A prospective study of non-invasive preimplantation genetic testing for Rh status, sex prediction and mtDNA copy number analysis using ddPCR on spent culture media

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Abstract. Presence of embryo-derived cell-free DNA (cfDNA) in spent culture medium (SCM) represents a promising non-invasive alternative to the conventional preimplantation genetic testing (PGT). The present study evaluated the feasibility of using cfDNA isolated from SCM to determine Rhesus (Rh) status through Rh blood group D antigen (*RHD*) gene analysis, predicting sex-linked disorders using sex determining region Y (*SRY*) gene detection and assessing the association between mitochondrial cfDNA (cf-mtDNA) levels and maternal age using mitochondrially encoded cytochrome b (*MT-CYB*) gene quantification. SCM samples collected during the IVF process were subjected to cfDNA isolation and target regions including *RHD* exon 7 and exon 10, *SRY* and *MT-CYB* were analyzed using droplet digital PCR. *RHD* gene amplification was detected in 6 of 15 samples for exon 7 and in 11 samples for exon 10, with partial concordance between exons. *SRY* amplification was observed in 16 of 31 samples and was largely consistent with clinical sex data. *MT-CYB* was detected in 19 of 31 samples, with higher cf-mtDNA/nuclear cfDNA ratios observed in women >40 years of age. A moderate positive correlation trend was noted between maternal age and *MT-CYB* levels (r=0.46; P=0.354). These findings demonstrate the potential applicability of SCM-derived cfDNA for non-invasive Rh and sex determination without compromising embryo integrity, while mtDNA quantification may provide additional insights into embryo quality. Overall, SCM-based cfDNA analysis exhibited promise as a complementary tool for embryo selection in IVF workflows.

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Introduction

In vitro fertilization (IVF) has proved beneficial for assisted reproductive technologies by opening new possibilities for couples experiencing infertility. An important step in this process is the selection of embryos with the highest survival rate and the lowest risk of genetic abnormalities (1). Preimplantation genetic testing (PGT) has serves an important role in this selection, allowing the detection of chromosomal aneuploidies and single-gene disorders before embryo transfer (2). However, conventional PGT methods, such as blastomere, polar body and trophectoderm biopsy, rely on invasive biopsy procedures, which not only raise ethical concerns but may also create a small risk to embryo viability and implantation potential. In previous years, the identification of cell-free DNA (cfDNA) in embryo spent culture media has proven a novel perspective in non-invasive PGT (niPGT) (3). During early embryonic development, fragments of nuclear and mitochondrial DNA are released into the culture medium of the embryo, providing a key source of genetic information without exposing the embryo to invasive procedures. This discovery represents a promising method for both research and clinical applications, as it enables genetic screening while preserving the structural integrity of the embryo. By using advanced molecular techniques such as digital PCR and next-generation sequencing, niPGT aims to provide a comprehensive and reliable assessment of the genetic profile of the embryo, offering the potential to improve implantation outcomes, reduce the risk of heritable diseases and optimize reproductive success in IVF programs (4).

Rhesus D (RhD) incompatibility between the mother and the fetus occurs when an Rh-negative mother carries an Rh-positive fetus. In this context, maternal antibodies cross

the placenta and destroy fetal RhD antigens (5). Without any treatment, this condition can result in fetal anemia, hydrops fetalis, neonatal jaundice (hyperbilirubinemia) and even fetal mortality. Rh-induced hemolytic disease of the fetus and newborn (HDFN) is a blood disorder that is triggered by the incompatibility between a mother and fetus; this disease remains an important clinical problem, with the worldwide prevalence decreasing from ~99 to 44 cases per 100,000 live births after the introduction of routine anti-D immunoprophylaxis, which is applied to prevent RhD incompatibility (6,7). In this approach, Rh-negative pregnant women receive anti-D immunoglobulin at ~28 weeks of gestation to reduce the risk of fetomaternal hemorrhage. The RhD antigen is considered one of the most important immunogenic antigens in transfusion medicine (8). RhD antigens are encoded by the *RHD* gene, which is located on chromosome 1, close to the Rh blood group CcEe antigens (*RHCE*) gene (Fig. 1). *RHD* and *RHCE* genes exhibit 92-97% sequence similarity and both genes contain 10 exons. This high similarity can make genetic testing more difficult due to the risk of cross-reaction (9).

In non-invasive *RHD* genotyping, exon 7 and exon 10 are the main targets. *RHD* exon 7 is important as it includes variations specific to *RHD*, while exon 10 is used as an additional control to improve accuracy and reliability. Using both exons together helps to separate *RHD* from *RHCE*, resulting in an advantageous method for non-invasive prenatal *RHD* genotyping. Non-invasive methods for fetal RhD determination also exhibit the potential to influence infertility treatments. In particular, cell-free DNA (cfDNA), found in embryo culture media during IVF, has emerged as a new biological source for PGT (4). These analyses are performed without harming the embryo and non-invasive preimplantation genetic testing has attracted great attention in recent years (4,10). Specifically, for RhD genotyping, this method provides the possibility to detect Rh incompatibility without physical intervention on the embryo (9).

An important application of IVF is the screening and prevention of sex-linked genetic disorders, which are inherited conditions caused by mutations on the X or Y chromosomes and often exhibit distinct patterns of inheritance and clinical manifestation between men and women (11). These disorders are influenced not only by the genome but also by lifestyle, nutrition, environment and sex hormones such as estrogen, progesterone and testosterone, which affect immune responses and gene expressions (12). The X chromosome carries ~867 genes that are not only involved in sex determination but also serve notable roles in the development and function of major organs such as the brain, heart, liver, kidneys and skin, as a number of these genes encode structural proteins, enzymes and regulatory molecules necessary for normal cellular and physiological processes. X-linked disorders are usually more severe in male patients as men carry only one X chromosome, while females may exhibit milder symptoms due to a second X chromosome being present. However, abnormal X chromosome inactivation or homozygosity can still cause serious disease in females, including >500 X-linked disorders, including Duchenne muscular dystrophy, hemophilia A and fragile X syndrome, which appear to be more severe in males (12).

Since the Y chromosome is present only in males, Y-linked disorders are inherited exclusively through the paternal line and their phenotypic expression is restricted to male patients, as females lack the Y chromosome. An important gene on the Y chromosome is sex-determining region Y (*SRY*), located at p11.2 (Fig. 2) (13). This gene produces the testis determining factor, which initiates testis development. During early development, *SRY* activates other genes such as Sox9, leading to Sertoli cell formation and anti-Müllerian hormone production, which directs male reproductive system formation. Mutations or deletions of the *SRY* gene can cause conditions such as gonadal dysgenesis or hypospadias. Therefore, *SRY* is a key biomarker for sex differentiation and genetic diagnosis (13).

As the mitochondrial function of reproductive cells deteriorates, the quality of the germ cells declines and in the majority of cases, it prevents healthy embryo development (14). Mitochondrially encoded cytochrome b (*MT-CYB*) makes it possible to check for the quality of the reproductive cells beforehand as a part of niPGT. *MT-CYB* encodes a key protein component of mitochondrial complex III of the respiratory chain (the cytochrome bc_1 complex), which serves a central role in cellular energy production through oxidative phosphorylation. Mitochondrial complex III is located in the mitochondrial inner membrane (15) and is responsible for catalyzing the electron transfer from ubiquinol to cytochrome *c* (16). Thus, it represents an important step in establishing the proton gradient across the inner mitochondrial membrane (17). This complex also has 11 subunits, of which only one subunit is encoded by mitochondrial DNA and is named cytochrome b. *MT-CYB* gene is located between the positions 14747 and 15887 of mitochondrial DNA (Fig. 3) and the cytochrome b protein, which is the only protein subunit that is encoded by mitochondrial DNA, is 380 amino acids long (17). In addition, the relatively high abundance of this gene in mitochondrial genomes, as well as its key function in oxidative phosphorylation, make *MT-CYB* an ideal target for quantification studies such as droplet digital PCR (ddPCR). Notably, pathogenic variants in the gene have been associated with mitochondrial complex III deficiency, impaired cellular energy production, and a broad spectrum of clinical disorders affecting tissue viability and organismal health (18).

The sensitivity of *MT-CYB* expression and mutations to cellular stress and metabolic changes makes it a potential biomarker of mitochondrial dysfunction, especially in IVF (19). In previous years, it has become common to analyze cfDNA samples in niPGT. Recent advancements in niPGT have allowed the use of cfDNA and mitochondrial cfDNA (cf-mtDNA) samples to identify the qualities of examined germ cells. Subsequently, this increases the chance of obtaining pregnancy with advanced maternal age by allowing the monitoring of reproductive cell quality (20).

Therefore, the aim of the present study was to identify Rh incompatibility, which can be lethal for the embryo, by analyzing the Rh factor in cfDNA obtained from spent culture medium (SCM) through niPGT and ensuring correct embryo selection before implantation. In addition, it was intended to use the identified genes associated with sex-linked diseases for screening, thereby providing an effective method for selecting the healthiest blastocyst. Furthermore, the present study sought to investigate whether there is an association between

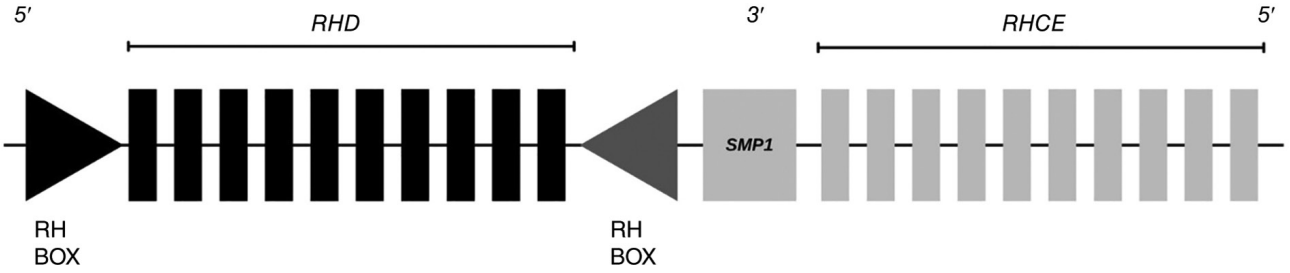

Figure 1. Schematic representation of 10 exons and Rh boxes of the *RHD* gene on chromosome 1. Rh, Rhesus; *RHD*, Rh blood group D antigen; *RHCE*, Rh blood group CcEe antigens; SMP1, small membrane protein 1.

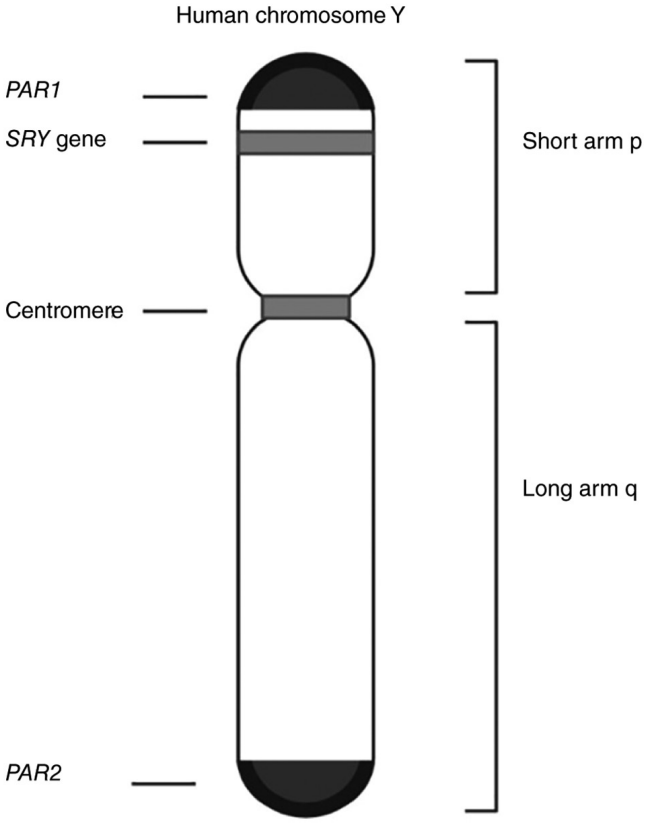

Figure 2. Schematic representation of the human Y chromosome, including the *SRY* gene. *PAR*, pseudoautosamal region; *SRY*, sex-determining region Y.

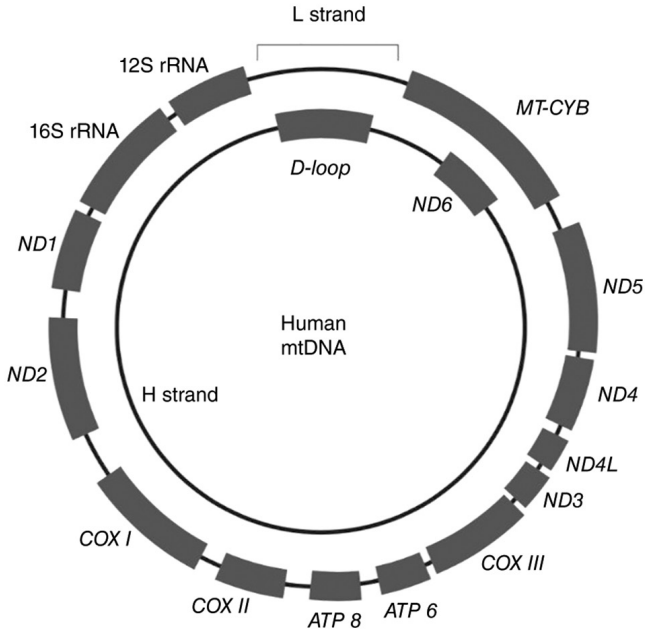

Figure 3. Schematic representation of 16,569 kb circular double-stranded human mitochondrial DNA molecule. The *MT-CYB* gene is represented within the mtDNA regions. *MT-CYB*, mitochondrially encoded cytochrome b; rRNA, ribosomal RNA; mtDNA, mitochondrial DNA; ND, NADH dehydrogenase subunit; COX, cytochrome *c* oxidase.

the amount of cfDNA in SCM and maternal age, with the aim of providing insights for future research as a part of promising biomarkers in niPGT.

Materials and methods

Study design. Embryos from routine clinical procedures of IVF treatment were used to conduct the present study. During the IVF procedure, fertilized embryos were cultured in individual droplets for incubation, observation and morphological assessment. For the present study, the SCM from each embryo was collected. After sample collection, cfDNA was isolated from SCM samples using a magnetic bead-based extraction method to improve cfDNA recovery. Primers were designed and validated by quantitative PCR (qPCR) for target genes. ddPCR was then performed to amplify the target sequences and the resulting data were analyzed accordingly. The overall workflow of the present study is presented in Fig. 4.

Study criteria. SCM samples from embryos were provided by the IVF Center of Acıbadem Fulya Hospital (Istanbul, Turkey). All samples were transferred to the Department of Molecular Biology and Genetics, Istanbul University (Istanbul, Turkey). All patients were requested to sign informed consent. The necessary documents were obtained from patients according to the ethical regulations approved by the Ethics Committee of Istanbul University Faculty of Medicine (Istanbul, Turkey; approval no. 2023/1488 in 2023). The present study was conducted without the use of embryos, human/animal tissues or any other biological material derived from living organisms; only cfDNA from the SCM of embryos was analyzed.

Couples who participated in the present study: i) Had a family history of genetic diseases or an infertility background; ii) were 29-45 years old; and iii) presented with infertility indications, as shown in Table I. Patients with systemic diseases, active infections or any condition that contraindicated IVF

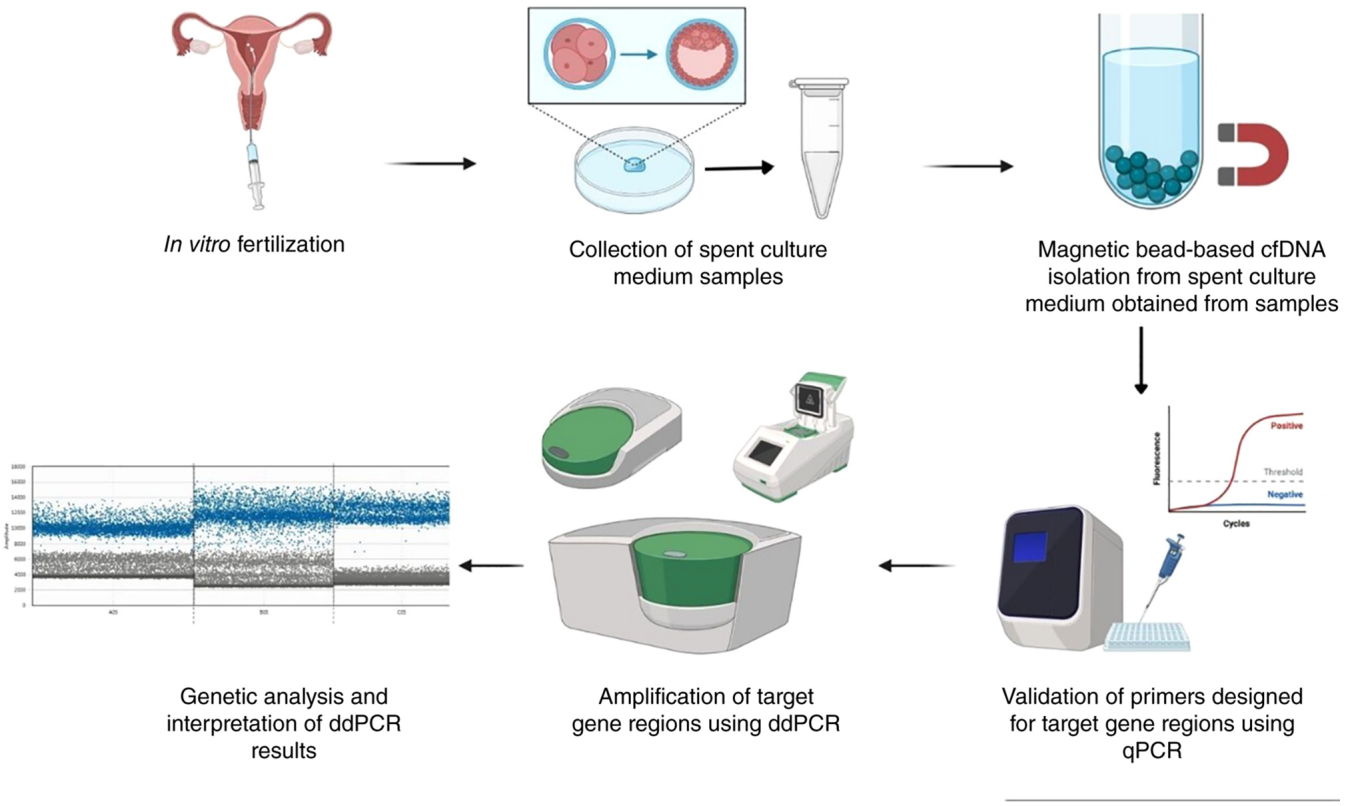

Figure 4. Study workflow. Schematic diagram of the experimental process, showing *in vitro* fertilization, collection of spent culture medium samples, cfDNA isolation using magnetic bead-based technology, primer validation by qPCR, ddPCR amplification of target genes and genetic analysis and interpretation of results. cfDNA, cell-free DNA; ddPCR, digital droplet PCR; qPCR, quantitative PCR.

treatment, such as diabetes, active malignancies and uncontrolled thyroid disorders, were excluded from the present study. The minimum sample size for the present study was calculated using G*Power version 3.1 (21) analysis using t-test for means, two independent means (two group) with a power of 0.85 (Fig. S1).

SCM collection. All samples were collected from the IVF Center of Acıbadem Fulya Hospital between January 2024 and December 2025. IVF procedures in the medical center were carried out as routine procedures; oocytes were inseminated by intracytoplasmic sperm injection (ICSI) and used for embryo culturing. Prior to ICSI, cumulus-corona cells were removed through standard denudation procedures to minimize residual maternal cell carryover. All embryology procedures were performed under validated IVF laboratory protocols in controlled clean-room conditions using certified sterile consumables and aseptic handling practices (22,23). Fresh embryos were placed in media containing human serum albumin (cat no: H5GT-010; LifeGlobal™). Each embryo was placed in a different culture dish to prevent samples from getting contaminated. Individual culture handling, dedicated pipettes/tips and single-embryo culture drops were used to reduce the risk of cross-contamination and environmental DNA exposure. Procedures, including sample transfer, were conducted in biosafety cabinets under sterile conditions. During the incubation process, embryo growth and division were carefully monitored. On day 5-6, the majority of embryos were expected to be in the blastocyst stage. Embryos were graded according to the Gardner Grading Scale based on their morphology (24).

A total of ~30 μl spent media was collected from each embryo culture after trophoectoderm biopsy on day 5-6. Samples were transferred into RNase-DNase-free PCR tubes containing 5 μl lysis buffer (cat no: 19075; Qiagen GmbH). Collection tubes were prepared in advance and samples were processed individually to further minimize exogenous DNA contamination. Negative control media droplets processed in parallel were included, where applicable, as contamination monitoring controls. All samples were stored at -20˚C for a day in the clinic at Acıbadem Fulya Hospital before being transported to Istanbul University, maintaining cold chain conditions. In the laboratory, all samples were frozen at -80˚C until experiments were initiated.

DNA purification. cfDNA from SCM samples was isolated using a QIAamp MinElute ccfDNA Kit (cat no: 55284; Qiagen GmbH), which utilizes magnetic bead technology for extra yield. Extracted cfDNA was eluted in 50 μl elution buffer (Buffer EB; cat. no. 19086; Qiagen GmbH) for subsequent steps according to the manufacturer's instructions. Immediately after purification, samples were quantified using a NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific, Inc.). Processed samples were frozen at -80˚C until amplification.

Primer design. For the detection of selected gene regions, including *GAPDH* and *β-globin* as housekeeping genes, 6

Table I. Demographic characteristics and clinical indications of the study population.

Indication	Total number of patients	Female patients	Male patients	Mean age ± SD, years
Advanced maternal age	12	12	-	41.6±2.8
Uterine-related miscarriage	8	8	-	38.3±4.8
Diminished ovarian reserve	5	5	-	38.8±3.5
Male related factors	5	-	5	38.4±3.5
Unexplained ınfertility	1	1	1	N/A
Recurrent pregnancy loss	1	1	1	N/A

For groups with a single participant (n=1), the age is reported as N/A due to insufficient sample size for statistical derivation. For the indications 'Unexplained infertility' and 'Recurrent pregnancy loss', the diagnosis refers to the couple as a unit; thus, for these specific categories, both partners are included in the demographic representation, explaining the count of '1' for both sexes within these couples. N/A, not applicable.

primer sets were designed using the PrimerQuest™ PCR tool (Integrated DNA Technologies, Inc.), targeting *RHD* exon 5, *RHD* exon 10, *SRY*, *MT-CYB*, *GAPDH* and *β-globin* (Table II). Primer specificity was validated using an *in silico* PCR tool (University of California Santa Cruz *In-Silico* PCR) (25), before primers determined to be specific were then outsourced for synthesis. The primer sequences for *RHD* genotyping were selected to ensure maximal specificity. The primers for *RHD* exon 10 were modified from research conducted by Hromadnikova *et al* (26), which underwent clinical validation for the detection of fetal RHD and was specifically tested on Rh-negative (dd) individuals to ensure the elimination of false-positive signals. For exon 7, the primers were carefully designed *in silico* to only target RHD-specific sequences that don't have any similarities with the *RHCE* gene. To avoid cross-reactivity, particular attention was paid to the 3' end of the primers. This made sure that the amplification was specific to the *RHD* gene, even when *RHCE* sequences were highly homologous. For further validation, whole-blood analysis was performed on Rh (-) and Rh (+) individuals. Genomic DNA isolated from whole-blood samples using the QIAamp MinElute Media Kit (Qiagen GmbH) was amplified with ddPCR for *RHD* exon 7, exon 10 and *GAPDH* genes.

ddPCR. A ddPCR protocol was prepared and validated for the present study using a QX Droplet Digital™ PCR system (Bio-Rad Laboratories, Inc.; Table III). Each sample and reaction mix was loaded into a cartridge well along with a droplet generator oil (Bio-Rad Laboratories, Inc.) for droplet generation, a process which separates DNA samples into ~20,000 droplets. Generated droplets were gently transferred to a 96-well plate for ddPCR before the plate was sealed. Amplification conditions were as follows: 5 min at 95˚C for enzyme activation, 30 sec at 95˚C for initial denaturation, 1 min at 58˚C (40 cycles of annealing and extension), 5 min at 4˚C and 5 min at 90˚C for signal stabilization. After thermal cycling was conducted, the plate was placed into a QX200 Droplet Reader (Bio-Rad Laboratories, Inc.) for signal detection.

Statistical analysis. The built-in droplet reader algorithm (QX Manager; version 2.2; Bio-Rad Laboratories, Inc.) was used for droplet analysis as it provides the positive and negative droplet counts for each well corresponding to the target gene region. Based on the number of positive droplets, the software applies the Poisson statistical model to automatically provide the absolute copy number of the target DNA (copies/μl). The threshold for the outlined result was determined as 3 positive droplets per sample. McNemar's tests were applied for *RHD* gene outcomes. For calculating the correlation between maternal age and mitochondrial DNA levels, Spearman's rank correlation tests were used. Statistical analyses were performed using R software (version 4.5.3; R Foundation for Statistical Computing) and two-sided $P<0.05$ was considered to indicate a statistically significant difference.

Results

Patient characteristics and the interpretation of the study results. A total of 33 SCM samples were collected from IVF patients between July 2024 and July 2025. Among these samples, 18 samples were used for *RHD* status prediction (targeting exons 7 and 10), 31 samples were analyzed for *SRY* gene detection and the same 33 samples were also examined for *MT-CYB* gene expression to assess embryo quality in association with maternal age. The total droplet counts exceeded 10,000 in all cases, ensuring the reliability of ddPCR data. No positive droplets were observed in negative controls. A sample was considered positive when ≥3 positive droplets were detected. This threshold was determined empirically based on repeated no-template control reactions, which consistently yielded 0-1 positive droplets only. Samples with detectable droplets above background were considered positive. Lower droplet counts (<50) were interpreted as low-abundance signals, whereas higher counts reflected robust target detection.

Determination of RH status from SCM. RHD assay results for ddPCR in Table IV demonstrated positive amplification in 8 samples for exon 7 (47.1%) and 10 samples for exon 10 (58.8%) out of 17 samples. The McNemar test was used to verify consistency between exon 7 and exon 10 detection. The result was a 52.94% (9/17) conformity rate. Statistical analysis indicated no significant discordance between the detection rates of the two exons ($P=0.7266$; 95% CI for the difference in proportions: -44.1% to +20.6%), implying that both genetic regions were identified with similar efficiency in cfDNA obtained from SCM.

Table II. Designed primers and their sequences.

Gene	Direction	Sequence
RHD exon 7	Forward	5'-GGTGTTGTAACCGAGTGCT-3'
	Reverse	5'-CACCAGCAGCACAATGTAGA-3'
RHD exon 10	Forward	5'-CCTCTCACTGTTGCCTGCATT-3'
	Reverse	5'-AGTGCCTGCGCGAACATT-3'
MT-CYB	Forward	5'-TGAAACTTCGGCTCACTCCT-3'
	Reverse	5'-AATGTATGGGATGGCGGATA-3'
SRY	Forward	5'-TCCTCAAAAGAAACCGTGCAT-3'
	Reverse	5'-AGATTAATGGTTGCTAAGGACTG GAT-3'
β-globin	Forward	5'-GCAGGTTGGTATCAAGGTTACA-3'
	Reverse	5'-GTGCCTATCAGAAACCCAAGAG-3'
GAPDH	Forward	5'-GGAGTGAGTGGAAGACAGAATG-3'
	Reverse	5'-GAGGGACACAAGGTTACCATATAC-3'

RHD, Rhesus blood group D antigen; *SRY*, sex-determining region Y; *MT-CYB*, mitochondrially encoded cytochrome b.

Table III. Reaction mix preparation for ddPCR.

Component	Volume per reaction, μl	Final concentration
2x QX200™ ddPCR™ EvaGreen® Supermix	10	1X
Forward primer	1	100 nM
Reverse primer	1	100 nM
DNA template and RNase-/DNase-free water	8	Up to 100 ng
Total	20	

ddPCR, digital droplet PCR.

Available clinical follow-up data for the five embryos that resulted in live births are shown in Table V. Among these, one newborn was RhD-negative and four were RhD-positive. ddPCR correctly predicted the Rh status of the RhD-negative newborn, while false-negative results were obtained in two cases (ddPCR negative and clinically RhD-positive). The validation data of RHD exon 7 and 10 in whole blood presented in Fig. 5 demonstrated that the primer sets were suitable for distinguishing between *RHCE* and *RHD*.

Determination of embryonic sex region from SCM. A total of 31 samples were analyzed by ddPCR for the detection of the *SRY* gene. The presence of the *SRY* gene, indicated as either positive or negative are demonstrated in Table VI. According to this analysis, 16 samples (51.6%) were classified as *SRY* positive, whereas 15 samples (48.4%) were *SRY* negative. Among the *SRY*-positive samples, the proportion of positive droplets was consistently clustered around 0.38-0.44, indicating a reproducible detection threshold for true positivity. By contrast, *SRY*-negative samples exhibited ratios close to 0 (0.00-0.02), consistent with the absence of the target gene. Notably, two cases (sample 1: ratio 0.020 and sample 3: ratio 0.005) exhibited very low levels of positive droplets, which fell between the negative and clearly positive ranges (Fig. S2). These borderline results may have represented either low-level true positives or technical difficulties such as non-specific amplification or contamination. Overall, the ddPCR assay demonstrated a clear distinction between *SRY*-positive and *SRY*-negative samples, with the majority of results exhibiting conclusive classification.

Detection of the *SRY* gene by ddPCR analysis demonstrated complete concordance with the pregnancy determination results in Table VII. Specifically, samples 3, 14 and 18 were identified as *SRY*-positive, whereas samples 29 and 30 were *SRY*-negative. In all five cases, the molecular findings were fully consistent with the clinical confirmation of pregnancy outcomes, corresponding to an accuracy rate of 100%. Although the sample size was limited (n=5), these results provided strong preliminary evidence supporting the reliability and specificity of ddPCR for the detection of the *SRY* gene in this experimental setting.

Determination of the correlation between maternal age and cf-mtDNA amount from SCM. In the present study, cfDNA samples derived from 33 different embryo SCMs were examined. The maternal age for the examined embryos

Table IV. *RHD* exon 7 and exon 10 digital droplet PCR positive droplet counts and predicted Rh status.

Sample no.	*RHD* exon 7 positive droplet count	Total droplet count	*RHD* exon 10 positive droplet count	Total droplet count	*RHD* exon 7	*RHD* exon 10
1	11,785	17,097	0	14,358	Positive	Negative
2	11,762	15,434	0	16,063	Positive	Negative
3	0	13,165	0	15,170	Negative	Negative
4	58	13,621	0	16,269	Borderline positive	Negative
5	0	13,574	0	15,843	Negative	Negative
6	42	12,527	91	17,445	Borderline positive	Borderline positive
7	0	12,563	35	17,719	Negative	Borderline positive
8	0	12,626	13,912	16,815	Negative	Positive
9	164	11,514	12,774	17,507	Positive	Positive
10	0	12,791	8,857	13,715	Negative	Positive
11	0	11,142	7,008	10,863	Negative	Positive
12	10	17,707	7,780	11,224	Borderline positive	Positive
13	0	16,333	11,762	17,897	Negative	Positive
14	12,030	17,411	9,516	15,487	Positive	Positive
15	10,778	16,338	13,420	17,340	Positive	Positive
16	12	4,226			Borderline positive	
17	0	13,605	0	14,785	Negative	Negative
18	0	9,347	0	12,635	Negative	Negative
NTC	0	10,079	0	10,355		

Samples yielding <50 positive droplets were categorized as borderline positive to indicate low-abundance signals near the detection threshold. NTC, no-template control; Rh, Rhesus; *RHD*, Rh blood group D antigen.

Table V. Correlation between *RHD* exon 7 and *RHD* exon 10 ddPCR results and clinical data.

	ddPCR Rh status prediction results		Clinical records	
Sample no.	*RHD* exon 7	*RHD* exon 10	Pregnancy confirmation	Compatibility
3	Negative	Negative	Rh positive	Not compatible
15	Negative	Negative	Rh negative	Compatible
16	Negative	Negative	Rh positive	Not compatible
17	Positive	Positive	Rh Positive	Compatible
18	Positive		Rh Positive	Compatible

Rh, Rhesus; RHD, Rh blood group D antigen; ddPCR, digital droplet PCR.

ranged between 31 and 48 years, allowing for the evaluation of age-related changes in embryo quality across a broad spectrum. The *MT-CYB* gene was successfully amplified in 19 samples and the *β-globin* gene was successfully amplified in 12 samples, as shown in Table VIII. Furthermore, in 6 of the samples, both *MT-CYB* and *β-globin* genes were detected during ddPCR analysis. Of the 6 results obtained, one extreme result was excluded from the analysis as the positive droplets of the reference gene were considered low, indicating insufficient template DNA for reliable quantitative analysis. The cf-mtDNA/cf-DNA ratio was subsequently evaluated using a threshold maternal age of 40 years, as it was stated that advanced maternal age is considered to be >35 years in previous studies (27,28). In Fig. 6, the cf-mtDNA/cf-nDNA ratio was found to be moderately higher in the ≥40 years group. In SCM samples obtained from women undergoing IVF, women aged ≥40 years exhibited a moderately higher cf-mtDNA/cf-nDNA ratio compared with those aged <40 years, although these results did not reach significance.

In Fig. 7, the association between maternal age and the ratio of *MT-CYB* copy number per μl was examined, which was obtained from 6 out of 33 samples collected from SCM. A Spearman's rank correlation test was conducted to evaluate the results, revealing a coefficient of r=0.464, indicating a

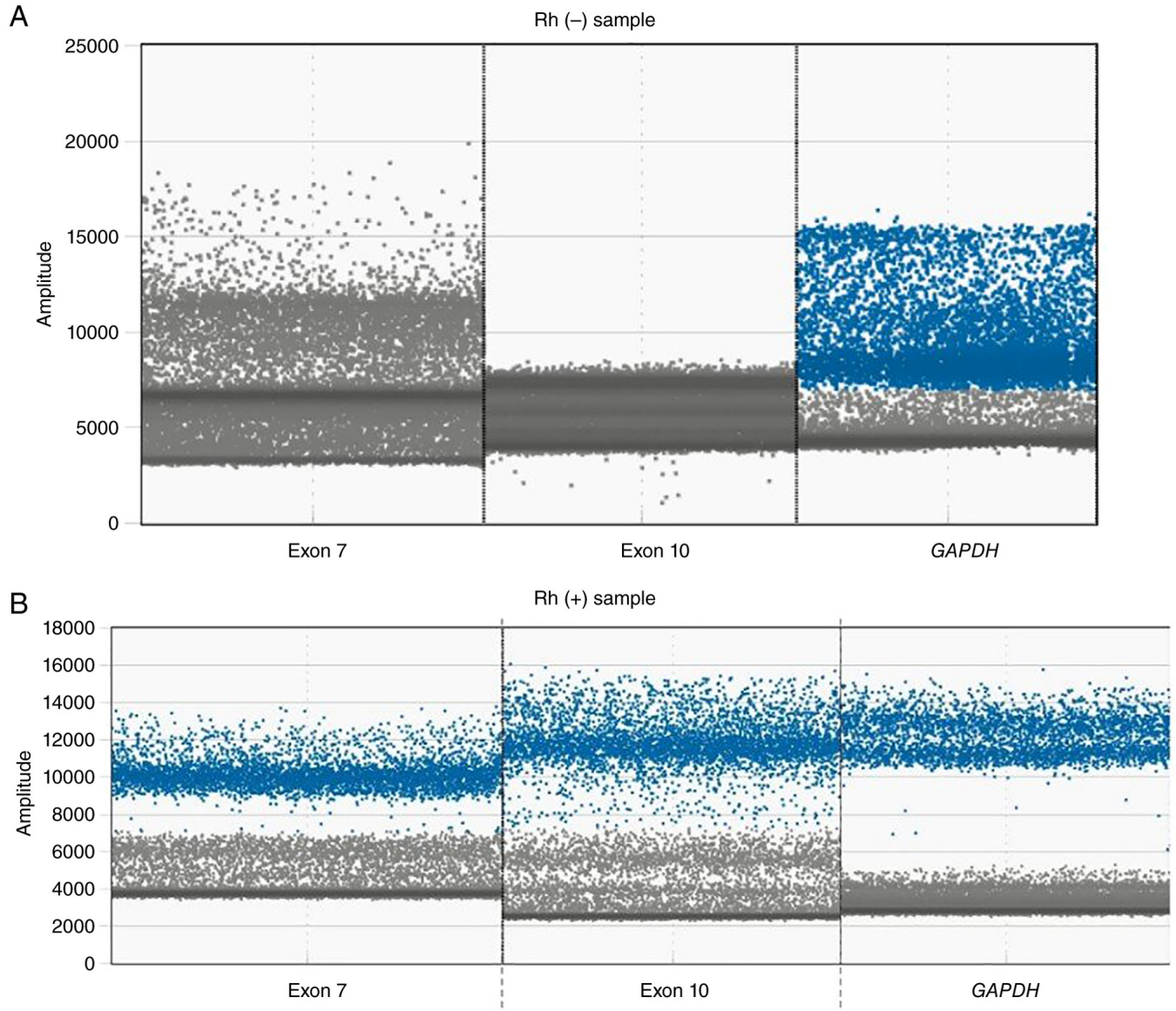

Figure 5. Validation data of *RHD* exons. Digital droplet PCR amplitude plots of *RHD* exon 7, *RHD* exon 10 and *GAPDH* assays obtained from validation samples. (A) RhD-negative control and (B) RhD-positive controls are represented. *GAPDH* was used as the internal control. RhD, Rhesus D; *RHD*, Rh blood group D antigen.

moderate positive trend. However, since P=0.354 and P>0.05, the difference was not statistically significant. Although the data suggested that the copy number ratio may have increased with advancing age, the limited sample size prevented this association from reaching statistical significance. Under these conditions, this ratio only served as a hypothesis-generating preliminary evidence for it to be considered as a biomarker for mitochondrial dysfunction as the maternal age increases, especially in the experiment groups with >30 samples.

Discussion

niPGT has emerged as a new approach in reproductive medicine that avoids invasive techniques and reduces risks, such as structural damage to the embryo or mosaicism associated with biopsy. Since the first discovery of cell-free fetal DNA in maternal plasma in 1997 (29), researchers have started to move towards less invasive methods for genetic analysis in assisted reproductive technologies. The identification of cfDNA and cf-mtDNA in SCM represented new possibilities for analyzing embryonic genetic status without compromising embryo viability. With the help of technological advancements such as ddPCR, these techniques established the way for highly sensitive and precise quantification of genetic material from minimal and fragmented DNA sources, offering marked clinical potential for embryo selection in IVF.

In the present study, cfDNA and cf-mtDNA isolated from SCM were analyzed using ddPCR to understand their potential in niPGT. The present findings showed that ddPCR offered a highly sensitive detection of low-input genetic targets, an important advantage in niPGT applications whereby cfDNA quantities are often limited and highly fragmented.

Despite ddPCR having demonstrated high analytical sensitivity for *RHD* detection in low-input samples, the clinical concordance rate remained moderate. The clinical data concordance rate was 60% for five samples, considering the

Table VI. Digital droplet PCR results of the *SRY* gene.

Sample no.	Positive droplet count	Negative droplet count	Total droplet count	*SRY* gene presence
1	30,000	16,905	16,935	Borderline positive
2	0	15,149	15,149	Negative
3	63.000	11,727	11,790	Positive
4	0	16,720	16,720	Negative
5	12,238	19,188	31,426	Positive
6	9,525.000	15,856	25,381	Positive
7	0	16,669	16,669	Negative
8	0	15,062	15,062	Negative
9	0	16,929	16,929	Negative
10	0	16,011	16,011	Negative
11	0	18,734	18,734	Negative
12	14.086	17,902	31,988	Positive
13	71.000	16,743	16,814	Positive
14	11.794	17,066	28,860	Positive
15	0	14,589	14,589	Negative
16	12,214	16,416	28,630	Positive
17	0	16,397	16,397	Negative
18	13,741	18,758	32,499	Positive
19	10,435	15,054	25,489	Positive
20	0	19,163	19,163	Negative
21	9,995.000	14,910	24,905	Positive
22	0	12,151	12,151	Negative
23	9,707	15,905	25,612	Positive
24	12,840	17,687	30,527	Positive
25	0	19,238	19,238	Negative
26	9,511.000	13,542	23,507	Positive
27	9,631.000	13,996	23,627	Positive
28	11,170	15,511	26,681	Positive
29	0	13,438	13,438	Negative
30	0	12,032	12,032	Negative
31	0	8,529	8,529.000	Negative

Samples yielding <50 positive droplets were categorized as borderline positive to indicate low-abundance signals near the detection threshold. *SRY*, sex-determining region Y.

Table VII. ddPCR *SRY* gene results and clinical data.

Sample no.	ddPCR *SRY* prediction results	Clinical records
3	Positive	Compatible
14	Positive	Compatible
18	Positive	Compatible
29	Negative	Compatible
30	Negative	Compatible

ddPCR, digital droplet PCR; *SRY*, sex-determining region Y.

challenging nature of SCM-derived cfDNA. This discrepancy may have partly stemmed from the intrinsic characteristics of SCM-derived cfDNA, which contain a variable mixture of embryonic and maternal DNA fragments. The present findings are clinically relevant given that accurate RhD genotyping is important in preventing HDFN. Previous studies have shown that maternal DNA contamination and allelic drop-out are major limiting factors in RhD genotyping when fetal or embryonic DNA input is low (29,30). In addition, the *RHD* gene exhibits structural complexity, including pseudogenes and hybrid alleles, which may lead to false-positive or false-negative results depending on the region being targeted (31). These biological and technical constraints may explain why exon 7 and exon 10 amplification results were not always consistent across samples. To reduce these diagnostic risks in future clinical applications, the present study recommends employing a more effective multiplexing approach. Targeting at least three exons of the *RHD* gene at the same time in a single ddPCR reaction (such as exons 5, 7 and 10)

Table VIII. Digital droplet PCR results of the *MT-CYB* gene.

Sample no.	Maternal age, years	*MT-CYB* positive droplet count	*β-globin* positive droplet count
1	38	0	0
2	31	0	0
3	45	0	0
4	38	0	0
5	31	8	0
6	34	0	0
7	48	13	0
8	41	11,764	9,463
9	42	76	0
10	37	11,887	0
11	41	35	0
12	40	7	0
13	44	23	0
14	37	5	0
15	44	12	12,679
16	37	0	0
17	37	0	0
18	45	0	10,630
19	31	99	0
20	37	25	0
21	38	0	46
22	41	0	12,860
23	36	0	0
24	38	0	12,814
25	37	0	10,649
26	41	10,373	8,137
27	39	28	11,100
28	40	9,389	9,575
29	44	6,962	0
30	40	11,563	0
31	38	0	49
32	33	9,312	10
33	41	10,556	0

MT-CYB, mitochondrially encoded cytochrome b.

could make detection notably more precise, compared with just relying on one or two exons.

Despite these limitations, the fact that ddPCR was able to detect *RHD* in fragmented cfDNA samples highlights its robustness and supports its potential for future niPGT applications, particularly when combined with contamination-reduction strategies or embryo-specific molecular markers. Previous studies using qPCR have reported reduced sensitivity at low cfDNA concentrations and in early gestation stages (32). By contrast, ddPCR partitions the DNA template into thousands of nanoliter-sized droplets, allowing absolute quantification of target sequences without the need for standard curves, thus providing a higher tolerance to PCR inhibitors (33). This may explain why, in the present study, ddPCR could reliably detect *RHD* sequences even in samples with low cfDNA input, supporting its advantages compared with conventional PCR methods for niPGT.

SRY gene amplification was successful in 65.3% of samples, with 100% concordance in the five cases with available pregnancy outcome data. *SRY* detection exhibited a higher clinical concordance compared with *RHD*, which may be attributed to its male-specific nature and the absence of maternal background interference. However, the overall amplification rate may indicate that *SRY* detection is still challenged by the low abundance of Y-chromosome fragments in SCM. Studies have reported that Y-derived cfDNA is often less abundant due to both biological factors (for example, fewer copies of Y-chromosomal sequences) and technical factors

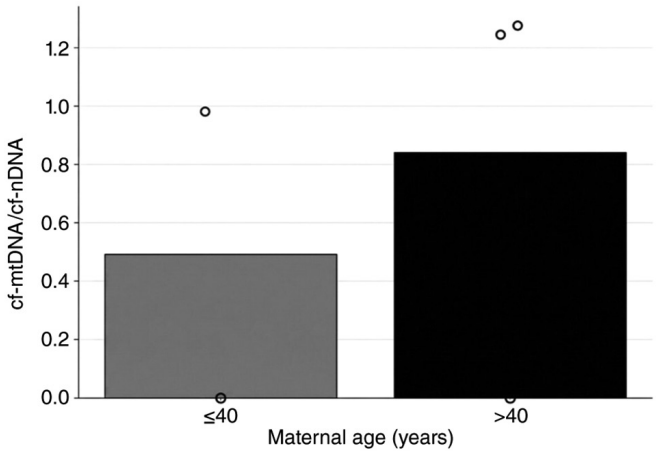

Figure 6. Comparison of mean ratio between cf-mtDNA/cf-nDNA and maternal age. The bars represent the mean values for mothers aged ≤40 and >40 years. The open circles represent individual data points (outliers). cf-mtDNA, mitochondrial cell-free DNA; cf-nDNA, nuclear cell-free DNA.

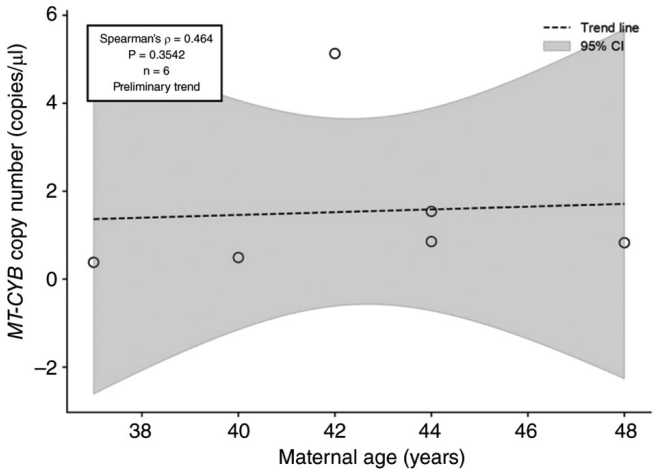

Figure 7. Scatter plot showing the correlation between *MT-CYB* gene copy number per μl and maternal age. The dashed line represents the Spearman's rank correlation trend line and the shaded area indicates the 95% CI. Due to the limited sample size, this correlation is presented strictly as a preliminary and exploratory trend. *MT-CYB*, mitochondrially encoded cytochrome b.

such as preferential degradation of longer Y-chromosome segments (34,35). The 100% concordance in the clinically validated samples nevertheless suggested that when detectable, ddPCR provided highly reliable sex determination for niPGT. It also supports that ddPCR may improve the precision of fetal sex determination, particularly in pregnancies at risk of X-linked disorders, by detecting male-specific DNA at earlier gestational ages and with greater sensitivity compared with qPCR.

It has previously been determined that the condition of mitochondrial DNA serves a key role in understanding the quality of the embryo and that this quality decreases as the maternal age advances (20). In the present study, the quality of cf-mtDNA samples derived from SCM was identified and evaluated with the *MT-CYB* gene as a part of a potential biomarker in niPGT. Mitochondrial cfDNA analysis with *MT-CYB* gene droplet count using ddPCR revealed a positive correlation between maternal age and cf-mtDNA levels, suggesting that mitochondrial stress and dysfunction increase with advancing maternal age, ultimately impacting embryo quality. In a study by May-Panloup *et al* (36), it was stated that as the age of the mother progresses, accumulation of the mtDNA mutations in oocytes increases and not only deteriorates the quality of the oocyte but also increases the risk of allowing mitochondrial abnormalities inherited by the offspring (36).

Given the central role of mitochondria in embryo quality and development, this finding may indicate an age-associated increase in mitochondrial dysfunction in oocytes, which in turn could contribute to reduced fertility. An elevated cf-mtDNA/cf-nDNA ratio may reflect enhanced release of damaged or dysfunctional mitochondria into the extracellular space, potentially impairing the overall health and developmental competence of the oocyte, unless there is embryo fragmentation or degeneration. In the case of embryo fragmentation and degeneration, release of mt-DNA into the SCM may increase regardless of embryo quality and maternal age. Under optimal conditions, which state physiologically intact and healthy embryo samples, the cf-mtDNA/cf-nDNA ratio effect, as presented in Fig. 6, was consistent with the findings of Liu *et al* (37), further supporting the evidence that cf-mtDNA levels increase with maternal age. The cf-mtDNA/cf-nDNA ratio graph shown in Fig. 6 has also been reported in a study by Tsirka *et al* (38), where the ratio was investigated in the context of IVF treatment. In this study, follicular fluid samples were analyzed using qPCR with the ribulose-5-phosphate-3-epimerase gene for cf-nDNA detection. The study found a direct association between increasing maternal age and elevated cf-mtDNA/cf-nDNA ratios. Although the amplification methods and primers used in the present study differed from those employed by previous studies (37,38), the present results corroborate their findings. Nevertheless, this observation should be considered an exploratory finding due to the limited sample size (n=6) and thus requires validation in larger cohorts.

In recent studies, >100 different samples have been analyzed (37,38). Compared with these studies, the reliability of the present findings was statistically less powerful in terms of detecting significant correlations due to the smaller dataset. Thus, obtained results of the present hypothesis should be valued as preliminary and require larger sample data set analysis to eliminate the main limitation in the present research for further studies. The relatively low detection rate of β-globin (12/33 samples) suggested that nuclear cfDNA may be present at lower abundance or may be more susceptible to degradation compared with mitochondrial DNA in SCM. Therefore, apparent increases in mtDNA relative to β-globin should be interpreted cautiously, as they may reflect reduced recovery or detectability of nuclear cfDNA rather than true mitochondrial enrichment alone. Despite these limitations, the present research holds marked value as it was conducted within a Turkish patient group and provides a basis for future studies with larger datasets. Expanding this research within the Turkish patient population has the potential to introduce a notable advancement in reproductive medicine. Anticipating embryo quality prior to implantation may increase the likelihood of achieving successful pregnancies in couples undergoing IVF for certain indications. Consequently, IVF success rates and embryo selection strategies may be improved.

Particularly in cases where age-associated declines in oocyte quality are anticipated, cf-mtDNA level measurements could guide the selection of the most viable embryos for implantation, thereby enabling successful pregnancies, particularly in women of advanced maternal age. Overall, the application of niPGT utilizing cf-mtDNA demonstrates potential clinical utility with minimal invasiveness.

The sample size in the present research was concordant with the G*Power analysis, however was below the threshold for the specificity and sensitivity. Due to the fragmented structure of the cfDNA samples, the amplification rate, despite being higher compared with qPCR, was not satisfactory in all targets. The reliability of ddPCR results when cfDNA is fragmented beyond amplification was evaluated by Liu (39), who demonstrated how some genes, although present, can be silent during the early embryonic stages. In the early stages of embryonic development, even housekeeping genes may preserve a heterochromatin structure, therefore are not part of transcription.

Furthermore, early-stage embryos may release a limited quantity of nuclear DNA and the presence of mitochondrial DNA-rich but nuclear DNA-poor droplets could lead to stochastic loss of the target genes during partitioning, such as housekeeping genes. This limitation may also explain the inconsistent amplification observed between different targets, particularly between RHD exon 7 and exon 10, whereby unequal representation of fragmented genomic regions may result in exon-specific drop-out. Therefore, negative amplification results should be interpreted cautiously, as they may reflect technical limitations associated with low-input fragmented cfDNA rather than the true absence of the target sequence. Genes with high embryo-specificity, such as *SRY*, demonstrate higher clinical concordance, whereas targets susceptible to maternal background noise or genomic structural variation, such as *RHD*, require additional optimization. Inclusion of additional RHD targets, such as multiplexing methods of at least three exons, may improve detection reliability and concordance in future studies. In addition, the incorporation of internal embryonic DNA markers or polymorphic markers may function as a quality control mechanism to determine the presence of adequate embryonic DNA, thereby differentiating a genuine Rh-negative result from a technical amplification failure. This indicates that although niPGT exhibits good potential for diagnosis based on biomarkers, in the present context, it may be insufficient to determine a precise conclusion and should instead be used as a predictive model before further analysis.

An additional limitation of the present study was the absence of systematic validation using paired conventional embryo biopsy-based PGT results. Since embryo sex determination is not permitted to be reported under ethical regulations in Turkey and RHD status is not routinely evaluated within standard PGT protocols (40), clinical data were not available for all embryos included in the present study. Therefore, outcome information could only be obtained from a limited number of transferred embryos that resulted in live birth, where postnatal sex and/or Rh status could be determined. Although these limited follow-up data showed general concordance with SCM-based findings, larger prospective studies incorporating paired biopsy-PGT validation and broader clinical follow-up are required to determine the diagnostic accuracy and clinical applicability of this non-invasive approach. In addition, the correlation between the cf-mtDNA/cfDNA ratio and maternal age warrants further investigation. The subsample used for this specific correlation consisted of only 6 samples (n=6), which was markedly below the minimum sample size established by the present G*Power analysis. Therefore, the findings for this endpoint are underpowered and should be considered strictly preliminary. Nevertheless, cf-mtDNA-based parameters may still serve as promising non-invasive biomarkers in future studies.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

TG, EGA, SS and EB conceptualized and designed the study. SS, MC, ZS, EK, BKB, HGC, EB and FB prepared materials and performed the study. SS, EGA and MC performed data collection and analysis. TG, SS, MC, ZS and EK prepared the original draft. EGA, BKB, HGC, EB, FB and TG reviewed and edited the manuscript. TG supervised the study. EGA and TG confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was conducted in accordance with institutional and national guidelines for research involving human biological materials. Ethical approval was obtained from the Ethics Committee of Istanbul University Faculty of Medicine (Istanbul, Turkey; approval no. 2023/1488). Written informed consent to participate and consent for the use of anonymized biological samples were obtained in accordance with institutional regulations.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Leaver M and Wells D: Non-invasive preimplantation genetic testing (niPGT): The next revolution in reproductive genetics? Hum Reprod Update 26: 16-42, 2020.

2. Kuznyetsov V, Madjunkova S, Abramov R, Antes R, Ibarrientos Z, Motamedi G, Zaman A, Kuznyetsova I and Librach CL: Minimally invasive cell-free human embryo aneuploidy testing (miPGT-A) utilizing combined spent embryo culture medium and blastocoel fluid-towards development of a clinical assay. Sci Rep 10: 7244, 2020.
3. Vera-Rodriguez M, Diez-Juan A, Jimenez-Almazan J, Martinez S, Navarro R, Peinado V, Mercader A, Meseguer M, Blesa D, Moreno I, *et al*: Origin and composition of cell-free DNA in spent medium from human embryo culture during preimplantation development. Hum Reprod 33: 745-756, 2018.
4. Chen K, Hu Z, Lian Y, Han Y, Zhou X, Li Y, Xiang L, Jiang W, Li M, Zeng P, *et al*: The diagnostic accuracy of preimplantation genetic testing (PGT) in assessing the genetic status of embryos: A systematic review and meta-analysis. Reprod Biol Endocrinol 23: 39, 2025.
5. Günel T, Kalelioğlu I, Ermiş H and Aydınlı K: Detection of fetal RhD gene from maternal blood. J Turk Ger Gynecol Assoc 11: 82-85, 2010.
6. Hall V, Vadakekut ES, Maines J and Avulakunta ID: Hemolytic disease of the fetus and newborn. [Updated 2025 Jan 22]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing, 2026.
7. Drozdowska-Szymczak A, Łukawska S, Mazanowska N, Ludwin A and Krajewski P: Management and treatment outcomes of hemolytic disease of the fetus and newborn (HDFN)-a retrospective cohort study. J Clin Med 13: 4785, 2024.
8. Alalshaikh MA, Alsughayir AH, Alsaif AS, Ababtain SA, Aloyouni SY, Aldilaijan KE and Alsubaie SF: Molecular background of RhD-positive and RhD-negative phenotypes in a Saudi population. Saudi J Med Med Sci 12: 210-215, 2024.
9. Clausen FB: Lessons learned from the implementation of non-invasive fetal RHD screening. Expert Rev Mol Diagn 18: 423-431, 2018.
10. Brouillet S, Martinez G, Coutton C and Hamamah S: Is cell-free DNA in spent embryo culture medium an alternative to embryo biopsy for preimplantation genetic testing? A systematic review. Reprod Biomed Online 40: 779-796, 2020.
11. Migeon BR: X-linked diseases: Susceptible females. Genet Med 22: 1156-1174, 2020.
12. Sinclair AH, Berta P, Palmer MS, Hawkins JR, Griffiths BL, Smith MJ, Foster JW, Frischauf AM, Lovell-Badge R and Goodfellow PN: A gene from the human sex-determining region encodes a protein with homology to a conserved DNA-binding motif. Nature 346: 240-244, 1990.
13. Graves JA: Sex chromosome specialization and degeneration in mammals. Cell 124: 901-914, 2006.
14. Emmanuele V, Sotiriou E, Rios PG, Ganesh J, Ichord R, Foley AR, Akman HO and Dimauro S: A novel mutation in the mitochondrial DNA cytochrome b gene (MTCYB) in a patient with mitochondrial encephalomyopathy, lactic acidosis, and strokelike episodes syndrome. J Child Neurol 28: 236-242, 2013.
15. Barel O, Shorer Z, Flusser H, Ofir R, Narkis G, Finer G, Shalev H, Nasasra A, Saada A and Birk OS: Mitochondrial complex III deficiency associated with a homozygous mutation in UQCRQ. Am J Hum Genet 82: 1211-1216, 2008.
16. Saleh Jaweesh M, Hammadeh ME, Dahadhah FW, Al Zoubi MS and Amor H: Association between the single nucleotide variants of the mitochondrial cytochrome B gene (MT-CYB) and the male infertility. Mol Biol Rep 49: 3609-3616, 2022.
17. Wallace DC: Mitochondria and cancer. Nat Rev Cancer 12: 685-698, 2012.
18. Blakely EL, Mitchell AL, Fisher N, Meunier B, Nijtmans LG, Schaefer AM, Jackson MJ, Turnbull DM and Taylor RW: A mitochondrial cytochrome b mutation causing severe respiratory chain enzyme deficiency in humans and yeast. FEBS J 272: 3583-3592, 2005.
19. Glick I, Kadish E and Rottenstreich M: Management of pregnancy in women of advanced maternal age: Improving outcomes for mother and baby. Int J Womens Health 13: 751-759, 2021.
20. Fragouli E, Spath K, Alfarawati S, Kaper F, Craig A, Michel CE, Kokocinski F, Cohen J, Munne S and Wells D: Altered levels of mitochondrial DNA are associated with female age, aneuploidy, and provide an independent measure of embryonic implantation potential. PLoS Genet 11: e1005241, 2015.
21. Faul F, Erdfelder E, Buchner A and Lang AG: Statistical power analyses using G*Power 3.1: Tests for correlation and regression analyses. Behav Res Methods 41: 1149-1160, 2009.
22. ESHRE Guideline Group on Good Practice in IVF Labs; De los Santos MJ, Apter S, Coticchio G, Debrock S, Lundin K, Plancha CE, Prados F, Rienzi L, Verheyen G, *et al*: Revised guidelines for good practice in IVF laboratories (2015). Hum Reprod 31: 685-686, 2016.
23. ESHRE PGT Consortium Steering Committee; Carvalho F, Coonen E, Goossens V, Kokkali G, Rubio C, Meijer-Hoogeveen M, Moutou C, Vermeulen N and De Rycke M: ESHRE PGT consortium good practice recommendations for the organisation of PGT. Hum Reprod Open 2020: hoaa021, 2020.
24. Gardner DK and Schoolcraft WB: In vitro culture of human blastocyst. In: Jansen R and Mortimer D (eds), Towards Reproductive Certainty: Infertility and Genetics Beyond 1999: The Plenary Proceedings of the 11th World Congress on In Vitro Fertilization and Human Reproductive Genetics, Parthenon Press, Pearl River, pp378-388, 1999.
25. Casper J, Speir ML, Raney BJ, Perez G, Nassar LR, Lee CM, Hinrichs AS, Gonzalez JN, Fischer C, Diekhans M, *et al*: The UCSC genome browser database: 2026 update. Nucleic Acids Res 54 (D1): D1331-D1335, 2026.
26. Hromadnikova I, Vechetova L, Vesela K, Benesova B, Doucha J, Kulovany E and Vlk R: Non-invasive fetal RHD exon 7 and exon 10 genotyping using real-time PCR testing of fetal DNA in maternal plasma. Fetal Diagn Ther 20: 275-280, 2005.
27. Lo YM, Corbetta N, Chamberlain PF, Rai V, Sargent IL, Redman CW and Wainscoat JS: Presence of fetal DNA in maternal plasma and serum. Lancet 350: 485-487, 1997.
28. American College of Obstetricians and Gynecologists Committee on Gynecologic Practice and Practice Committee: Female age-related fertility decline. Committee opinion No. 589. Fertil Steril 101: 633-634, 2014.
29. Ontario Health (Quality): Noninvasive fetal RhD blood group genotyping: A health technology assessment. Ont Health Technol Assess Ser 20: 1-160, 2020.
30. Thornhill AR and Snow K: Molecular diagnostics in preimplantation genetic diagnosis. J Mol Diagn 4: 11-29, 2002.
31. Flegel WA: The genetics of the Rhesus blood group system. Blood Transfus 5: 50-57, 2007.
32. Witchel SF, Rajkovic A and Yatsenko SA: Discrepancies between sex prediction and fetal sex after prenatal noninvasive cell-free DNA screening. J Endocr Soc 9: bvaf007, 2025.
33. Lo YM, Tein MS, Lau TK, Haines CJ, Leung TN, Poon PM, Wainscoat JS, Johnson PJ, Chang AM and Hjelm NM: Quantitative analysis of fetal DNA in maternal plasma and serum: Implications for noninvasive prenatal diagnosis. Am J Hum Genet 62: 768-775, 1998.
34. Hindson BJ, Ness KD, Masquelier DA, Belgrader P, Heredia NJ, Makarewicz AJ, Bright IJ, Lucero MY, Hiddessen AL, Legler TC, *et al*: High-throughput droplet digital PCR system for absolute quantitation of DNA copy number. Anal Chem 83: 8604-8610, 2011.
35. Chiu RWK, Chan KCA, Gao Y, Lau VYM, Zheng W, Leung TY, Foo CHF, Xie B, Tsui NBY, Lun FMF, *et al*: Noninvasive prenatal diagnosis of fetal chromosomal aneuploidy by massively parallel genomic sequencing of DNA in maternal plasma. Proc Natl Acad Sci USA 105: 20458-20463, 2008.
36. May-Panloup P, Boucret L, Chao de la Barca JM, Desquiret-Dumas V, Ferré-L'Hotellier V, Morinière C, Descamps P, Procaccio V and Reynier P: Ovarian ageing: The role of mitochondria in oocytes and follicles. Hum Reprod Update 22: 725-743, 2016.
37. Liu Y, Shen Q, Zhao X, Zou M, Shao S, Li J, Ren X and Zhang L: Cell-free mitochondrial DNA in human follicular fluid: A promising bio-marker of blastocyst developmental potential in women undergoing assisted reproductive technology. Reprod Biol Endocrinol 17: 54, 2019.
38. Tsirka G, Zikopoulos A, Papageorgiou K, Kostoulas C, Tsigkas I, Moustakli E, Kaltsas A, Sarafi E, Michaelidis TM and Georgiou I: The ratio of cf-mtDNA vs cf-nDNA in the follicular fluid of women undergoing IVF is positively correlated with age. Genes (Basel) 14: 1504, 2023.
39. Liu Y: At the dawn: Cell-free DNA fragmentomics and gene regulation. Br J Cancer 126: 379-390, 2022.
40. Republic of Turkey Ministry of Health: Regulation on assisted reproductive technology applications and assisted reproductive technology centers. Official Gazette of the Republic of Turkey. Legislation no. 29135, Turkey, 2014. Available from: https://www.mevzuat.gov.tr/mevzuat?MevzuatNo=20085&MevzuatTur=7&MevzuatTertip=5 (In Turkish). Accessed 23/06/2026.