day 6/7 samples showing total concordance for ploidy and sex of 84%, and sensitivity and specificity of 95.2% and 82.1%, respectively. Ongoing implantation rates in euploid TE/euploid SBM showed a threefold increase compared with euploid TE/aneuploid SBM (52.9% vs. 16.7%, respectively), without reaching significant differences. Interestingly, no miscarriages were observed when TE and SBM were euploidy concordant.
Conclusion(s): These results offer a better understanding of the dynamics of cfDNA during embryo development and despite more basic research being needed, they are reassuring to consider in the future this noninvasive approach as an alternative to TE biopsy for PGT-A. (Fertil Steril® 2019;112:510–9. ©2019 by American Society for Reproductive Medicine.)
El resumen está disponible en Español al final del artículo.

Key Words: Embryo, spent blastocyst media, trophectoderm biopsy, aneuploidy, noninvasive PGT-A

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The high incidence of aneuploid embryos in IVF (ranging from 20% to 100%) is an important biologic burden (1–3). Preimplantation genetic testing for aneuploidy (PGT-A) is, at present, the most reliable method to assess the chromosomal status of preimplantation embryos. Currently, DNA isolated and amplified from trophectoderm (TE) biopsies and

analyzed by means of next-generation sequencing (NGS) is the state of the art for this technique (4–8).

Despite the value of PGT-A, there are two main unsolved problems causing controversies in the scientific community. First, embryo biopsy requires specialized equipment and expertly trained operators to maintain standard protocols to protect embryo viability (3, 9). However, possible harm to the embryo is always a concern of both doctors and patients. Despite similar ongoing implantation rates having been reported in a study comparing transfer of nonmanipulated blastocysts and blastocysts with TE biopsy (10), the putative variability associated with the different expertise of operators that were not properly trained should not be dismissed. Second, mosaicism, a biologic event occurring at any stage of embryo development, can have an impact on the accuracy of diagnosis based on the analysis of only 5–10 cells from a blastocyst with >100 cells, introducing uncertainty (3, 11, 12).

There is increasing evidence for a "true" noninvasive approach consisting of the analysis of cell-free DNA (cfDNA) released by the embryo into the spent blastocyst medium (SBM) during the late stages of preimplantation development (13). SBM, in which the embryo is cultured, is routinely discarded at the time of transfer or freezing. Several publications have compared the chromosomal results of the criterion-standard PGT-A from TE biopsies and those of the cfDNA in the SBM to establish concordance rates. All reports have obtained high cfDNA amplification rates, ranging from 80% to 100%. However, the concordance rates have been variable, with 3.5% (14) reported in a proof of concept study and 30.4% (15), 65% (16), and 85.7% (17) reported in later studies. Another study reported 72.2% concordance rate between polar body biopsies and media collected on day 5, including also arrested embryos (18). These discrepancies could be related to different methodologies applied during embryo culture (drop volume and time in culture), blastocyst manipulation (assisted hatching, vitrification), and DNA analysis (amplification and detection methods) as well as to the different criteria used to define the concordance rates. In addition, how the embryo is handled during its development is extremely important because it can determine not only the quantity and quality of cfDNA, but also, and most importantly, its origin, i.e., DNA coming from residual cumulus cells could lead to maternal DNA contamination (MCC) and therefore a high rate of false negative results (13, 15, 16, 19, 20).

The aim of this study was to estimate the concordance rates between TE biopsies and SBM with an optimized protocol incorporating technical improvements at two levels: the culture conditions in the IVF laboratory and the NGS protocol applied to the analysis of the SBM. Importantly, embryos were not subjected to any intervention during in vitro culture, such as previous assisted hatching, vitrification, or blastocentesis. In addition, to test the functional relevance of embryonic cfDNA testing, we retrospectively compared the clinical outcome of euploid single embryo transfers (SETs) with concordant or discordant results in TE biopsy versus SBM, considering that the selection of embryos for transfer was always performed according to the results obtained by TE biopsies and that the SBM was subsequently analyzed.

MATERIALS AND METHODS

Study Design

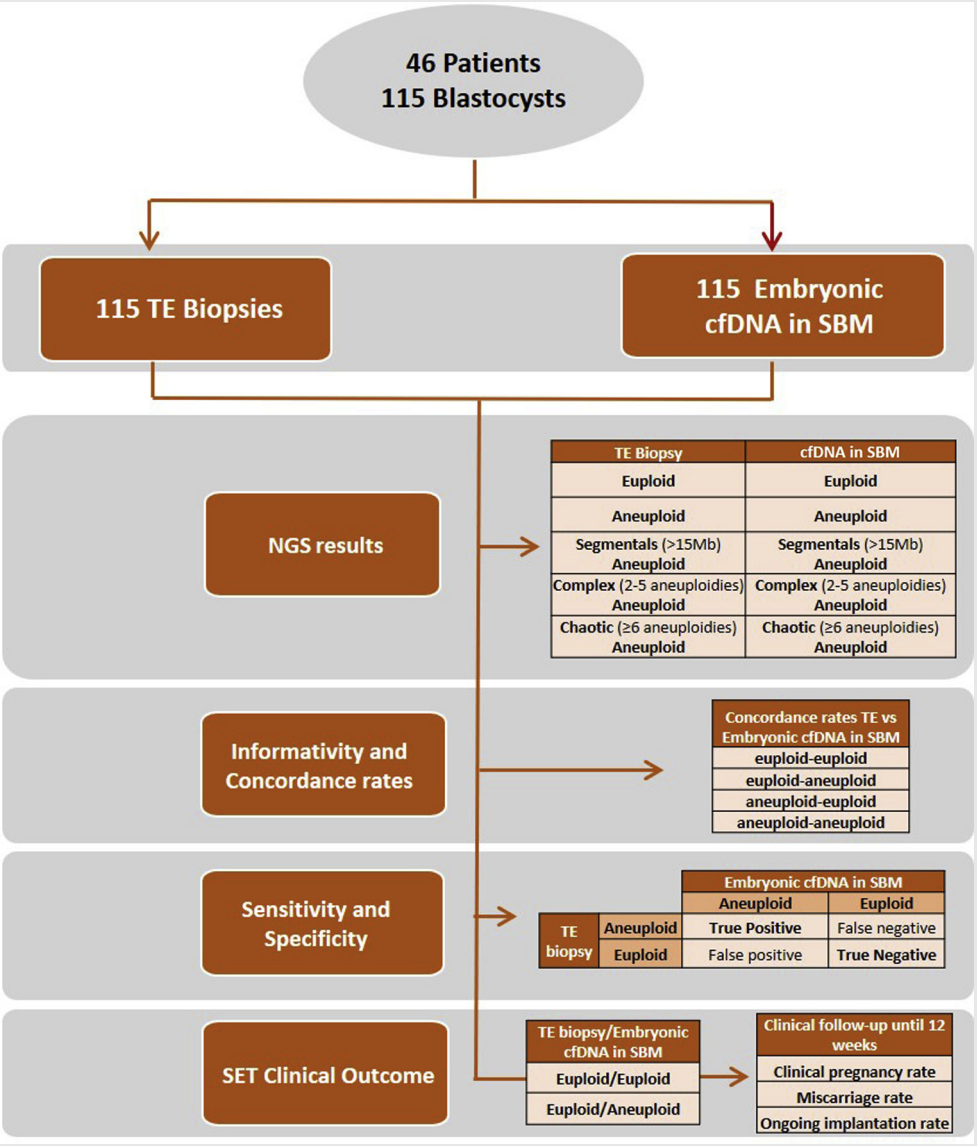
This was a prospective validation study performed from November 2017 to March 2018, including 115 blastocysts from 46 couples undergoing PGT-A at Genera (Rome, Italy). PGT-A indications were advanced maternal age (>35 years; n = 41), repeated implantation failure (>2 previous failed embryo transfers; n = 1), and repeated pregnancy loss (>2 previous miscarriages; n = 4). All patients underwent karyotyping before IVF, and carriers of structural abnormalities or monogenic diseases were excluded. Mean female age was 38.8 (SD 3.3) years, and mean number of blastocysts biopsied per patient was 2.5 (SD 1.6). TE biopsy was performed in all cases, and the results were compared blindly with the analysis of embryonic cfDNA released by the same embryo to the SBM. A negative control sample consisting in a drop of media without previous contact with any embryo was included for each patient.

Patients yielding oocytes that did not undergo vitrification and assisted hatching before the collection of the SBM were included. TE biopsy was performed on day 5, 6, or 7 according to the morphologic development of the embryo, and SBM was removed immediately after transferring the embryo to the biopsy dish. After SBM collection, samples were stored for a period of 1–4 weeks before shipping to the genetics laboratory.

Embryo transfer was guided by the results of TE biopsy, and blinded embryonic cfDNA analysis was always performed after TE biopsy assessment. The flow-chart of the study is shown in Figure 1. NGS results were analyzed with the use of an Igenomix proprietary algorithm to identify whole-chromosome aneuploidies and segmental aneuploidies (del/dup >15 Mb). This algorithm was designed with the use of gDNA from cell lines (Coriell Institute, NJ, USA) with known aneuploidies (trisomies 2, 9, 13, 15, 18, 20, 21, and 22). Once the samples were sequenced, the thresholds were calculated with the use of the raw data. For this pilot study, the thresholds were <50% aneuploid DNA for euploidy and ≥50% aneuploid DNA for aneuploidy.

Informativity rates (successfully amplified samples/total analyzed samples) were estimated individually for TE biopsies and embryonic cfDNA samples and for blastocysts, with informative results for both types of samples. Four scenarios in terms of concordance were possible when comparing the ploidy results of TE versus embryonic cfDNA: euploid-euploid; euploid-aneuploid, aneuploid-euploid, and aneuploid-aneuploid. The analysis of ploidy concordance included the matches euploid-euploid and aneuploid-aneuploid and for discordances euploid-aneuploid and vice versa. We subdivided the aneuploid-aneuploid matches into full (concordance for all 24 chromosomes), and partial concordance (some concordant chromosomes) categories (Fig. 2). Also, the percentage of false positives (FPs) was estimated considering the number of euploid TE with aneuploid SBM regarding the total informative results for both type of samples. And false negatives (FNs) were calculated considering the number of aneuploid TE with euploid SBM regarding the total informative results for both type of samples. For

FIGURE 1



Flow-chart of the study. cfDNA = cell-free DNA; NGS = next-generation sequencing; SBM = spent blastocyst medium; SET = single embryo transfer; TE = trophectoderm.

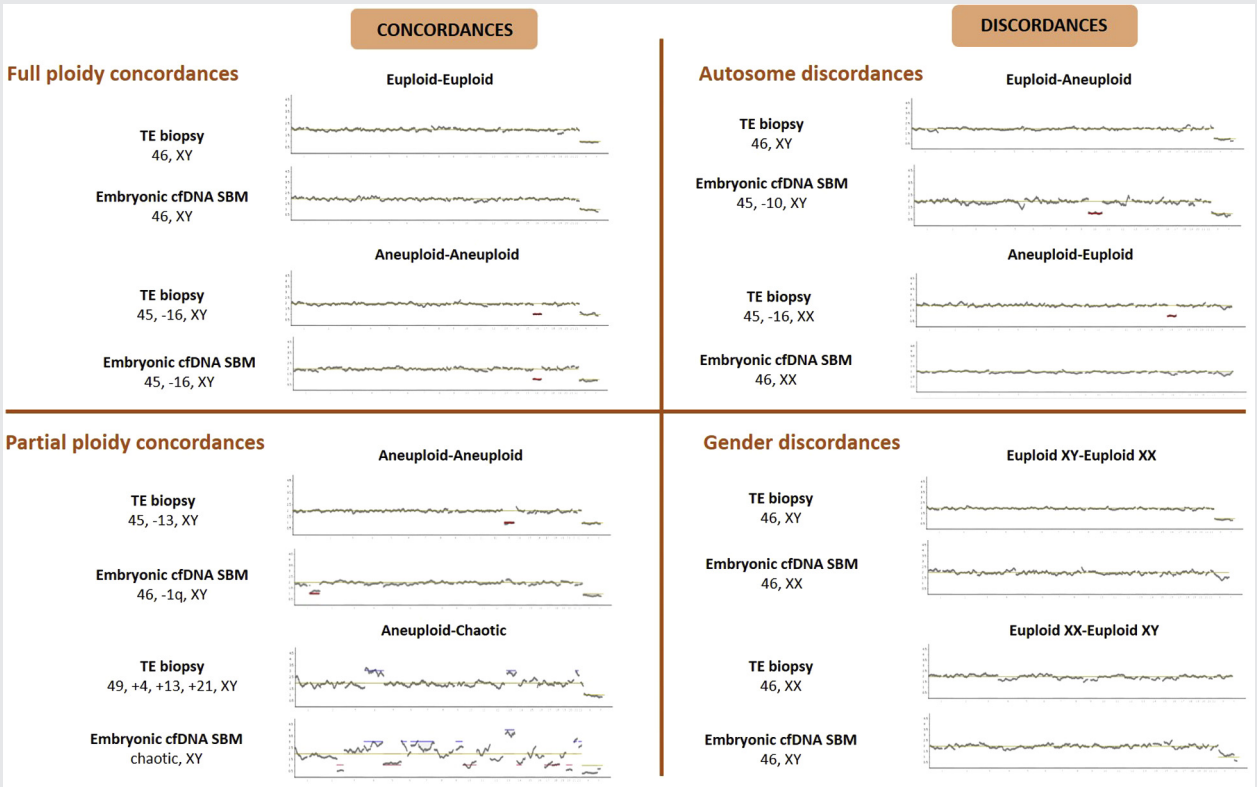
Rubio. Embryonic cfDNA analysis for niPGT-A. Fertil Steril 2019.

individual chromosome concordance, chaotic embryos (≥6 aneuploidies) were excluded (n = 17/108; 15.7%), because low DNA quantity or quality can also result in this type of profiles.

Sensitivity and specificity were determined for the embryonic cfDNA in the SBM related to the TE biopsy as follows. Sensitivity: the proportion of aneuploid embryos that have been correctly identified (aneuploid SBM–aneuploid TE): true positive/(true positive + false negative) × 100. Specificity: the proportion of euploid embryos that have been correctly identified (euploid SBM–euploid TE): True negative/(true negative + false positive) × 100.

Follow-up of all SETs was performed until 20 weeks of pregnancy according to the World Health Organization (21). Biochemical pregnancy loss was defined as serum β-hCG levels ≥50 IU/L in at least two pregnancy tests but not associated with any ultrasound evidence of pregnancy. A clinical pregnancy was defined as the presence of a gestational sac with fetal heart beat. A pregnancy loss earlier than 20 gestational weeks was considered to be a miscarriage, otherwise the pregnancy was considered to be ongoing. The rate of positive pregnancy tests, the clinical pregnancy rate, and the ongoing implantation rate were calculated based on the number of vitrified-warmed embryo transfers performed. The rate of

FIGURE 2



Profiles representing different concordant and discordant profiles between TE biopsies and cfDNA in SBM. Abbreviations as in Figure 1.

Rubio. Embryonic cfDNA analysis for niPGT-A. Fertil Steril 2019.

biochemical pregnancy losses was calculated based on the number of positive pregnancy tests, and the clinical miscarriage rate was calculated on the number of clinical pregnancies. Clinical outcome was compared in two scenarios, when both TE biopsy and embryonic cfDNA were euploid and when TE biopsy was euploid but the embryonic cfDNA was aneuploid.

Ethical Approval

The project was approved by the Institutional Review Board of Genera (IGX1-NIP-CS-18-02; CVG-06112017), and all included patients signed written informed consents.

IVF Cycle and Embryo Culture

Controlled ovarian stimulation and induction of ovulation were conducted as previously described (22). Thirty-six hours later, oocyte retrieval was performed under ultrasound vaginal guidance. After 2–3 hours, intracytoplasmic sperm injection (ICSI) was performed as described in Rienzi et al. (23). Careful denudation of surrounding cumulus cells was conducted before ICSI. Immediately after ICSI, the oocytes were washed in 0.6 mL Multipurpose Handling Medium with 5% human serum albumin and finally transferred to individual 30-μL drops of preequilibrated continuous single-culture medium (CSCM; Irvine Scientific) under mineral oil (Irvine Scientific). Single embryo culture was conducted in a controlled humidified atmosphere containing 5% O_2 and 6% CO_2.

On day 4, each compacted embryo or morula was thoroughly washed in three sequential 20-μL drops of CSCM and finally moved to an individual 10-μL drop. A negative control sample of media that followed the same protocol but without being in contact with the embryo, was included for each patient. If the embryos reached the fully expanded blastocyst stage on day 5–7, they were moved to a biopsy dish and the SBM were collected in polymerase chain reaction (PCR) tubes kept on ice. The SBM corresponded to conditioned culture media collected after 1 day in culture (day 4 to day 5) or 2 or 3 days in culture (day 4 to day 6 or 7).

The samples were stored at −20°C for at least 24 hours. The samples were analyzed in a blinded fashion. The blastocysts were instead biopsied through a method that entails simultaneous zona pellucida opening and TE fragment retrieval (24). No zona drilling on day 3 was conducted, and all embryos reaching the fully expanded blastocyst stage were biopsied regardless of their morphologic quality and day of full development. Embryo grading was performed according to a method described by Capalbo et al. (24) and Cimadomo et al. (25), adapted from Gardner and Schoolcraft (26). Accordingly, each blastocyst was classified as excellent, good, average, or poor quality.

Static morphologic evaluation was conducted, and the presence of cells excluded from the body of the embryo after compaction was registered (27). We also recorded the hours of culture elapsed from medium change-over to sample collection and whether the embryo was spontaneously hatching or not as well as the diameter of the blastocyst and the thickness of its zona pellucida before biopsy. These last two parameters were calculated through Cronus video capture and embryo analysis software at ×40 magnification (Research Instrument). Blastocysts were vitrified within 30 minutes after biopsy as previously described (25, 28, 29). The samples, tools, and devices were handled under sterile conditions during the whole process to prevent contamination. Vitrified-warmed euploid embryo transfer was performed as previously described (22). β-hCG was measured 11 days later.

NGS in TE Biopsies and in SBM-Derived Embryonic cfDNA

In both sample types, TE biopsies and SBM, whole-genome amplification (WGA) and DNA barcoding was performed with the use of the Ion Reproseq PGS Kit (Thermo Fisher Scientific), with a modified protocol for the SBM. Automated template preparation and chip loading were automated with the use of Ion Chef. For TE biopsies, the standard NGS protocol for 96 samples (530 chip) was used. For SBM, all volumes collected (8–10 μL) were preamplified and amplified by means of extended thermal cycles to increase the DNA yield after WGA, followed by individual purifications. For SBM, runs of 24 samples were performed (520 chip) according to the number of samples received. Finally, the 520 and 530 chips were placed in an S5TM XL sequencer (Thermo Fisher Scientific). Sequencing data were processed and sent to the Ion Reporter Software version 5.4 (Thermo Fisher Scientific) for data analysis. Quality parameters of both the run and individual samples were evaluated.

Statistical Analysis

Comparison of quantitative values for NGS results from TE biopsies, SBM, and clinical outcomes was done with the use of independent-sample Student *t* tests, with a *P* value of $<.05$ obtained for a two-tailed test considered to be statistically significant. A normal distribution was confirmed with the use of the Levene test for equality of variants, and for this reason a parametric test was chosen.

For comparing categoric data, chi-square tests were performed, and nonparametric Fisher exact tests were applied in cases of low number. $P<.05$ was considered to be statistically significant. In the tables, analysis is expressed as the percentage of probability and odds ratio (OR) with 95% confidence interval (CI). A multivariate analysis was also performed for concordance rates, including the variables of embryo quality (number of cells on day, fragmentation degree on day 3, inner cell mass, and TE grade at blastocyst stage), time in culture, and presence of MCC. These statistical analyses were performed with the use of SPSS 25.0 software (IBM).

RESULTS

In this study, embryonic DNA from TE biopsies and embryonic cfDNA from SBM corresponding to 115 blastocysts were amplified and sequenced. Informative results (successful amplification and interpretable NGS results) were obtained for 99.1% of TE biopsies and 94.8% of SBM. In total, 108 blastocysts out of 115 analyzed (93.9%) had informative results in both TE and SBM samples. TE biopsies showed similar informativity rates independently from the day of biopsy and hours in culture. In contrast, SBM informativity was significantly increased on day 6/7 compared with day 5 sample collection ($P=.0004$; Table 1).

Overall ploidy concordance per embryo was 78.7% and was significantly higher for day 6/7 than for day 5 samples (84.0% vs. 63.0%; $P=.0299$). Full concordance rates per embryo showed a similar trend (71.6% vs. 40.7%; $P=.0055$). However, partial concordance rates (14.8%) and concordance rates per autosomes (97.0%) and per sex chromosomes (95.1%) were similar at any time of sample collection. The overall concordance results for day 6 (71 informative samples) and day 7 (10 informative samples) were similar and both significantly different from day 5. For this reason, day 6 and day 7 samples were grouped in a single category as day 6/7, as in Table 1.

In the discordant cases, no significant differences were observed when comparing FN rates between day 5 (3.7%) and day 6/7 (2.5%), whereas FP rates were significantly higher for day 5 (29.6%) compared with day 6/7 (8.6%; $P=.0108$). Several FP cases corresponded to chaotic profiles in the SBM (53.3%), suggesting that low DNA input or degraded DNA could be partly responsible for the FP rates. If SBM with chaotic results were removed from the calculation, the average FP rates were reduced almost by half (14.8% for day 5, 3.7% for day 6/7). The sensitivity and specificity of the embryonic cfDNA in predicting chromosomal status for day 5 and day 6/7 blastocysts were estimated according to the incidence of aneuploidy in TE biopsies and SBM (Supplemental Fig. 1, available online at www.fertstert.org). The presence of MCC interfering with final diagnosis was observed in nine SBM samples: Two were female FP, two were female FN, and five were sex mismatch in euploid samples. In addition, we observed one female euploid TE biopsy with a male SBM, suggesting contamination in the media or plasticware or during embryo handling. Detailed results for TE biopsy and SBM are presented in Supplemental Table 1 (available online at www.fertstert.org). The overall sensitivity was 94.5%, without any statistical differences between day 5 and day 6/7. Lower overall specificity was observed (71.7%), with a significant increase for day 6/7 compared with day 5 (82.1% vs. 42.9%; $P<.0001$).

Concordance rates were calculated according to the number of aneuploid chromosomes in the SBM with a significant decrease when the number of aneuploid chromosomes increased ($P<.0001$). Interestingly, on day 6/7 the concordance rate of TE biopsies and SBM samples with only one aneuploid chromosome was 100%. Concordance rates per individual chromosome excluding chaotic profiles in the SBM ($n = 17$; 15.7% of the total informative SBM), revealed no

TABLE 1

Results according to sample type and day of biopsy, n (%).

Result		Day 5	Day 6/7	Total	*P* value	OR (95% CI)
General Information	No. of blastocysts analyzed, n	33	82	115	–	–
	Female age, y, mean ± SD	37.3 ± 2.8	38.4 ± 3.2	38.8 ± 3.3	NS	–
	Hours in culture, mean ± SD	26.0 ± 2.8	50.8 ± 8.8	44.6 ± 13.3	< .0001	24.800 (21.697–27.903)
Informative NGS results	Informative TE biopsies	33 (100%)	81 (98.2%)	114 (99.1%)	NS	–
	Informative SBM	27 (81.8%)	82 (100%)	109 (94.8%)	.0004	0.02564 (0.001398–0.4704)
	Blastocyst s with informative TE + SBM	27 (81.8%)	81 (98.8%)	108 (93.9%)	.0022	0.05556 (0.006395–0.4826)
Embryo concordances[a]	Total concordance for ploidy with different sex	18 (66.7%)	72 (88.9%)	90 (83.3%)	.0144	0.7500 (0.5682–0.9900)
	Total concordances for ploidy and sex	17 (63.0%)	68 (84.0%)	85 (78.7%)	.0299	0.3250 (0.1218–0.8669)
	Full concordances for ploidy and sex	11 (40.7%)	58 (71.6%)	69 (63.9%)	.0055	0.2726 (0.1100–0.6754)
	Partial concordances for ploidy and sex	6 (22.3%)	10 (12.4%)	16 (14.8%)	NS	–
Chromosome concordances[b]	Concordant autosomes	399/418 (95.5%)	1,542/1,584 (97.3%)	1,941/2,002 (97.0%)	NS	–
	Concordant sex chromosomes	35/38 (92.1%)	138/144 (95.8%)	173/182 (95.1%)	NS	–

Note: NGS = next-generation sequencing; NS = nonsignificant ($P \geq .5$); SBM = spent blastocyst media; TE = trophectoderm.

[a] For embryo concordance, total concordance includes embryos with full + partial concordances; full concordance is defined as TE and SBM both euploid or aneuploid for the same chromosomes, and partial concordance is defined as aneuploid embryos but with at least one different aneuploid chromosome in TE and SBM. All results were calculated considering the blastocysts with informative TE + SBM.

[b] For chromosome concordance, SBM with chaotic profiles were excluded for calculations.

Rubio. Embryonic cfDNA analysis for niPGT-A. Fertil Steril 2019.

significant differences in concordances, with the lowest being for sex chromosomes (90%) and the highest for chromosome 21 (100%). Finally, we analyzed concordance rates for female and male TE on day 6/7 and they were similar: 85% for female TE (n = 40) and 82.9% for male TE (n = 41). FN rates also were similar for female TE (2.5%) and male TE (2.4%), indicating that the impact of overall MCC was not underestimated.

Additional analysis considering embryo concordance as a dichotomous variable (yes/no) showed significant findings for the hours of culture elapsed from medium change-over to sample collection (P=.025; mean difference 6.97 [0.89–13.05], range 20–76 hours) and the presence of MCC also as a dichotomous variable (P<.0001; relative risk 1.93 [1.26–2.94]). For the number of aneuploid chromosomes in SBM, a negative significant correlation with the percentage of embryo concordance was found by means of a linear regression (P=.003; Pearson correlation coefficient −0.83). The multivariate analysis for concordance rates was performed for all embryo quality variables, with no significant findings for the other variables described.

Finally, 29 SETs were performed, guided by euploid results of the TE biopsy, and clinical outcomes were retrospectively calculated in two different scenarios: when euploid TE was concordant with euploid SBM and when euploid TE was discordant with aneuploid SBM. Nonconclusive cases with sex mismatch were excluded for the calculation of clinical outcomes. The ongoing implantation rate for euploid TE/euploid SBM embryos was threefold greater than the rate for euploid TE/aneuploid SBM embryos (52.9% vs. 16.7%, respectively). Interestingly, no clinical miscarriages were observed when TE biopsies and SBM were both euploid, suggesting that this new approach might have an important role complementing the current TE biopsy for embryo aneuploidy testing. However, because of the low number of SETs performed, differences were not significant (Table 2).

DISCUSSION

This study has shown the consistency of aneuploidy testing with the use of a noninvasive approach compared with TE biopsy, advancing a step toward understanding the added

TABLE 2

Clinical outcome after single embryo transfer of thawed blastocysts diagnosed as euploid after TE biopsy.

Outcome	Euploid TE/euploid SBM	Euploid TE/aneuploid SBM	Total
No. of transfers	17	12	29
Mean female age, y	37.5 ± 2.5	37.4 ± 2.3	37.5 ± 2.4
Positive pregnancy test	11 (64.7%)	4 (33.3%)	15 (51.7%)
Biochemical pregnancy loss	2 (18.2%)	0	2 (13.3%)
Clinical pregnancy rate	9 (52.9%)	4 (33.3%)	13 (44.8%)
Clinical miscarriage	0	2 (50.0%)	2 (15.4%)
Ongoing implantation rate	9 (52.9%)	2 (16.7%)	11 (37.9%)

Note: Abbreviations as in Table 1.

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clinical value of noninvasive (ni) PGT-A. With the incorporation of a noninvasive approach, we aimed to address the two main limitations of current PGT-A, namely invasiveness and diagnosis of mosaicism with only 5–8 cells retrieved from the whole blastocyst in a TE biopsy.

The concordance between PGT-A and niPGT-A has already been reported by other groups but with remarkable variation (14–18). The number of embryos analyzed in our study was 108, which is at least twice the number of embryos analyzed in previous studies. Also, those studies analyzed embryonic cfDNA from day 3 to day 5 (14–17) or even from day 1 (18). In our case, we scheduled media collection from embryos cultured under specific conditions from day 4 to day 5, and from day 4 to days 6/7. Moreover, our approach was completely noninvasive without the assisted hatching conducted before TE biopsy in other studies (14–16) or without previous embryo vitrification (16, 17). Feichtinger et al. (18) performed polar body biopsy and then cultured the embryo to obtain the SBM; that process could be categorized as assisted hatching, because the effects of the opening of the zona pellucida on the release of embryonic cfDNA might be similar. Despite these differences, our overall amplification rate was 93.9% and reached 100% in the SBM when embryos were cultured more than 48 hours from day 4 onward. It is noteworthy that all SBM samples with failed amplification or noninformative profiles had shorter times in culture from day 4 to sample collection, on average 26 hours. Ploidy concordance rate between TE biopsies and embryonic cfDNA was 78.7%, reaching 84% in extended culture, when SBM was collected on day 6/7. Despite the different criteria used to define ploidy concordance rates in other studies, we reached a high level of concordance without previous intervention. Only Xu et al. (17) had similar ploidy concordance rates (85.7%), but with previous vitrification and warming steps, that could enhance the release of embryonic cfDNA. We previously reported concordance rates of only 30.4% (15), and other groups obtained 3.5% (14), 65% (16), and 72.2% (18), but in all these studies previous interventions were performed on the embryos before SBM collection.

When we analyzed the discordances, 13.9% were FP and 2.8% FN. Therefore, our sensitivity and specificity were 94.5% and 71.7%, respectively. Those values were even better when considering day 6/7 SBM (with a mean of 50.8 hours in culture from day 4) with only 8.6% FP and 2.5% FN and increased sensitivity and specificity of 95.2% and 82.1%, respectively. Therefore, the longer the time of the embryo in the specific culture conditions, the higher the specificity, without a significant impact on sensitivity. These results are very promising, especially compared with the literature where sensitivity and specificity were, respectively, 73.3% and 66.7% (18), 88.2% and 84.0% (17), or 80.0% and 61.0% (16). Besides, we observed that in almost one-half of the FPs that the SBM profiles were chaotic ($\geq$6 aneuploidies), where low- or poor-quality DNA could lead to noisy NGS profiles and therefore be partly responsible for the FP results. Regarding FNs, they were ostensibly higher in our previous study (15) and in others (16, 18) and were related to the presence of residual cumulus cells not completely stripped from the oocyte. Therefore, maternal DNA contamination would correlate with decreased sensitivity values. We have also observed male SBM results coming from a TE-diagnosed female embryo, indicating that external DNA contamination resulting from plasticware, media, or manipulation during IVF is crucial, and caution should be taken to prevent this. Also, the presence of residual PBs has been suggested as a potential source of discrepancies, related to sex or complementary aneuploidies (15). Interestingly, this phenomenon is minimized when delaying the placement of embryos in the final culture drop (13, 30).

We studied the potential impact of embryo quality, maternal age, and sperm quality, and no influence was observed as suggested by others (20, 31). Regarding the methodology developed for our niPGT-A approach, the small volume of medium used to culture the embryos (10 μL) was an initial concern. Nevertheless, it has been reported by Minasi et al. (32) that reduced volume of medium is not harmful to the embryo but instead actually improves their development into blastocysts. This is thought to be a response to the increase of autocrine factors released by the preimplantation embryos (32).

Moreover, our NGS protocol has been substantially modified from our previous publication (15). In the present study, we provide evidence that modifications of the culture conditions, embryo handling, and NGS protocol can improve informativity and concordance rates, decreasing the impact of MCC in the accuracy of the diagnosis. This was a pilot study testing a new approach with different hours of embryo culture in the 10-μL drop to explore the optimal time in culture. Improved results were observed with culture time from day 4 to day 6 compared with shorter time in culture and previous publications.

In previous studies, different methodologies have been applied for WGA and aneuploidy testing. A single WGA step was used by several authors (14, 16–18), whereas Vera-Rodríguez et al. (15) conducted a double amplification. Also, array comparative genome hybridization was the technology applied in some studies (14, 18), whereas NGS has been applied in the latest publications (15–17).

Regarding clinical outcomes, we had follow-up data on a subset of patients after SET performed according to TE biopsy results and compared the clinical outcome retrospectively according to cfDNA results. Interestingly, ongoing implantation rates were three times higher when both TE and cfDNA were euploid than when euploid TE was paired with aneuploid cfDNA (52.9% vs. 16.7%, respectively), indicating that embryonic cfDNA might open a new avenue for the understanding of embryo ploidy. The origin of embryonic cfDNA is still unclear, and we do not know if it comes preferentially from ICM or TE, as also suggested by some authors (33, 34). Nonetheless, there are many open questions related to the origin of the embryonic cfDNA and the underlying mechanism that may explain potential differences in its origin in the inner cavity or SBM. To further explore the origin of embryonic cfDNA, a multicenter study adding ICM biopsy is ongoing, extending the analysis to eight centers worldwide (clinicaltrials.gov: NCT03520933).

In the present study, and in most previous ones dealing with the analysis of SBM, FNs and FPs have been determined by comparing them with TE biopsy, the criterion standard. In fact, TE biopsies are surely representative of meiotic errors. However, for detection of mitotic errors and the understanding of mosaicism, new sources of DNA, such as embryonic cfDNA that is potentially released by the cells of the whole embryo, could provide additional valuable information and help to elucidate the real impact of mosaicism on clinical outcomes, as suggested by our preliminary clinical outcomes with the use of niPGT-A.

CONCLUSION

We can conclude that a noninvasive or a "liquid biopsy" approach to study the chromosomal status of embryos could have several important advantages compared with current invasive PGT-A with the use of TE biopsy, primarily in avoiding invasiveness with potential embryo harm while extending the feasibility of PGT-A in a larger number of clinics and increasing its accessibility from a wider population of patients by minimizing laboratory and personnel expenses. Therefore, this approach would be accessible worldwide. Nevertheless, more studies are needed with new strategies to minimize external DNA contamination and to understand why euploid TE biopsies with aneuploid SBM showed poorer clinical outcomes.

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Adn embrionario libre comparado con biopsia de trofectodermo para análisis de aneuploidía: tasa de concordancia e implicaciones clínicas

Objetivo: Estudiar si el ADN embrionario libre (cfDNA) en medio de cultivo de blastocisto es representativo de la constitución cromosómica de un blastocisto.

Diseño: Estudio ciego prospectivo piloto.

Marco: Centro de fecundación in vitro y laboratorio de genética.

Pacientes: Un total de 115 biopsias de trofectodermo (TE) y medio de cultivo de blastocisto (SBM) de 46 pacientes con edades entre 32 y 46 años, cuyas indicaciones para diagnóstico genético preimplantacional para aneuploidía (PGT-A) eran edad materna avanzada, aborto de repetición, o fallo de implantación recurrente.

Intervención: Obtención de embriones tras biopsia de TE.

Medidas de resultados principales: Tasa de concordancia, sensibilidad y especificidad entre biopsia de TE y SBM. Resultados clínicos en casos de biopsia de TE euploide y SBM euploide comparados con casos de TE euploide y SBM aneuploide.

Resultados: En general, la tasa de concordancia total en ploidía y sexo fue de 78.7% y la sensibilidad y especificidad fueron 94.5% y 71.7%, respectivamente. Se observó un aumento significativo en todos los parámetros al comparar las muestras de días 6 ó 7 con las muestras de día 5, con las muestras de días 6 ó 7 mostrando una concordancia total de ploidía y sexo de 84%, y una sensibilidad y especificidad de 95.2% y 82.1%, respectivamente. La tasa de implantación en TE euploide/SBM euploide fue tres veces superior a la de TE euploide/SBM aneuploide (52.9% vs 16.7%, respectivamente), sin llegar dicha diferencia a ser significativa. Interesantemente, no se observaron abortos en casos en que el TE y el SBM eran concordantes.

Conclusiones: Estos resultados ofrecen un mejor entendimiento de las dinámicas del cfDNA durante el desarrollo embrionario y, a pesar de necesitarse más investigación básica, son alentadores para el futuro a la hora de considerar este método no invasico como alternativa a la biopsia de TE para PGT-A.