OBSTETRICS

# Multicenter prospective study of concordance between embryonic cell-free DNA and trophectoderm biopsies from 1301 human blastocysts

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**BACKGROUND:** The recent identification of embryonic cell-free DNA in spent blastocyst media has opened a new era of possibilities for noninvasive embryo aneuploidy testing in assisted reproductive technologies. Yet, previous studies assessing a limited number of embryos reported variable concordance between embryonic cell-free DNA and trophectoderm biopsies, thus questioning the validity of this approach.

**OBJECTIVE:** This study aimed to evaluate the concordance and reproducibility of testing embryonic cell-free DNA vs trophectoderm DNA obtained from the same embryo in a large sample of human blastocysts and to assess the contribution of the inner cell mass and trophectoderm to embryonic cell-free DNA released to the culture media.

**STUDY DESIGN:** This is an interim analysis of a prospective, observational study among 8 in vitro fertilization centers in 4 continents to assess consistency between noninvasive embryo aneuploidy testing of embryonic cell-free DNA and conventional trophectoderm biopsy. The analysis included 1301 day-6/7 blastocysts obtained in 406 in vitro fertilization cycles from 371 patients aged 20—44 years undergoing preimplantation genetic testing for aneuploidy. Fresh oocytes underwent intracytoplasmic sperm injection or in vitro fertilization. No previous assisted hatching or vitrification was allowed before media collection. Individual spent blastocyst medium was collected from embryos cultured at least 40 hours from day 4. After media collection, conventional preimplantation genetic testing for aneuploidy, comprising trophectoderm biopsy and blastocyst vitrification, was performed. Embryonic cell-free DNA was analyzed blindly after embryo transfer. Inner cell mass and trophectoderm biopsies were also performed in a subset of 81 aneuploid blastocysts donated for research.

**RESULTS:** Embryonic cell-free DNA analyses were 78.2% (866/1108) concordant with the corresponding trophectoderm biopsies. No significant differences were detected among centers ranging from 72.5% to 86.3%. Concordance rates exceeded 86% when all defined steps in the culture laboratory were controlled to minimize the impact of maternal and operator contamination. Sensitivity per center ranged from 76.5% to 91.3% and specificity from 64.7% to 93.3%. The false-negative rate was 8.3% (92/1108), and false-positive rate was 12.4% (137/1108). The 2 fertilization techniques provided similar sensitivity (80.9% vs 87.9%) and specificity (78.6% vs 69.9%). Multivariate analysis did not reveal any bias from patient clinical background, ovarian stimulation protocols, culture conditions, or embryo quality on testing accuracy of concordance. Moreover, concordances of embryonic cell-free DNA with trophectoderm and inner cell mass suggest that the embryonic cell-free DNA originates from both compartments of the human embryo.

**CONCLUSION:** Noninvasive analysis of embryonic cell-free DNA in spent blastocyst culture media demonstrates high concordance with trophectoderm biopsy results in this large multicenter series. A noninvasive approach for prioritizing embryo euploidy offers important advantages such as avoiding invasive embryo biopsy and decreased cost, potentially increasing accessibility for a wider patient population.

**Key words:** embryo aneuploidy testing, embryonic cfDNA, IVF, noninvasive PGT-A, spent blastocyst media

Since the isolation of fetal cell-free DNA (cfDNA) from the peripheral maternal blood,[1] interest has grown in the potential for its use in prenatal diagnosis. In fact, noninvasive prenatal testing (NIPT) through the analysis of fetal cfDNA has dramatically changed clinical practice in prenatal care for the detection of fetal aneuploidies during the first trimester of pregnancy.[2,3] The use of NIPT has brought sharp decreases in the use of invasive procedures such as amniocentesis or chorionic villus biopsy,[4—6] and indications for its use continue to expand.[7]

In the setting of assisted reproduction, embryonic cfDNA is offering a new strategy to assess the preimplantation embryo. Current practice for preimplantation genetic testing of aneuploidies (PGT-A) requires embryo manipulation, specifically a trophectoderm (TE) biopsy, with the retrieval of 4—8 cells and the analysis of chromosome copy number by next-generation sequencing (NGS).[8] However, biopsy-based approaches on preimplantation embryos entail both technical and embryo viability challenges together with the criticism that in some cases normal embryos may be discarded.[9] A noninvasive method for detecting aneuploidies in the preimplantation embryo would avoid the technical challenges of biopsy. In particular, analyzing the cfDNA released by the embryo to the culture media during the latest stages of in vitro

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## AJOG at a Glance

### Why was this study conducted?

Variable concordance rates have been reported for aneuploidy analysis of embryonic cell-free DNA (cfDNA) in spent blastocyst media vs trophectoderm (TE) biopsy for the same blastocyst in a series with small sample sizes.

### Key findings

In 1301 human blastocysts from 8 reproductive centers in 4 continents, the chromosomal analysis concordance rate for embryonic cfDNA with the corresponding TE DNA was 78.2% (866/1108). Simultaneous analyses of TE and inner cell mass suggest that the embryonic cfDNA originates from both compartments of the human embryo.

### What does this add to what is known?

This is the largest study to date assessing ploidy concordance per embryo between invasive and noninvasive preimplantation genetic testing for aneuploidies. This noninvasive approach could help avoid embryo biopsy while still enabling embryo euploidy prioritization and reduce costs, thereby democratizing accessibility for a wider patient population.

development, namely the blastocyst stage, could identify any aneuploidies in the embryo before its transfer into the uterine cavity.[10]

Initial isolation of embryonic cfDNA from the spent blastocyst media (SBM) indicated the potential to identify embryonic chromosome copy number.[10] Subsequent studies explored the concordance between embryonic cfDNA in SBM and DNA from the human embryo obtained from polar bodies,[11] TE biopsies,[12–15] and whole blastocysts.[16] These studies reported informativity rates for the embryonic cfDNA (ie, successful cfDNA amplification and interpretable NGS results) ranging from 80% to 100%. However, concordance rates varied from 3.4% to 85.7% in polar bodies and TE biopsies.[10–15] Yet, concordance reached 93.8% when compared with the whole blastocyst, suggesting that embryonic cfDNA in spent media could be more representative of the embryonic chromosomal status than the TE biopsy.[16] In these previous studies of noninvasive PGT-A (niPGT-A) from other groups[10–16] before media collection, embryos were manipulated by assisted hatching, biopsy, and vitrification.

For clinical translational purposes, we modified the strategy to isolate and amplify embryonic cfDNA without previous embryo manipulation by collecting spent media from embryos with intact zona pellucida. Our pilot study achieved high concordance rates between embryonic cfDNA from spent media and TE biopsies after optimizing the protocol for media collection and embryonic cfDNA amplification at day 6.[17] Of note, in those cases with discrepancies between euploid TE biopsies and aneuploid embryonic cfDNA, clinical results were affected in terms of a decrease in ongoing pregnancies and increase in rates of miscarriages.[17] Furthermore, our pilot study and the earlier studies noted previously were limited by the number of embryos analyzed, with sample sizes ranging from 22[11] to 115[17] from individual assisted reproductive centers.

To determine the clinical utility of niPGT-A, we report a prospective multicenter study to evaluate the concordance and reproducibility of the analysis of embryonic cfDNA vs TE biopsies from the same embryo in a large sample size: 1301 blastocysts from 8 assisted reproductive centers in 4 continents. Furthermore, we assessed the contribution of the inner cell mass (ICM) and TE to the embryonic cfDNA in a subset of 81 aneuploid blastocysts.

## Materials and Methods

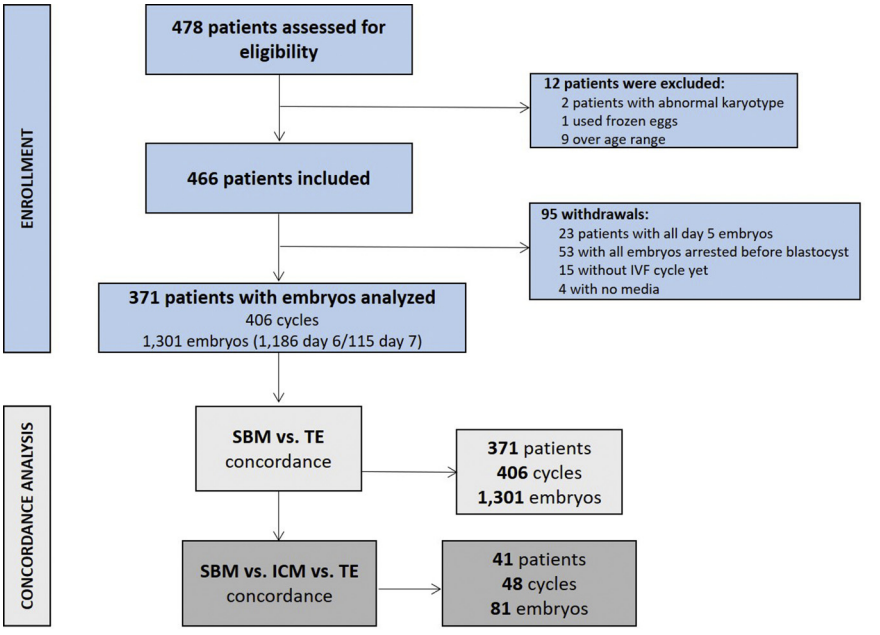
Detailed descriptions of the materials and methods used in this study are provided in the Supplemental Materials and Methods section. We report the interim analysis of a prospective, observational, multicenter study performed in 8 assisted reproductive centers; the study was approved by each local institutional review board. The study design is presented in Figure 1. TE biopsies and SBM were collected from April 2018 to September 2019 for 1301 day-6/7 blastocysts, from 406 cycles (371 patients). After intracytoplasmic sperm injection (ICSI) or in vitro fertilization (IVF) in fresh oocytes, embryos were cultured using the standard culture conditions of each laboratory up to day 4. On day 4, embryos were individually transferred to a 10-$\mu$L drop of fresh media. No previous assisted hatching or embryo vitrification was allowed before media collection. Individual SBM was collected at day 6/7 and frozen until analysis. Embryonic cfDNA samples were analyzed by NGS. Results were evaluated with a specific algorithm developed for embryonic cfDNA and compared with the NGS results obtained from TE biopsies. ICM biopsy was also performed in a subset of 81 aneuploid blastocysts donated for research. Concordance rates, false-positive (FP) and false-negative (FN) rates, specificity, sensitivity, positive predictive value (PPV), and negative predictive value (NPV) were determined.

## Results

From 371 patients and 406 PGT-A cycles (337 patients with 1 cycle, 33 patients with 2 cycles, 1 patient with 3 cycles), 1301 embryos were assessed. The most common indication for PGT-A was advanced maternal age (66.2% of cases). Baseline demographic and clinical background details are presented in Supplemental Table 1.

In 98.7% of cases, antagonist protocol was employed for gonadal suppression; the most common stimulation protocol included follicle-stimulating hormone plus luteinizing hormone (53.6%) and gonadotropin-releasing hormone agonist for triggering ovulation (52.7% of the

**FIGURE 1**
**Flow diagram of the patients included in the study**




*ICM*, inner cell mass; *SBM*, spent blastocyst medium; *TE*, trophectoderm.

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cycles). A mean (±standard deviation [SD]) of 10.3 (7.1) metaphase II oocytes were aspirated and 8.1 (6.0) were normally fertilized. Most (88.2%) cycles used the patient's own oocytes, and ICSI was conducted in 90.6% of cycles. One center also performed routine insemination of oocytes. Ovarian stimulation and cycle characteristics are presented in Supplemental Table 2.

Embryonic culture conditions used in this study were diverse. A 1-step system (varying in commercial culture media) was used in 93.1% of cycles. MIRI (ESCO, Egaa, Denmark) (33.5%) and K-Systems (CooperSurgical, Målov, Denmark) (32.3%) were the most used incubators, and 5% albumin supplementation was incorporated in 72% of cycles. Biopsies for most of the 1301 blastocysts were conducted on day 6 (91.2%); at the time of biopsy, most at the expanded blastocyst stage (68.8%). Blastocyst quality is described in Table 1.

## Embryonic cfDNA concordance with TE biopsies

To compare chromosome copy number results between embryonic cfDNA and TE biopsy, stratification was applied according to the Standards for Reporting of Diagnostic Accuracy Studies criteria (Figure 2), where the index test was the embryonic cfDNA analyzed from spent media and the reference standard was the TE biopsy. DNA amplification failure (AF) was observed in 34 (2.6%) embryonic cfDNA samples and 14 (1.1%) TE biopsies. Noninformative (NI) results were obtained in 70 (5.4%) embryonic cfDNA samples and 26 (2.0%) TE biopsies. A chaotic profile (>5 aneuploidies) was observed in 55 (4.2%) embryonic cfDNA samples and considered as NI because the percentage of euploid TE biopsies for this type of samples (49%) was similar to the percentage for AF (47%) and for NI (57%). Therefore, the overall percentage of NI SBM samples including chaotic samples was significantly higher than that for PGT-A ($P<.0001$), where TE biopsies with >5 aneuploidies were considered as chaotic abnormal. In total, 1108 blastocysts (85.2%) had informative results in both TE and embryonic cfDNA samples.

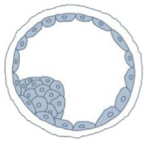
The embryo ploidy concordance rate between informative samples for TE biopsies and cfDNA was 78.2% (866/1108), ranging from 72.5% to 86.3% in different centers. An additional 1.1% (13/1108) were concordant for embryo ploidy but not sex chromosomes. The total concordance rate for all chromosomes was 67.7%, and partial concordance for some chromosomes was 10.5%. Overall concordance rate for trisomies was 77.3% and 70.1% for monosomies.

Regarding subchromosomal abnormalities, we were able to detect duplications and deletions in the SBM above 10 Mb, and the concordance rates with TE biopsies were significantly lower ($P<.0001$) than those for whole-chromosome abnormalities: 52.2% for deletions and only 10.3% for duplications. For this reason, in the prioritization system we are developing, subchromosomal abnormalities, more specifically duplications, would be prioritized for transfer before other abnormalities.

We analyzed the concordance rates according to female age (stratified into 5 groups), which revealed a significant linear trend with increasing age ($P=.02$) (Figure 3, A). In contrast, we did not detect significant differences in concordance when stratifying by culture media (Figure 3, B) or incubator model (Figure 3, C).

Overall sensitivity per center ranged from 76.5% to 91.3% and specificity from 64.7% to 93.3% (Table 2; also shows PPV and NPV). In the center with higher concordance rate, 86.3%, sensitivity and specificity were 86.7% and 87.5%, respectively, indicating that optimal results can be achieved when strictly following the defined steps to minimize external and maternal contamination. The FN rate was 8.3% (92/1108), half of which (46/92) corresponded to female TE biopsies and the other half (46/92) to male TE biopsies. The FP rate was 12.4% (137/1108), 43.8% (60/137) of which corresponded to female TE biopsies and 56.2% (77/137) to male TE biopsies. Stratified results for FP indicated 16 SBM with duplications (11.7%), 6 SBM with deletions (4.4%), 28 SBM with single monosomies (20.4%), 33 SBM with single trisomies

**TABLE 1**
**ICM and TE quality of day-6 and day-7 blastocysts**

| Blastocyst quality assessment | | Day 6 | Day 7 | Total |
|---|---|---|---|---|
| | Total no. of blastocysts, n (%) | 1186 (91.2) | 115 (8.8) | 1301 (100) |
|  Blastocyst grading[a] | Early blastocyst | 8/1186 (0.7) | 0 | 8/1301 (0.6) |
| | Blastocyst (cavitated) | 47/1186 (4.0) | 3/115 (2.6) | 50/1301 (3.8) |
| | Expanded blastocyst | 809/1186 (68.2) | 86/115 (74.8) | 895/1301 (68.8) |
| | Hatching blastocyst | 213/1186 (17.9) | 14/115 (12.2) | 227/1301 (17.5) |
| | Fully hatched | 90/1186 (7.6) | 11/115 (9.5) | 101/1301 (7.8) |
| | N/A | 19/1186 (1.6) | 1/115 (0.9) | 20/1301 (1.5) |
| ICM quality | A | 550/1186 (46.4) | 19/115 (16.5) | 569/1301 (43.7) |
| | B | 494/1186 (41.6) | 60/115 (52.2) | 554/1301 (42.6) |
| | C | 142/1186 (12.0) | 36/115 (31.3) | 178/1301 (13.7) |
| TE quality | A | 591/1186 (49.8) | 15/115 (13.0) | 606/1301 (46.6) |
| | B | 417/1186 (35.2) | 60/115 (52.2) | 477/1301 (36.6) |
| | C | 178/1186 (15.0) | 40/115 (34.8) | 218/1301 (16.8) |

Values are provided as n/N (%) unless indicated otherwise.

*ICM*, inner cell mass; *N/A*, not available; *TE*, trophectoderm.

[a] Blastocyst classification following modified Gardner's criteria.

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(24.1%), and the remaining 54 with combinations of aneuploidies with more than 1 chromosome (39.4%). However, no significant differences were observed in sensitivity (85.0% and 78.4%) and specificity (75.0% and 79.7%) between cfDNA diagnosed as male and female, respectively. Moreover, ICSI and IVF techniques provided similar sensitivity (80.9% vs 87.9%) and specificity (78.6% vs 69.9%).

### Multivariate logistic regression analysis

Multivariate logistic regression was applied to assess the impact of patient characteristics, ovarian stimulation, embryonic culture, and blastocyst quality on informativity and concordance rates. None of these factors significantly affected informativity or concordance.

### Contribution of the ICM and TE to the embryonic cfDNA

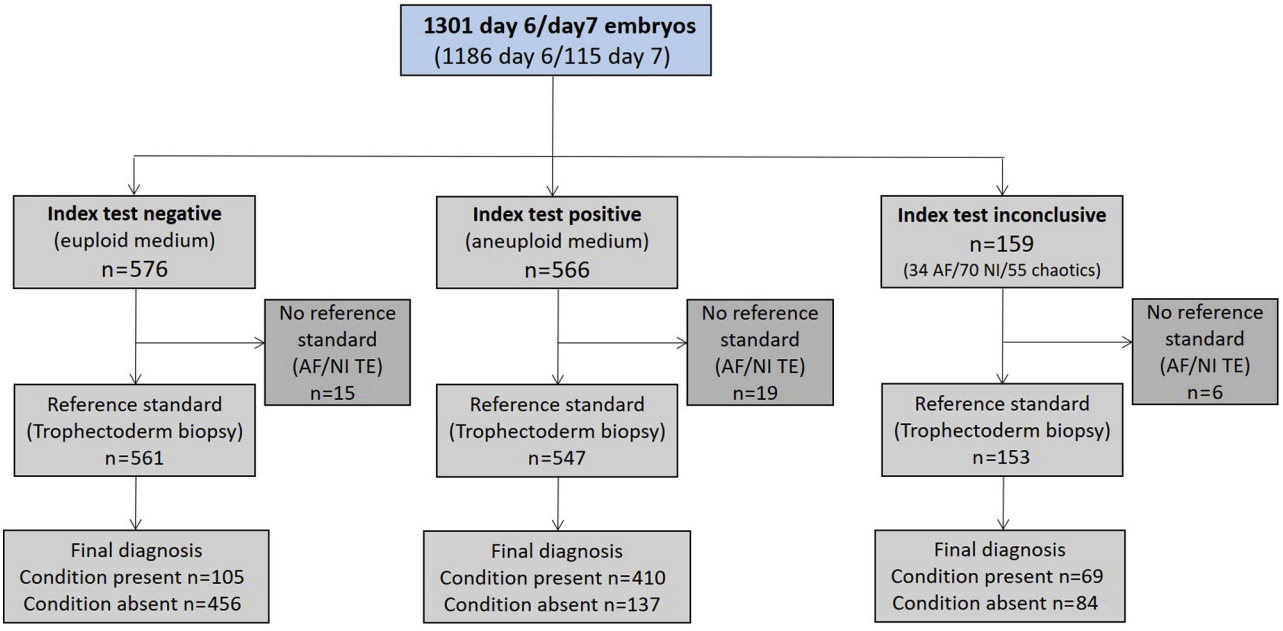
Among blastocysts deemed as aneuploid by initial TE biopsy donated for assessment of concordance to ICM, informative results were obtained in 97.5% (79/81) of TE biopsies, 90.1% (73/81) of embryonic cfDNA samples, and 90.1% (73/81) of ICM biopsies. All 3 sample types were informative in 79.0% (64/81) (Table 3). Briefly, embryonic cfDNA ploidy concordances with TE and with ICM were similar (87.5% and 84.4%, respectively). Among 10 discrepancies between embryonic cfDNA and ICM, 4 corresponded to aneuploid cfDNA and euploid ICM, which could be attributed to the presence of mosaicism in the blastocyst (TE biopsies were also aneuploid). The 6 remaining discrepancies corresponded to euploid cfDNA and aneuploid ICM, mostly caused by the presence of maternal contamination masking the aneuploidies. Considering ICM as the gold standard, ploidy concordance rates for embryonic cfDNA and TE was 84.4% and 87.5%, respectively, which were not statistically significant different. FP rates were similar for embryonic cfDNA and TE biopsy (6.2% and 10.9%, respectively); FN rates did not significantly differ but were higher in embryonic cfDNA (9.4%) than in TE (1.6%) owing to potential maternal contamination. Examples of concordance among embryonic cfDNA (SBM), TE, and ICM are shown in Figure 4.

## Discussion

### Principal findings

An efficient noninvasive approach can offer the benefits of invasive PGT-A, such as improved live birth at the first embryo transfer, reduction of miscarriage rates and multiple pregnancies, and shortened time to pregnancy,[18] without the limitations of embryo manipulation and biopsy. Our multicenter study assessing a niPGT-A approach in a total of 1301 blastocysts found high concordance rates between embryonic cfDNA and corresponding TE biopsies (78.2%). The lack of significant differences in results among the 8 centers indicates reproducibility under varied laboratory conditions. Moreover, the concordances of embryonic cfDNA with TE and ICM suggest that the embryonic cfDNA could originate from both compartments of the human embryo.

FIGURE 2
**Standards for Reporting of Diagnostic Accuracy Studies diagram for accuracy comparison SBM between TE**




*AF*, amplification failure; *NI*, noninformative; *SBM*, spent blastocyst medium; *TE*, trophectoderm.

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## Results

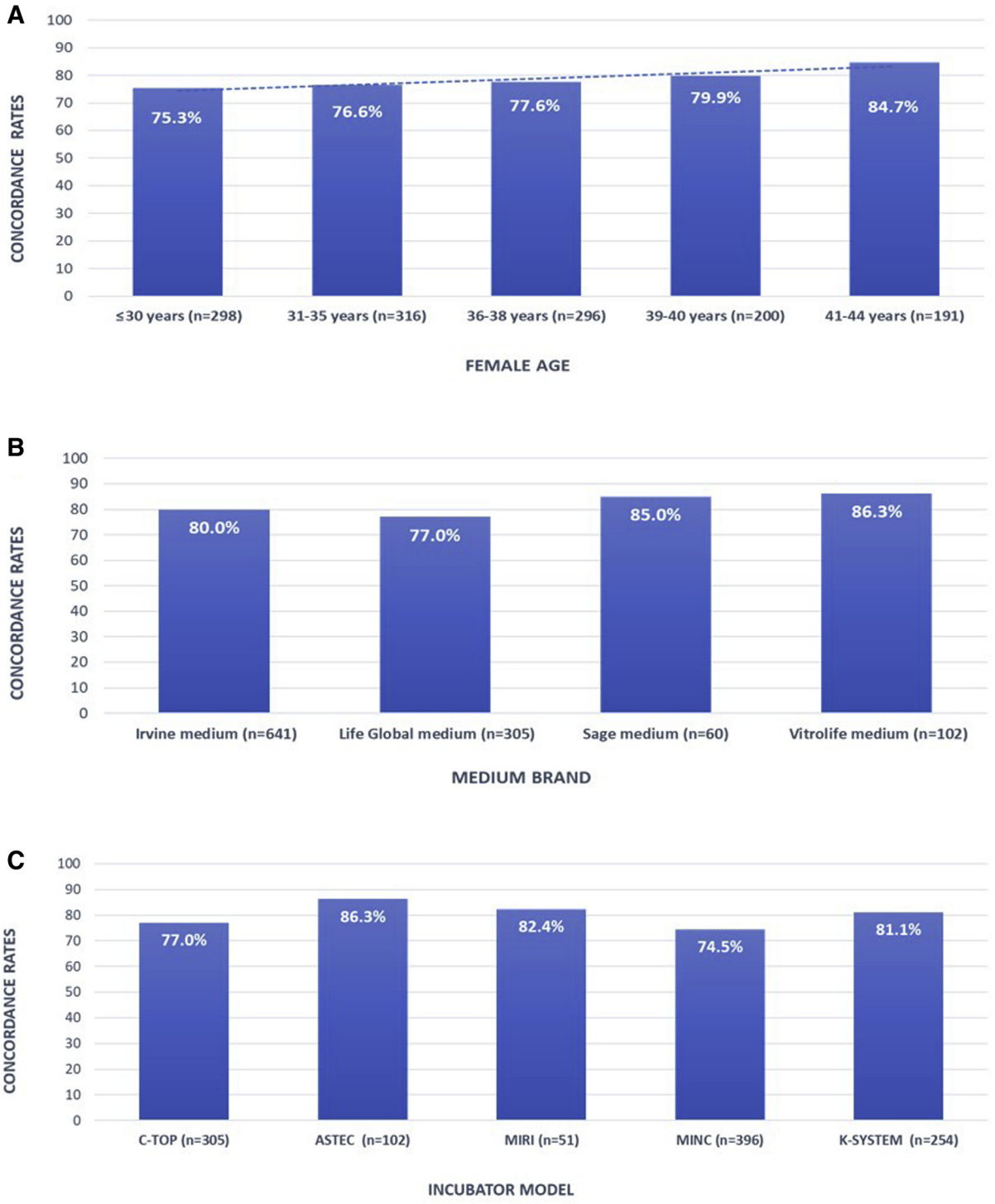
This is the largest study to date to assess concordance of embryo ploidy between embryonic cfDNA and TE biopsy. The findings align with recent publications indicating concordance rates between 62.1% and 85.7%.[12,14–17] At the same time, our study differs from these previous reports. First, the analysis of this large sample of 1301 human embryos incorporated variable culture conditions among the 8 participating centers. Second, our study protocol was truly noninvasive, without any previous manipulation of the embryos (eg, day-3 assisted hatching, previous vitrification). Third, other studies used a minimally invasive approach combining SBM and blastocoelic fluid and requiring an extra manipulation step (blastocyst collapse).[19–21] These small-sample studies reported variable concordance of embryonic cfDNA with TE (45.0%, 87.5%, and 95.3%).

In addition, our study investigated the origin of embryonic cfDNA using 81 blastocysts donated for research. Embryonic cfDNA ploidy was concordant with both TE and ICM biopsies (87.5% and 84.4%, respectively). The discordant findings reflected either mosaicism in the blastocyst or the presence of maternal contamination. Embryonic cfDNA ploidy was concordant not only with TE biopsies, as in previous studies, but also with ICM biopsies, suggesting that both TE and ICM could contribute to the embryonic cfDNA. However, our results are in contrast with other publications suggesting a superiority of the embryonic cfDNA over the TE biopsies when compared with whole blastocysts.[16,18,19]

## Clinical implications

Laboratory and culture conditions vary between different reproductive centers worldwide, which could have implications for clinical testing. Of note, we observed consistent results for niPGT-A among centers. These 8 centers used 4 different culture media, sequential and continuous, and 2 different percentages of albumin supplementation (5% and 10%), without significant effects on the accuracy of embryonic cfDNA results. These findings support the potential applicability of embryonic cfDNA analysis to every IVF laboratory, when working under the specific protocol. A previous study comparing 3 commercial culture media without previous contact with an embryo reported consistently low DNA concentration. Moreover, the minimal traces of DNA were mostly derived from protein supplementation (albumin 5 mg/mL).[22] Continuous culture systems (embryos left in the same medium from day 1 to day 6) have also been proposed to impair the yield and quality of embryonic cfDNA.[22,23] Across these 8 centers, culture media were changed at day 4, which discarded potentially degraded DNA secreted from days 1 to 3. In addition, the day and volume of media collection were optimized to achieve a higher embryonic cfDNA concentration without compromising embryo quality. In a previous study, we observed that concordance rates of embryonic cfDNA and TE biopsies were significantly better when the spent media were collected at day 6/7 rather than day 5.[17] This enabled us to standardize the media collection protocol for clinical practice.

FIGURE 3
**Concordance rates**




**A,** Concordance rates according to female age. This figure shows no significant differences among the 5 age groups, but a significant linear trend with increasing female age (chi-square test for trend; $P$=.0212). **B,** Concordance rates between embryonic cell-free DNA and trophectoderm biopsies according to different culture media brands. No significant differences were found. **C,** Concordance rates between embryonic cell-free DNA and trophectoderm biopsies according to different incubator models. No significant differences were found.

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**TABLE 2**
**Percentages of concordance between TE biopsies and embryonic cfDNA, sensitivity, specificity, PPV, and NPV per center**

| Statistical measures | Center 1 | Center 2 | Center 3 | Center 4 | Center 5 | Center 6 | Center 7 | Center 8 | Total |
|---|---|---|---|---|---|---|---|---|---|
| Concordance | 75.6 | 77.1 | 81.8 | 86.3 | 84.2 | 85.0 | 72.5 | 77.0 | 78.2 |
| Sensitivity | 80.5 | 84.8 | 88.2 | 86.7 | 91.3 | 76.7 | 76.5 | 78.9 | 81.7 |
| Specificity | 69.9 | 72.7 | 85.2 | 87.5 | 80.0 | 93.3 | 64.7 | 78.1 | 77.4 |
| PPV | 83.7 | 66.9 | 79.0 | 74.3 | 87.5 | 92.0 | 81.3 | 65.1 | 75.0 |
| NPV | 65.2 | 88.0 | 92.0 | 94.0 | 85.7 | 80.0 | 57.9 | 87.7 | 83.6 |

*cfDNA*, cell-free DNA; *NPV*, negative predictive value; *PPV*, positive predictive value; *TE*, trophectoderm.
*Rubio. Embryonic cell-free DNA released to the spent blastocyst media. Am J Obstet Gynecol 2020.*

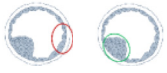
## Strengths

Our study provides a strong analysis of concordance rates through a large sample size (1301 embryos), a variety of culture conditions (8 centers from 4 continents), and different insemination techniques. Furthermore, we compared the results of embryonic cfDNA with ICM biopsies (the real representation of the embryo) in a subset of blastocysts, with high concordance. The findings bring closer the clinical application of a noninvasive approach that would help avoid embryo manipulation, decreasing the complexity of the IVF laboratory and the cost of analysis.

## Limitations

Two potential concerns were raised by embryologists participating in this study: the small volume of embryo culture medium (10 $\mu$L) and the expanded culture to day 6 regardless of morphology at day 5. First, a reduced volume of medium could positively

**TABLE 3**
**Concordance rates between embryonic cfDNA from SBM, ICM, and TE biopsies**

| | Results | SBM vs TE  | SBM vs ICM  | TE vs ICM  |
|---|---|---|---|---|
| | ICM biopsies, N | 81 | 81 | 81 |
| Informativity | Informative ICM | — | 73/81 (90.1) | 73/81 (90.1) |
| | Informative TE | 79/81 (97.5) | — | 79/81 (97.5) |
| | Informative SBM | 73/81 (90.1) | 73/81 (90.1) | — |
| | Informative all | 64/81 (79.0) | 64/81 (79.0) | 64/81 (79.0) |
| Embryo concordances | Global concordance | 56/64 (87.5) | 54/64 (84.4) | 56/64 (87.5) |
| | Concordance for all chromosomes | 44/64 (68.8) | 44/64 (68.8) | 48/64 (75.0) |
| | Concordance for some chromosomes | 12/64 (18.7) | 10/64 (15.6) | 8/64 (12.5) |
| Embryo discordances | False-positive rate | 0 | 4/64 (6.2) | 7/64 (10.9) |
| | False-negative rate | 8/64 (12.5) | 6/64 (9.4) | 1/64 (1.6) |
| | Only sex discordance euploid | 0 | 0 | 0 |
| Contamination | Maternal contamination | 8/64 (12.5) | 8/64 (12.5) | 0 |
| | Male contamination | 0 | 0 | 0 |
| | Polar body contamination | 0 | 0 | 0 |

Values are provided as n/N (%) unless indicated otherwise.

No significant differences in concordance rates among groups. Maternal and male contamination identified when discordant sex was observed in SBM compared with TE and ICM.

*cfDNA*, cell-free DNA; *ICM*, inner cell mass; *SBM*, spent blastocyst medium; *TE*, trophectoderm.
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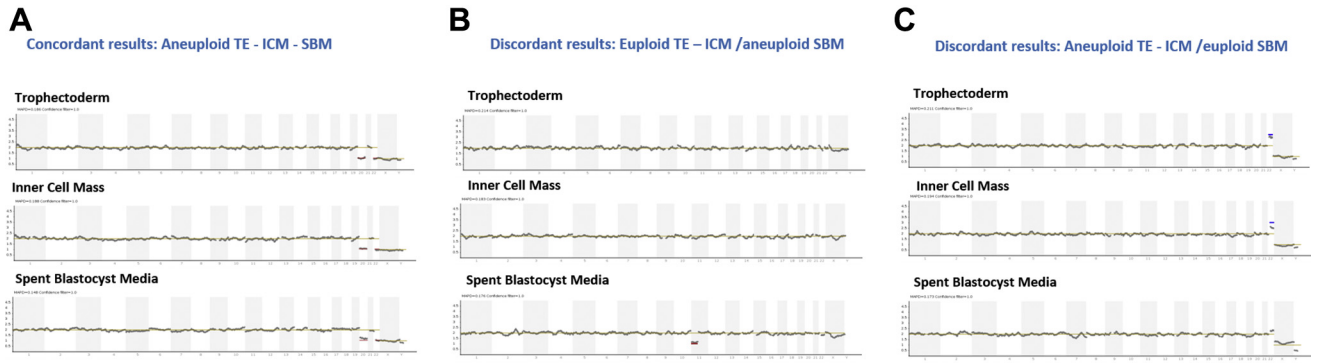
FIGURE 4
**Profiles showing concordant (A) and discordant results (B, C) of SBM vs TE and ICM**




*ICM*, inner cell mass; *SBM*, spent blastocyst medium; *TE*, trophectoderm.

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affect blastocyst development because the autocrine factors produced by the embryo could exert a positive effect on its development.[24] Second, we noted that >75% of blastocysts were expanded blastocysts, classified as good quality for ICM and TE, indicating that expanded culture did not affect embryo quality. In a previous study,[25] slow embryos at day 5 had better prognosis when transferred at day 6. In our study, embryos with good quality at day 5 were also good-quality embryos at day 6. The correlation between the day of blastocyst transfer and clinical outcome was addressed in a recent meta-analysis that concluded that implantation, clinical pregnancy, live birth, and ongoing pregnancy rates are better for day-5 transfer than for day-6 transfer in untested embryos.[26] However, when PGT-A is performed, clinical outcomes are similar for euploid day-5 vs euploid day-6 blastocyst transfer.[26] In fact, when the chromosomal content rather than the day of transfer is considered, no significant differences are detected in the sustained implantation rates between day-5 and day-6 single embryo transfers.[27] These differences in outcome for tested vs untested day-5 and day-6 embryos could be attributed to a higher euploidy rate of day-5 than day-6 embryos, and therefore, the selection of euploid embryos would eliminate the differences. In addition, in most of the published studies, day-6 embryos were slow embryos; in our study, all the embryos were cultured until day 6 independent of their developmental stage at day 5. Therefore, niPGT-A could be applied to all day-6 embryos, good-quality embryos, and slow-developing embryos, as long as they reach blastocyst stage. Slower embryos, if they are chromosomally normal, will benefit the most from this strategy. The clinical outcomes for blastocyst transfer according to morphology in untested embryos compared with those for blastocyst transfer according to cfDNA analysis of day-6 embryos will be addressed in an ongoing randomized controlled trial (ClinicalTrials.gov#NCT4000152).

## Conclusion

A niPGT-A approach could be reliably applied under defined embryo handling and culture conditions with a customized NGS protocol and diagnostic algorithm. Furthermore, differing ovarian stimulation protocols, culture conditions, and embryo quality do not affect the accuracy of niPGT-A results. In addition, concordance rates of embryo cfDNA with ICM were similar to TE biopsies. We also identified different concordance rates for different types of chromosomal and subchromosomal abnormalities, which allowed us to develop a prioritization system that could be applied to a wider scope of patients undergoing IVF and doctors reluctant to apply an invasive approach, with or without indication for PGT-A, to rank embryos with higher chances of euploidy, to decrease miscarriage, and increase live birth rates.

niPGT-A could have several important advantages compared with current invasive PGT-A using TE biopsy, primarily in avoiding invasiveness and potential embryo harm, while extending testing feasibility to a larger number of clinics and increasing accessibility for a wider population of patients through reduced costs. Nevertheless, more studies are needed to understand the origin of the embryonic cfDNA and the mechanisms involved. ■

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## References

1. Lo YM, Corbetta N, Chamberlain PF, et al. Presence of fetal DNA in maternal plasma and serum. Lancet 1997;350:485–7.
2. Lefkowitz RB, Tynan JA, Liu T, et al. Clinical validation of a noninvasive prenatal test for genomewide detection of fetal copy number variants. Am J Obstet Gynecol 2016;215:227.e1–16.
3. Qiao L, Yu B, Liang Y, et al. Sequencing shorter cfDNA fragments improves the fetal DNA fraction in noninvasive prenatal testing. Am J Obstet Gynecol 2019;221:345.e1–11.
4. Bianchi DW, Parker RL, Wentworth J, et al. DNA sequencing versus standard prenatal

aneuploidy screening. N Engl J Med 2014;370:799–808.

**5.** Williams J 3rd, Rad S, Beauchamp S, et al. Utilization of noninvasive prenatal testing: impact on referrals for diagnostic testing. Am J Obstet Gynecol 2015;213:102.e1–6.

**6.** Petersen AK, Cheung SW, Smith JL, et al. Positive predictive value estimates for cell-free noninvasive prenatal screening from data of a large referral genetic diagnostic laboratory. Am J Obstet Gynecol 2017;217:691.e1–6.

**7.** Di Renzo GC, Bartha JL, Bilardo CM. Expanding the indications for cell-free DNA in the maternal circulation: clinical considerations and implications. Am J Obstet Gynecol 2019;220:537–42.

**8.** Rubio C, Rodrigo L, Garcia-Pascual C, et al. Clinical application of embryo aneuploidy testing by next-generation sequencing. Biol Reprod 2019;101:1083–90.

**9.** Gleicher N, Vidali A, Braverman J, et al. Accuracy of preimplantation genetic screening (PGS) is compromised by degree of mosaicism of human embryos. Reprod Biol Endocrinol 2016;14:54.

**10.** Shamonki MI, Jin H, Haimowitz Z, Liu L. Proof of concept: preimplantation genetic screening without embryo biopsy through analysis of cell-free DNA in spent embryo culture media. Fertil Steril 2016;106:1312–8.

**11.** Feichtinger M, Vaccari E, Carli L, et al. Non-invasive preimplantation genetic screening using array comparative genomic hybridization on spent culture media: a proof-of-concept pilot study. Reprod Biomed Online 2017;34:583–9.

**12.** Xu J, Fang R, Chen L, et al. Noninvasive chromosome screening of human embryos by genome sequencing of embryo culture medium for in vitro fertilization. Proc Natl Acad Sci USA 2016;113:11907–12.

**13.** Vera-Rodriguez M, Diez-Juan A, Jimenez-Almazan J, et al. Origin and composition of cell-free DNA in spent medium from human embryo culture during preimplantation development. Hum Reprod 2018;33:745–56.

**14.** Ho JR, Arrach N, Rhodes-Long K, et al. Pushing the limits of detection: investigation of cell-free DNA for aneuploidy screening in embryos. Fertil Steril 2018;110:467–75.e2.

**15.** Yeung QSY, Zhang YX, Chung JPW, et al. A prospective study of non-invasive preimplantation genetic testing for aneuploidies (NiPGT-A) using next-generation sequencing (NGS) on spent culture media (SCM). J Assist Reprod Genet 2019;36:1609–21.

**16.** Huang L, Bogale B, Tang Y, Lu S, Xie XS, Racowsky C. Noninvasive preimplantation genetic testing for aneuploidy in spent medium may be more reliable than trophectoderm biopsy. Proc Natl Acad Sci USA 2019;116:14105–12.

**17.** Rubio C, Rienzi L, Navarro-Sánchez L, et al. Embryonic cell-free DNA versus trophectoderm biopsy for aneuploidy testing: concordance rate and clinical implications. Fertil Steril 2019;112:510–9.

**18.** Rubio C, Bellver J, Rodrigo L, et al. In vitro fertilization with preimplantation genetic diagnosis for aneuploidies in advanced maternal age: a randomized, controlled study. Fertil Steril 2017;107:1122–9.

**19.** Li P, Song Z, Yao Y, et al. Preimplantation genetic screening with spent culture medium/blastocoel fluid for in vitro fertilization. Sci Rep 2018;8:9275.

**20.** Kuznyetsov V, Madjunkova S, Antes R, et al. Evaluation of a novel non-invasive preimplantation genetic screening approach. PLoS One 2018;13:e0197262.

**21.** Jiao J, Shi B, Sagnelli M, et al. Minimally invasive preimplantation genetic testing using blastocyst culture medium. Hum Reprod 2019;34:1369–79.

**22.** Hammond ER, McGillivray BC, Wicker SM, et al. Characterizing nuclear and mitochondrial DNA in spent embryo culture media: genetic contamination identified. Fertil Steril 2017;107:220–8.e5.

**23.** Belandres D, Shamonki M, Arrach N. Current status of spent embryo media research for preimplantation genetic testing. J Assist Reprod Genet 2019;36:819–26.

**24.** Minasi MG, Fabozzi G, Casciani V, et al. Improved blastocyst formation with reduced culture volume: comparison of three different culture conditions on 1128 sibling human zygotes. J Assist Reprod Genet 2015;32:215–20.

**25.** Tannus S, Cohen Y, Henderson S, et al. Fresh transfer of day 5 slow-growing embryos versus deferred transfer of vitrified, fully expanded day 6 blastocysts: which is the optimal approach? Hum Reprod 2019;34:44–51.

**26.** Bourdon M, Pocate-Cheriet K, Finet de Bantel A, et al. Day 5 versus day 6 blastocyst transfers: a systematic review and meta-analysis of clinical outcomes. Hum Reprod 2019;34:1948–64.

**27.** Tiegs AW, Sun L, Patounakis G, Scott RT. Worth the wait? Day 7 blastocysts have lower euploidy rates but similar sustained implantation rates as day 5 and day 6 blastocysts. Hum Reprod 2019;34:1632–9.

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## Supplemental Materials and Methods

### Study design

We performed a prospective, observational, multicenter study to validate noninvasive preimplantation genetic testing of aneuploidies (PGT-A) by sequencing the embryonic cell-free DNA (cfDNA) obtained from the spent blastocyst media (SBM) vs the most common (invasive) PGT-A approach using trophectoderm (TE) biopsy from a blastocyst.

### Study participants

TE biopsies and spent media collection were performed from April 2018 to September 2019 at 8 different reproductive centers. Women aged 20−44 years enrolled in the PGT-A program for different indications at the participating centers in Europe, North and South America, and Asia were included. Ovum and sperm donation cases were included; fertilization techniques were intracytoplasmic sperm injection or in vitro fertilization (IVF) in fresh oocytes; sperm used was fresh or frozen. Carriers of structural abnormalities or monogenic diseases were excluded. Patients who fulfilled inclusion criteria provided their written informed consent to participate in the study.

At the time of interim analysis, 478 patients had been assessed for eligibility; 12 patients were excluded for not meeting the inclusion criteria at the time of PGT-A (2 patients had abnormal karyotypes, cycle oocytes were frozen in 1 patient, and 9 women were older than the defined female age range), and 95 patients were classified as early withdrawal. Finally, 371 patients were included, corresponding to 406 cycles from which 1301 day-6/7 blastocysts were obtained.

In addition, the contribution of the inner cell mass (ICM) to the embryonic cfDNA in spent media was assessed using 81 aneuploid blastocysts (48 cycles in 41 patients, with biopsies performed between December 2019 and March 2020) (Figure 1).

### Ethical approval

Ethical approval for the study was obtained from the Western Institutional Review Board (August 16 and 24, 2018) for Boston IVF and San Diego Fertility Center (San Diego, CA); Institutional review board from Clinica Valle Giulia (March 8, 2018) for Genera (Italy); Istanbul Bilim Üniversitesi Klinik Arastirmalari Ethics Committee (September 25, 2018) for Bahçeci Health Group (Turkey); institutional review board from the Centro de Educación Médica e Investigaciones Clínicas "Norberto Quirno" (November 11, 2018) for Pregna Medicina Reproductiva (Argentina); La Princesa Ethics Committee (July 26, 2018) for Procreatec (Spain); Universidade Federal do Rio Grande do Sul e CONEP Ethics Committee (April 17, 2019) for Nilo Frantz − Centro de Reproduçao Humana (Brazil); and Hospital ABC Ethics Committee (October 8, 2018) for Nascere clinic (Mexico). The study protocol was registered at ClinicalTrials.gov (NCT03520933); as previously planned, we report here the interim results in 1301 embryos.

### IVF cycle, embryonic culture, blastocyst media collection, TE biopsy, and vitrification

Controlled ovarian stimulation (COS) and induction of ovulation were performed using standard protocols in each of the participant sites according to female age, basal hormone levels, basal ovarian reserve, and body mass index. The COS protocol was determined by the enrolling physician. ICSI or IVF was performed according to the protocols of the participating sites.

Embryos were cultured according to the standard conditions of each IVF laboratory using 1- or 2-step culture systems employing culture media from Irvine Scientific (CA), Life Global (CT), Sage (Denmark), and Vitrolife (Sweden). Embryo culture was conducted in a controlled atmosphere containing 6% −7% $CO_2$ and 5%−6% $O_2$ (MINC, Cook Medical, IN; MIRI, ESCO, Egaa, Denmark; C-top, Labotec, Göttingen, Germany; ASTEC, Astec, Minamizato, Japan; K-System, CooperSurgical, Målov, Denmark) incubators. On day 4, each embryo was washed and moved to an individual 10-$\mu$L drop for at least 40 hours in culture. Thereafter, spent media were collected and TE biopsies performed. Spent medium from each embryo was loaded into a sterile 0.2-mL polymerase chain reaction (PCR) tube and stored at −20°C for at least 24 hours before shipping to the genetics laboratory. A negative control sample from each patient, consisting of a 10-$\mu$L drop of medium undergoing the same protocol, but without embryo exposure, was also collected and analyzed.

Morphology assessment encompassed developmental stage, blastocyst expansion, and quality of ICM and TE. Blastocysts were graded using published criteria.[1] To prevent DNA contamination owing to manipulation of samples, embryos and their culture media were handled under sterile conditions. Research personnel were properly trained in handling embryos and wore a mask, cap, and gloves while handling embryos, and the IVF laboratory material used was exclusively dedicated to the study. To minimize maternal contamination, careful denudation of surrounding cumulus cells was conducted before microinjection in ICSI cycles or at the moment of fertilization checking in IVF cycles.

Embryos did not undergo any extra manipulation during the culture period before biopsy, that is, no zona drilling at day 3. SBM was collected after removing the blastocysts from the culture drop to perform the TE biopsy. After TE biopsy, blastocysts underwent the vitrification procedures routinely used by each IVF center.

### Sample analysis

DNA from TE biopsies and spent media were analyzed in a blinded fashion. Whole-genome amplification (WGA) and DNA barcoding was performed using the Ion ReproSeq PGS Kit (Thermo Fisher Scientific, MA), with a modified protocol for spent media because of the specific characteristics of embryonic cfDNA.[2] WGA success was evaluated by loading 1.5 $\mu$L of amplified products on an agarose gel (Lonza, Switzerland). Samples were pooled in batches of 24 or 96 samples. Libraries were quantified using the Qubit High Sensitivity dsDNA Kit (Life Technologies, CA), diluted and

loaded onto the Ion Chef (Thermo Fisher Scientific) for automated template preparation and chip loading. For TE biopsies, the standard next-generation sequencing (NGS) protocol for 96 samples (530 chip) was used. For spent media, runs of 24 and 96 samples were performed (520 and 530 chip). Sequencing of the chips was done using S5 XL sequencer (Thermo Fisher Scientific).

## Contribution of ICM to embryonic cfDNA

ICM biopsies were performed in 81 thawed blastocysts previously diagnosed as aneuploid after invasive PGT-A. Results were compared with both TE biopsy and corresponding embryonic cfDNA. ICM biopsies were performed following a published protocol,[3] loaded into sterile 0.2-mL PCR tubes, and stored at −20°C for at least 24 hours. NGS was performed as for TE biopsies. All ICM biopsies included in this manuscript corresponded to a single IVF center where the research project included this additional analysis (Boston IVF).

## Interpretation of sequencing data and diagnosis

Quality was assessed for both the run and individual samples. Sequencing data were processed and sent to Ion Reporter Software v5.4 (Thermo Fisher Scientific). Samples with absence of a band in the agarose gel were considered as amplification failure; biopsies showing a band but without interpretable results after sequencing were classified as noninvasive.

For TE and ICM biopsies, data obtained from the BAM files for each embryo were analyzed by an Igenomix proprietary algorithm. For informative biopsies, whole-chromosome aneuploidies, mosaicism (between 30% −70%), and segmental aneuploidies (deletion or duplication >10 Mb) were identified.

For embryonic cfDNA samples, the difference value (DV) for each chromosome with 2 copy numbers was computed, and samples were categorized as euploid and aneuploid. DVs for the different aneuploidy types were established according to the best fit sensitivity and specificity values.

## Statistical analysis

Sample size was calculated using the McNemar test for 2 paired proportions comparison for applying 2 different tests to the same samples (invasive and noninvasive PGT-A), considering 60% aneuploidy rate for the age range of women participants. Estimated size for the study was 2496 blastocysts with paired TE biopsies and spent media samples. Adding a 30% dropout rate, the estimated number of samples needed was 3245. The interim analysis was planned at 30% of study completion.

Amplification rates (number of successfully amplified samples divided by total analyzed samples) and informativity rates (number of samples with interpretable results according to quality parameters divided by total analyzed samples) were estimated individually for TE biopsies, embryonic cfDNA samples, and ICM biopsies. When all types of samples were informative, concordance rates between sample types were calculated. False-positive and false-negative rates, specificity, sensitivity, positive predictive value, and negative predictive value were determined. Data are presented globally and by individual center.

Descriptive data for continuous variables are presented as mean and standard deviation and categorical data as number and percentage. For continuous variables, independent-sample Student *t*-tests were performed. A normal distribution was confirmed with the use of the Levene test for equality of variants; thus, a parametric test was chosen. For comparing categorical data, chi-square tests were performed, and nonparametric Fisher exact tests were applied in cases of low number. Statistical significance was set at $P<.05$. Multivariate logistic regression was applied to assess the impact of patient characteristics, ovarian stimulation, embryo culture, and blastocyst quality on informativity and concordance rates. Statistical analyses were performed using IBM SPSS v25.0 (IBM, MD).

## Supplemental References

1. Gardner DK, Stevens J, Sheehan CB, Schoolcraft WB. Analysis of blastocyst morphology. In: Elder K, Cohen J, eds. *Human Preimplantation Embryo Selection*. London: Informa Healthcare; 2007. p. 79–87.
2. Rubio C, Rienzi L, Navarro-Sánchez L, et al. Embryonic cell-free DNA versus trophectoderm biopsy for aneuploidy testing: concordance rate and clinical implications. Fertil Steril 2019;112:510–9.
3. Capalbo A, Rienzi L. Mosaicism between trophectoderm and inner cell mass. Fertil Steril 2017;107:1098–106.

SUPPLEMENTAL TABLE 1
**Patient demographics and clinical background**

| Characteristics | Cycles |
|---|---|
| No. of cycles (patients) | 406 (371) |
| Mean female age (SD), y | 36.4 (5.2) |
| Mean male age (SD), y | 39.9 (6.1) |
| Mean no. of previous implantation failures (SD) | 0.9 (1.5) |
| Mean no. of previous miscarriages (SD) | 0.8 (1.2) |
| Mean no. of previous live births (SD) | 0.3 (0.7) |
| Mean female BMI (SD), kg/m$^2$ | 24.3 (4.7) |
| Previous PCO history | 30/348 (8.6) |
| Previous endometriosis history | 31/348 (8.9) |
| Primary PGT-A indication: | |
| AMA | 268/405 (66.2) |
| RIF | 17/405 (4.2) |
| RPL | 14/405 (3.5) |
| SMF | 8/405 (2.0) |
| PTP | 3/405 (0.7) |
| Mixed causes | 12/405 (3.0) |
| GS | 3/405 (0.7) |
| GP | 32/405 (7.9) |
| OD | 48/405 (11.8) |

Values are presented as n/N (%) unless indicated otherwise. Percentages were calculated considering the number of cycles with informative data for each variable.

*AMA*, advanced maternal age; *BMI*, body mass index; *GP*, good prognosis; *GS*, gender selection; *OD*, ovum donation; *PCO*, polycystic ovary; *PGT-A*, preimplantation genetic testing for aneuploidy; *PTP*, previous trisomy pregnancy; *RIF*, repetitive implantation failure; *RPL*, recurrent pregnancy loss; *SD*, standard deviation; *SMF*, severe male factor.

*Rubio et al. Embryonic cell-free DNA released to the spent blastocyst media. Am J Obstet Gynecol 2020.*

SUPPLEMENTAL TABLE 2
**Ovarian stimulation and cycle characteristics**

| Characteristics | Cycles |
|---|---|
| ICSI cycles | 368/406 (90.6) |
| IVF cycles | 38/406 (9.4) |
| Type of gonadal suppression | |
| Antagonist protocol | 391/396 (98.7) |
| Long-agonist protocol | 1/396 (0.3) |
| Short-agonist protocol | 4/396 (1.0) |
| Type of stimulation protocol | |
| Clomiphene citrate | 1/390 (0.3) |
| FSH only | 94/390 (24.1) |
| FSH+hMG | 81/390 (20.8) |
| FSH+LH | 209/390 (53.6) |
| hMG | 5/390 (1.3) |
| Type of ovulation triggering | |
| GnRH agonist | 206/391 (52.7) |
| hCG | 136/391 (34.8) |
| Double triggering | 49/391 (12.5) |
| Mean stimulation days (SD) | 10.5 (1.7) |
| Mean number of MII oocytes (SD) | 10.3 (7.1) |
| Mean number of 2PN oocytes (SD) | 8.1 (6.0) |

Values are presented as n/N (%) unless indicated otherwise. Percentages were calculated considering the number of cycles with informative data for each variable.

*2PN*, 2 pronuclei; *FSH*, follicle-stimulating hormone; *GnRH*, gonadotropin-releasing hormone; *hCG*, human chorionic gonadotropin hormone; *hMG*, human menopausal gonadotropin; *ICSI*, intracytoplasmic sperm injection; *IVF*, in vitro fertilization; *LH*, luteinizing hormone; *MII*, metaphase II; *SD*, standard deviation.

*Rubio et al. Embryonic cell-free DNA released to the spent blastocyst media. Am J Obstet Gynecol 2020.*