Received: 12 July 2022 | Revised: 12 October 2022 | Accepted: 27 November 2022

DOI: 10.1002/pd.6277

ORIGINAL ARTICLE

PRENATAL DIAGNOSIS WILEY

Presence of fetal DNA in maternal exhaled breath condensate

Sıtkı Tıplamaz[1,2] | İrem Peker Eyüboğlu[1] | Canan Ünal[3,4] | Özge Soyer[3] | Mehmet Sinan Beksaç[3] | Mustafa Akkiprik[1]

[1]Department of Medical Biology and Genetics, Faculty of Medicine, Marmara University, İstanbul, Turkey

[2]Department of Forensic Medicine, Faculty of Medicine, Marmara University, İstanbul, Turkey

[3]Department of Obstetrics and Gynecology, Division of Perinatology, Faculty of Medicine, Hacettepe University, Ankara, Turkey

[4]Gazi Yaşargil Training and Research Hospital, Diyarbakır, Turkey

Correspondence
Sıtkı Tıplamaz, Department of Forensic Medicine, Faculty of Medicine, Marmara University, Pendik, İstanbul, Turkey.
Email: tiplamaz@gmail.com

Present address
Bostancı Mah, Bahçelerarası sok, No:2 Saray Apt, D:12 Kat:6 Kadıköy, İstanbul, Turkey.

Funding information
Marmara Üniversitesi, Grant/Award Number: SAG-C-YLP-130319-0094

Abstract

Objectives: Cell-free DNA has been found in all body fluids, but DNAs emerging from locations that are not in direct contact with breath in exhaled breath condensate (EBC) are yet to be found. The potential of EBC for prenatal and cancer screening prompted us to investigate whether fetal DNA is present in maternal EBC.

Method: A total of 20 pregnant women's EBC and blood samples were collected. Four Y chromosome-specific assays were tested on all EBC and plasma samples by quantitative PCR (qPCR). The best-performing assay was used for digital droplet PCR (ddPCR) on all EBC and the six plasma samples.

Results: The sex of the fetuses was accurately determined from plasma samples. DNA sequences could not be properly amplified in EBC samples by the qPCR. By ddPCR, the Y chromosome sequence was amplified in two of the 11 EBC samples, from women carrying male fetuses (2/11), and the Y chromosome sequence was not amplified in the EBC of women carrying female fetuses (9/9). Exhaled breath condensate ddPCR result's specificity was 100%, the detection rate of Y chromosome was 18.18% (2/11), and the corrected accuracy was 59.09%.

Conclusion: Our finding of "the presence of fetal DNA in maternal EBC", despite the low detection rate, might have a major impact on prenatal diagnosis and cancer screening.

Key points

What's already known about this topic?

- DNA/RNA sequences of lung cancer, *Mycobacterium tuberculosis*, and SARS-Cov-2 were detected in exhaled breath condensate (EBC). Cell-free DNA (cfDNA) has been detected in various body fluids like blood, urine, cerebrospinal fluid, sweat, and breast milk, and cell-free fetal DNA (cffDNA) has been shown in maternal plasma. Also, the lungs are thought to be filters of fetal cells in the maternal circulation.

What does this study add?

- This is the first study that showed the presence of Y chromosome sequence in the EBC of a woman carrying a male fetus. Additionally, the presence of a DNA sequence that does not belong to the gastrointestinal tract or respiratory system in the breath has not been shown until now. The findings of this study may have an impact on non-invasive prenatal diagnosis and cancer screening.

1 | INTRODUCTION

The exchange of nucleic acids and cells between the fetus and its mother is a well-known phenomenon. Schmorl is the first to report the presence of fetal cells in maternal circulation. He especially stated the presence of trophoblasts in the lungs of women whose cause of death was eclampsia.[1] Douglas GW, Thomas L, Carr M, et al. took their work one step further and showed the presence of trophoblasts in the uterine and inferior vena cava veins but not in the peripheral veins.[2] Potential fetal cell types entering maternal circulation are mainly trophoblastic cells, and these cells are most probably the origin of cffDNA existing in maternal fluids/tissues including lungs.[1–4] cfDNA has been demonstrated in various other body fluids like urine, saliva, breast milk, bronchial aspirate, etc., as a consequence of circulating plasma and serum throughout the whole body.[5] On the other hand, the rapid clearance of cffDNA from the maternal circulation makes it specific to the pregnancy.[6] Thus, the lungs could be one of the main places for the elimination of cffDNA and fetal cells. However, the current state of the literature on the metabolism/excretion of cffDNA and fetal cells in maternal circulation is still debated.

Exhaled breath condensate is a reliable biomarker of lung disorders, equivalent to blood, sweat, tears, urine, and saliva.[7] Various substances such as oxidative stress and inflammatory mediators, cfDNA, microRNAs, and mitochondrial DNA can be measured in EBC in various lung disorders, mainly in lung cancer.[8] The exact site of origin of substances in EBC is unknown, and they do not correlate with substances in bronchoalveolar lavage fluid.[9] In this study, we aimed to investigate whether fetal DNA is present in maternal EBC, which has never been studied before.

2 | METHOD

The study was performed according to the principles set out in the World Medical Association Declaration of Helsinki and was approved by the local ethics committee of the Medical Faculty of Marmara University (Protocol No: 09.2018.713). The most critical point to ensure the high quantity and quality of the samples is to maintain Y chromosome-free conditions. Specifically, the surfaces were cleaned with a solution containing 70% alcohol and 10% sodium hypochlorite before and after the sampling. The participants waited 10 min before the sampling. All the materials that came into contact with samples were sterilized before each sampling. Barrier-filter tips were used during all steps. All the sample collection and laboratory phases like cleaning, sample collection, DNA isolation, and amplification were performed by female staff only to avoid contamination with Y-chromosomal sequences. Males were not allowed to be in the same place as the participants in the waiting and sampling rooms.

2.1 | Patients

The pregnant women attending Hacettepe University Faculty of Medicine Department of Obstetrics and Gynecology (Ankara, Turkey) for routine control were recruited with informed consent. The inclusion criterion was to have a healthy singleton pregnancy between 16th and 40th gestational weeks, and the exclusion criteria were respiratory tract inflammation, organ transplantation, recent blood transfusion (<one year), and obstetric complications. The sample size was planned as 50 patients within a one-year period, and the collection of samples started on October 3, 2019. The COVID-19 pandemic erupted, so we had to terminate the sampling on February 28, 2020. For this reason, the study was continued with 20 participants' samples. The sex of the fetuses was determined postnatally. The sample collection phase was done at Hacettepe University Faculty of Medicine Department of Pediatric Allergy (Ankara, Turkey).

2.2 | Exhaled breath condensate

Exhaled breath condensate was collected using the EcoScreen™ instrument (Jaeger, Wurzburg, Germany) following the guidelines for EBC sampling by the ERS/ATS (Europian Respiratory Society/American Thoracic Society) Task Force.[10] Participants were requested to refrain from eating and drinking (except water) 1 hour before EBC collection. Prior to the sample collection, the participants were asked to rinse their mouths with sterile water and, then to breathe tidally through a mouthpiece for 10 min. An amount ranging from 1 to 3 ml of EBC was collected from each participant. After EBC collection, the samples were stored immediately at −21°C in 500 µL aliquots and then transferred to −80°C within four hours and stored there until the next procedure.

2.3 | Blood samples

5–10 ml of peripheral blood were obtained into a tube containing Sodium Ethylenediaminetetraacetic acid (EDTA) and stored at 4°C for no more than four hours for the next procedure. Blood was centrifuged at 1600 × g for 10 min at 4°C to separate the plasma from the cell components. The separated plasma was transferred into a fresh tube, centrifuged again at 16,000 × g for 10 min to remove any remaining cells, and then transferred into a fresh tube. The tubes were stored immediately at −21°C in 500 µL aliquots and then transferred to −80°C within four hours and stored there until the next procedure.

All the samples that were collected and stored at −80°C were transferred to Marmara University Faculty of Medicine, Department of Medical Biology and Genetics (Istanbul, Turkey) in a special case containing dry ice for maintaining the cold chain and immediately stored at −80°C. All laboratory staff was blinded to the sex of the fetuses.

2.4 | DNA extraction

Each aliquot was thawed only once. Cell-free DNA was extracted from all plasma and EBC samples using the DSP Virus Kit (Qiagen,

Hilden, Germany) according to the manufacturer's instructions. 200 µL plasma and 400 µL EBC were eluted in 40 µL elution buffer separately.

2.5 | Quantitative PCR (qPCR)

The extracted cfDNA was quantified using the LightCycler 480 Probes Master kit on Roche LightCycler 480 (Roche, Rotkreuz, Switzerland) instruments with their corresponding software using two forward and two reverse primers in four different ways. DYS14 was selected as a target gene region because it has multiple copies on the Y chromosome, thus increasing the probability of detection. Four assays targeting the DYS14 gene with final amplicon lengths of 84 bp, 177bp, 197bp, and 290 bp have been successful before.[11] The four assays were based on the combination of four primers and one common TaqMan probe with sequences. As a housekeeping gene sequence, the glyceraldehyde phosphate dehydrogenase (GAPDH) enzyme gene region was used. The forward and reverse primers and probes were as follows:

DYS14-F_1 5′-GGGCCAATGTTGTATCCTTCTC-3′, DYS14-R_1 5′-GCCCATCGGTCACTTACACTTC-3′,
DYS14-F_2 5′-GTTAATGCCCAAGCCAGGA-3′, DYS14-R_2 5′-CAATAGTACCCACGCCTGCT-3′,
FAM/TAMRA labeled DYS14 probe 5′-TCTAGTGGA-GAGGTGCTC-3′,
GAPDH F 5′ GAAGGTGAAGGTCGGAGT-3′, GAPDH R 5′ GAAGATGGTGATGGGATTTC-3′
FAM/TAMRA labeled GAPDH probe 5′-CAAGCTTCCCGTTCT-CAGCC-3′.

PCR amplification was performed according to a previously published study.[12] Briefly, 4 µL of template DNA was used in 20 µL reaction volume containing 0.8 µmol/L of each primer and 0.4 µmol/L of each probe. After the initial denaturation step at 95°C for 2 min, 50 amplification cycles were performed; each consisted of a denaturation step at 95°C for 10 s, followed by an annealing step at 60°C for 60 s, and a 1 s elongation step at 72°C with a single fluorescence read. The Ct value of 36 was set as the cutoff for a positive signal of amplification.

2.6 | Digital droplet PCR (ddPCR)

DNA was detected and quantified using a QX200™ Droplet Digital™ PCR system (Bio-Rad Laboratories, Hercules, CA, USA). Briefly, 11 µL of 2xddPCR Supermix for Probes (Bio-Rad Laboratories), 0.8 µmol/L of each primer, and 0.4 µmol/L of the probe were mixed with 8 µL of DNA template in a reaction volume of 22 µL. For the plasma sample, unlike the EBC, 2 µL of DNA template was used in a reaction volume of 22 µL. Just one primer pair (DYS14 F_1-DYS14 R_1) was used due to the low amount of EBC. This selected primer pair was the best-performing pair according to plasmas and EBCs quantitative PCR (qPCR) results, and the length of the amplicon was 84 bp. To generate the droplets, 20 µL of digital droplet PCR (ddPCR) reaction and 70 µL of Droplet Generation Oil for Probes (Bio-Rad Laboratories) were inserted in an eight-well cartridge using an Automated Droplet Generator (Bio-Rad Laboratories) according to the manufacturer's instructions. Then, 40 µL of the generated droplet emulsion was transferred to a new 96-well PCR plate (Eppendorf, Hamburg, Germany) and amplified in the C1000 Touch™ Thermal cycler (Bio-Rad Laboratories). Amplification conditions started with 10 min of activation of DNA polymerase at 95°C, followed by 40 cycles of a two-step thermal profile of 30 s at 94°C for denaturation and 1 min at 60°C for annealing and extension. A final hold of 10 min at 98°C was used for the enzyme inactivation. Digital droplet PCR does not need optimization with respect to qPCR annealing or probe concentration. After thermal cycling, plates were transferred to a QX200TM Droplet Reader (Bio-Rad Laboratories). The software provided with the ddPCR system (QuantaSoft 1.3.2.0; Bio-Rad Laboratories) was used for data acquisition to calculate the absolute concentration of target DNA in copies/µL of reaction using Poisson distribution analyses.

3 | RESULTS

A total of 20 pregnant women participated in this study. They gave birth to 11 male and 9 female babies. Their gestational ages at the time of sampling were between 16 and 37 weeks. The mean gestational age was 28.9 weeks, and the mean fetus weight was 1785.45 gr according to ultrasonographic measurements at the time of sampling. One pregnant woman had late-onset gestational diabetes mellitus. Detailed clinical information about participants is shown in Table 1.

Y chromosome sequences were detected in the plasma samples of all women carrying male fetuses (11/11) by the qPCR method. None of the Y chromosome sequences were detected in the plasma samples of women carrying female fetuses (9/9) by the qPCR method. DNA sequences (including the targeted housekeeping gene) could not be amplified efficiently from EBC samples by the qPCR method.

Three women carrying male fetuses and three women carrying female fetuses were randomly selected from their pools, and their plasma extracts were also run with ddPCR. The sex of these six fetuses was correctly determined with ddPCR. All the EBC extracts were run with ddPCR, and the Y chromosome sequence was amplified in two of the 11 EBC samples from women carrying male fetuses (2/11), but the Y chromosome sequence was not amplified in the EBC of women carrying female fetuses (9/9). The graphs of ddPCR are shown in Figures 1 and 2. The results of qPCR and ddPCR are shown in Table 2 and basic statistics of the results are shown in Table 3.

TABLE 1 Clinical data of the participants

Case number	Gestational weeks at sampling	Fetus weight at sampling (gr)	Age of mother (year)	BMI (kg/m^2)	Parity	Sex of the other child/ children	Disease of mother	Disease of newborn	Medical intervention
Male fetuses									
1	16	160	24	18.27	G1P0	x	Rheumatoid Arthritis	x	36 weeks, CS
3	20	337	26	19.49	G1P0	x	x	Prematurity	32 weeks, CS
4	37	3200	37	43.28	G4P2	Male, male	Gestational diabetes	x	40 weeks, CS
6	29	1330	25	28	G2P0	Female	x	Heart murmur	36 weeks, CS
8	34	2300	25	38.45	G1P0	x	Rheumatoid Arthritis	x	39 weeks, CS
9	36	3000	30	28.34	G4P2	Female, female	Rheumatoid Arthritis	x	38 weeks, CS
11	29	1350	25	25.40	G1P0	x	x	x	39 weeks, SD
16	29	1430	20	28	G1P0	x	x	x	38 weeks, CS
17	23	582	22	19.81	G1P0	x	x	x	39 weeks, CS
18	22	570	35	22	G4P1	Male	Sickle cell anemia	Sickle cell trait	33 weeks, CS
20	30	1830	41	26.73	G4P1	Male	x	x	37 weeks, CS
Female fetuses									
2	29	1365	40	22.23	G7P1	Male	Ankylosing spondylitis, ventricular septal defect	x	37 weeks, CS
5	37	2870	27	30.33	G1P0	x	Familial mediterranean fever		37 weeks, CS
7	36	2960	29	39.52	G2P1	Twin females	x	x	38 weeks, CS
10	34	2490	28	28.52	G2P0	x	x	Atrial septal defect	38 weeks, CS
12	35	2550	22	24.84	G1P0	x	Autoimmune hepatitis, ulcerative colitis	x	38 weeks, SD
13	20	355	24	17.93	G2P1	Female	x	Prematurity, intracerebral hemorrhage	24 weeks, CS
14	35	2760	40	26.17	G1P0	x	x	x	37 weeks, CS
15	32	2020	21	41.79	G1P0	x	Epilepsy	-	-
19	33	2250	35	29.72	G1P0	x	x		38 weeks, CS

Abbreviations: BDM, body mass index; CS, cesarean section; G, gravida; P, parity; SD, spontaneous delivery; "x", none.

4 | DISCUSSION

Clinical studies have already demonstrated the benefit of using cffDNA for prenatal screening.[6,13] We have shown that fetal DNA can be detected in EBC, and it is too early to say that this finding could be used for prenatal screening, but it is novel information and its mechanism should be explored by future studies. The presence of fetal DNA in EBC was validated by the amplification of the Y chromosome sequence in pregnancies with male fetuses, and the detection rate was 18.18% (2/11).

Exhaled breath condensate is a non-invasive method for sampling exhaled air. It has been reported that EBC is used in the

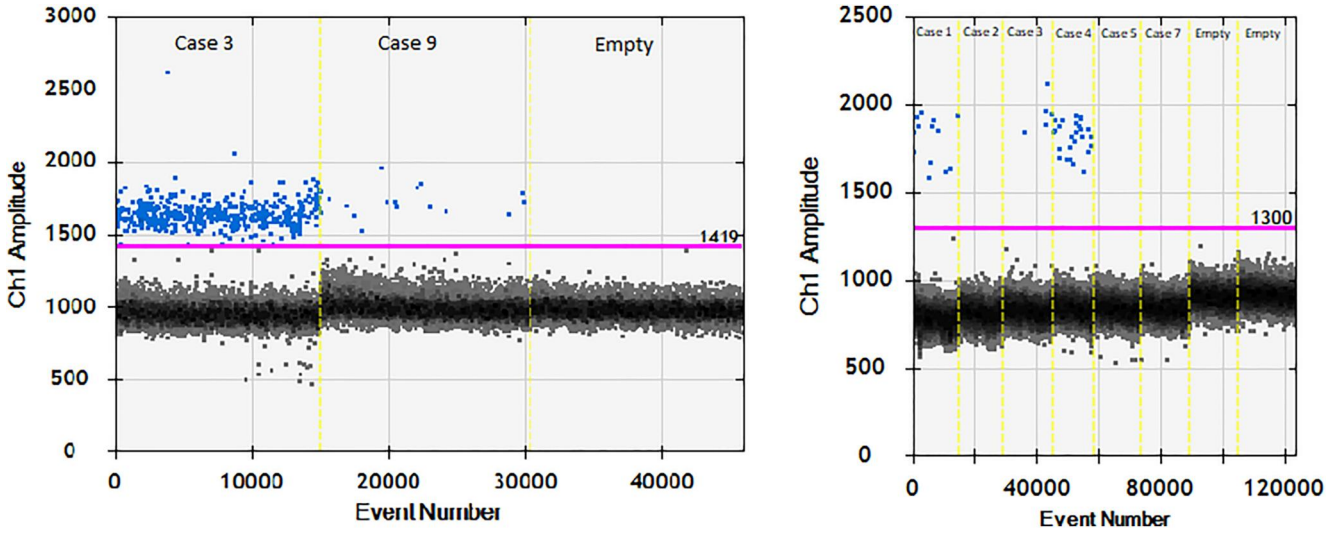

FIGURE 1 Representative graphs for cell-free fetal DNA (cffDNA) in samples analyzed by digital droplet PCR (ddPCR). Blue dots are the droplets that emit fluorescence, which means there is an amplification of DNA. Black dots are the droplets that do not emit fluorescence, which means there is no amplification of DNA. The threshold line is colored in Fuchsia. The left one is the graph of EBC's ddPCR. From left to right; Case 3, Case 9, and No Template Control (NTC) (Empty) respectively. The right one is the graph of plasma's ddPCR. From left to right; Case 1, Case 2, Case 3, Case 4, Case 5, Case 7, NTC (empty), and NTC, respectively.

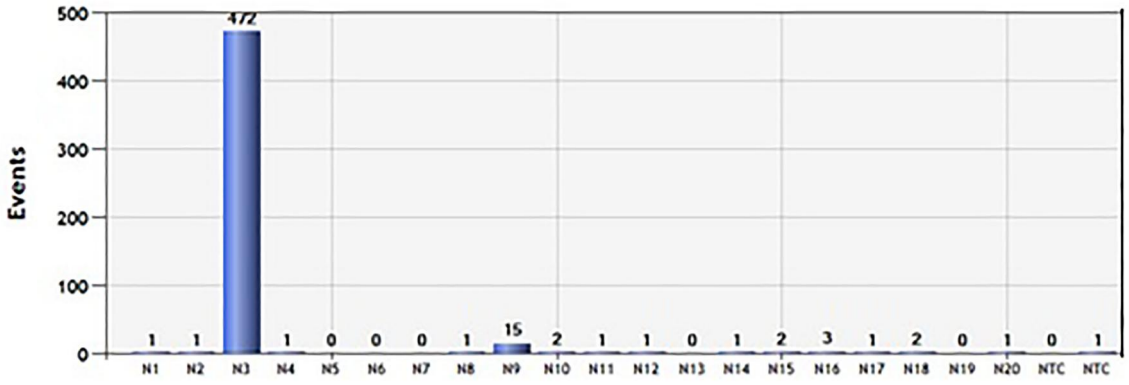

FIGURE 2 Bar chart of cell-free fetal DNA (cffDNA) in exhaled breath condensate (EBC) analyzed by digital droplet PCR (ddPCR). Samples are numbered in the same order as the case number. This chart shows the positive fluorescence signals that droplets emit. NTCs are No Template Control (NTC) (Empty sample). The threshold was calculated as 6.

detection of lung cancer, bronchial asthma, gastrointestinal disorders, inflammatory processes, and lung infections.[14–18] There have been no studies investigating DNAs from locations that are not in direct contact with breath in EBC. This is the first study to demonstrate the presence of fetal DNA in maternal EBC, and that means the EBC not only samples the respiratory and gastrointestinal systems but also samples the whole body. There are similarities between fetus and cancer. They both release cfDNA and living cells into the hosts' blood circulation, and that blood is filtrated and aerated by the lungs. This study showed that fetal DNA can emerge from the breath and may be used for prenatal and cancer screening just as cfDNA in plasma is used.[13,19] Nevertheless, fetal cell isolation will presumably be more precious for sequencing the whole genome and the longer sequences of DNA.

Obtaining an EBC sample is a non-invasive method that is easily repeatable, harmless, and gives a chance for longitudinal sampling. The limitations of this method are the lack of standardization, the lack of evidence for the origin of the aerosol particles, and the lack of enough information about the effects of the disease. There are two well-known EBC collection systems; the EcoScreen™ device is the most commonly known EBC collection system that was used in this study.[20] The Rtube™ is the second method frequently used. Both systems have pros and cons, but using the most frequently used one could diminish the effect of the lack of standardization. EBC-based methods require a strict protocol to reduce variability, increase sensitivity, and prevent contamination.[10] The standard operating procedures have been established for EBC collection, storage, and laboratory analysis. Additionally, special care was taken not to

TABLE 2 Amplification of Y chromosome sequences in plasma and exhaled breath condensate (EBC) using quantitative PCR (qPCR) and digital droplet PCR (ddPCR)

		qPCR DYS14 amplicons				ddPCR DYS14 amplicon	
		Plasma				Plasma	EBC
Case number	Gestational weeks at sampling	84bp	177 bp	197 bp	290 bp	84 bp	84 bp
Male fetuses							
1	16	+	+	+	+	+	-
3	20	+	+	+	-	+	+ (37·5 copies/µL)
4	37	+	+	+	+	+	-
6	29	+	+	+	+	x	-
8	34	+	+	+	+	x	-
9	36	+	+	+	+	x	+ (1·2 copies/µL)
11	29	+	+	+	+	x	-
16	29	+	+	+	+	x	-
17	23	+	+	+	+	x	-
18	22	+	+	+	+	x	-
20	30	+	+	+	+	x	-
Female fetuses							
2	29	-	-	-	-	-	-
5	37	-	-	-	-	-	-
7	36	-	-	-	-	-	-
10	34	-	-	-	-	x	-
12	35	-	-	-	-	x	-
13	20	-	-	-	-	x	-
14	35	-	-	-	-	x	-
15	32	-	-	-	-	x	-
19	33	-	-	-	-	x	-

Note: "+", detected; "-", not detected; "x", not run.

TABLE 3 Sensitivity, specificity, and accuracy comparison between plasma and EBC results

	Plasma (according to qPCR results)	EBC (according to ddPCR results)
Sensitivity	100% (95% CI: 0·7151–1)	18.18% (95% CI: 0·228–0·5178)
Specificity	100% (95% CI: 0·6637–1)	100% (95% CI: 0·6637–1)
PPV	100%	100%
NPV*	100%	50% (95% CI: 0·4305–0·6176)
Accuracy*	100% (95% CI: 0·8316–1)	59·09% (95% CI: 0·3522–0·8018)

Note: *These values were corrected to the prevalence of males (50%). PPV; positive predictive value, NPV; negative predictive value, 95% CI; 95% confidence interval.

contaminate the samples. Thus, there was no false-positive result in both plasma and EBC samples in our study.

The Y chromosome sequence was amplified by ddPCR but not by qPCR, and that shows the importance of using appropriate technology to obtain more precise results. It was also previously reported that ddPCR is more accurate and sensitive than qPCR.[21] We thought that the Y chromosome sequence could not be amplified in nine of 11 samples due to the very low amount of fetal DNA in

EBC. The ddPCR shared similar results with the other sensitive PCR methods, like the BEAMing enhanced digital PCR. A study that used the BEAMing obtained a fetal DNA detection rate of 80% in plasma on average 15.4 days after embryo transfer,[22,23] so the BEAMing-enhanced digital PCR might be an alternative to the ddPCR Additionally, previous technologies, until recently, were not as sensitive as today's to show the presence of cffDNA in EBC. This is probably one of the reasons why it has not been explored before.

In this study, the DSP virus kit was used to extract cfDNA. Studies showed that there was no significant difference in the yield of cfDNA between the DSP virus kit and the Circulating Nucleic Acid (CNA) kit (Qiagen, Hilden, Germany).[12,24] but some studies have recommended using the CNA kit due to better cfDNA yield and larger volume usability.[12,25] Using a larger EBC volume may also increase the detection rate of cffDNA, so the CNA kit could be preferable for further studies.

In Case 3, a 290-bp amplicon could not be amplified in the plasma by qPCR. One possible explanation for this is that there may be a critical mutation where the primer binds, so this fragment cannot be amplified. Another possible explanation is that the cffDNA of Case 3 in plasma may be highly fragmented, not long enough up to 290 bp for detection. The presence of cffDNA in maternal plasma increases during the progression of pregnancy. The portion of cffDNA in plasma increases by 0.1% every week between the 10th and 21st week of gestation, then by 1% every week after the 21st week of gestation.[26] The ddPCR result for Case 3 is inconsistent with this data. At an earlier gestational age (20th gestational age), according to other samples, the Y chromosome sequence was detected in her EBC. Additionally, although Case 9's gestational age was older, there was a discordance in the detected yield of the Y chromosome sequence between Case 3 (37.5 copies/μL) and Case 9 (1.2 copies/μL) by ddPCR. Schlütter et al. showed that a high level of IL-5 is associated with a higher number of isolated fetal cells in maternal blood.[27] Also, another study showed that a high level of IL-5 in maternal serum is associated with preterm delivery.[28] Case 3 had a preterm delivery. These observations may explain why we detected the higher yield of the Y chromosome sequence at an early gestational age. Samples from Case 9 were obtained at the 36th gestational week, and the Y chromosome sequence was detected in her EBC by ddPCR. This case was the second oldest according to the gestational age of the women carrying male fetuses. The oldest one was Case 4 but the Y chromosome sequence was not detected in her EBC by ddPCR, and neither was it in the third oldest one. Their body mass indexes (BMIs) were different. The BMI of Case 9 was 28.34, the BMI of Case 4 was 43.28, and the BMI of Case 8 was 38.45. A negative association was observed between maternal BMI and the yield of cffDNA in plasma.[26] There is also a huge variation in fetal fractions between patients at the same gestational age. These could be the reasons why we could not detect the Y chromosome sequence in those EBC samples.

The underlying mechanisms for the presence of cffDNA in maternal EBC have yet to be established. One possible mechanism is the circulating cfDNA in plasma throughout the whole body.[5] The other one is that the lungs are the places that eliminate fetal cells.[4] We have previously shown fetal cell microchimerism in maternal liver and skin but not in the lung tissue, probably because lungs eliminate cffDNA and fetal cells.[4,29]

Due to the nature of the used method, there are some drawbacks such as mosaicism, chimerism, vanished twin syndrome, and multiple gestations that affect the accuracy of the test as in Non-Invasive Prenatal Tests.[29,30]

The limitations of this study were the small sample size due to the COVID-19 pandemic that stopped us from obtaining more samples, and that we could not run other amplicons except 84 bp in ddPCR due to the low volume of EBC. We hypothesize that the low detection rate can be improved by increasing the volume of EBC, adding extra centrifugation steps before DNA extraction, using better extraction methods, shortening the amplicon size (due to cffDNA clearance from the lungs), targeted sequencing, or more advanced PCR techniques, etc.

Although these limitations, this study is the first to report fetal DNA in maternal EBC. Once the methodology is established, cffDNA in the EBC will be detected earlier in pregnancy, and this method/approach will be feasible for prenatal screening in the future.

5 | CONCLUSION

The presence of fetal DNA in the maternal EBC is shown, despite the low detection rate. But this rate can likely be improved by modifications to the method. The underlying mechanism of this finding should be further investigated. Although it is too early to rely on this method and more work is needed to improve, these findings might be the start of a new line of biological exploration.

ACKNOWLEDGMENTS

We would like to thank Health Technicians, Nagihan Dumlu and Dilara Çalıker, from Hacettepe University Faculty of Medicine Department of Pediatric Allergy for their contributions to the sampling and data collection of the participants. Also, we appreciate Assist. Prof. Aikaterini Panteli, MD for her precious review and comments. This study was funded by the Research Fund of Marmara University (Project Number: SAG-C-YLP-130319-0094). All the needed laboratory materials were purchased by this fund.

CONFLICT OF INTEREST

Marmara University and Hacettepe University have filed a patent application for described method. All authors are named inventors on the patent application. All authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Requests for data should be directed to the lead author (tiplamaz@gmail.com). Patient-level data will be made available within 6 months of publication. Requests will be assessed for scientific rigor before being granted. Data will be anonymized and securely transferred. A data-sharing agreement may be required.

ORCID

Sıtkı Tıplamaz https://orcid.org/0000-0001-9015-940X
İrem Peker Eyüboğlu https://orcid.org/0000-0003-0764-9841
Canan Ünal https://orcid.org/0000-0003-3783-5637
Özge Soyer https://orcid.org/0000-0002-7444-251X
Mehmet Sinan Beksaç https://orcid.org/0000-0001-6362-787X
Mustafa Akkiprik https://orcid.org/0000-0002-1100-765X

REFERENCES

1. Lapaire O, Holzgreve W, Oosterwijk JC, Brinkhaus R, Bianchi D. Georg Schmorl on trophoblasts in the maternal circulation. *Placenta*. 2007;28:1-5. https://doi.org/10.1016/j.placenta.2006.02.004
2. Douglas GW, Thomas L, Carr M, Cullen NM, Morris R. Trophoblast in the circulating blood during pregnancy. *Am J Obstet Gynecol*. 1959;78(5):960-973. https://doi.org/10.1016/s0002-9378(16)36649-2
3. Holzgreve W, Garritsen HSP, Ganshirt-Ahlert D. Fetal cells in the maternal circulation. *J Reprod Med*. 1992;37:410-418.
4. Sargent IL, Johansen M, Chua S, Redman CWG. Clinical experience: isolating trophoblasts from maternal blood a. *Ann N Y Acad Sci*. 1994;731(1):154-161. https://doi.org/10.1111/j.1749-6632.1994.tb55762.x
5. Hui L, Maron JL, Gahan PB. *Other Body Fluids as Non-invasive Sources of Cell-free DNA/RNA*. Springer. Kluwer Academic Publishers; 2015:295-323.
6. Rafi I, Chitty L. Cell-free fetal DNA and non-invasive prenatal diagnosis. *Br J Gen Pract*. 2009;59(562):e146-e148. https://doi.org/10.3399/bjgp09x420572
7. Hunt J. Exhaled breath condensate: an overview. *Immunol Allergy Clin*. 2007;27(4):587-596. https://doi.org/10.1016/j.iac.2007.09.001
8. Youssef O, Sarhadi VK, Armengol G, Piirila P, Knuuttila A, Knuutila S. Exhaled breath condensate as a source of biomarkers for lung carcinomas. A focus on genetic and epigenetic markers-A mini-review. *Gene Chromosome Cancer*. 2016;55(12):905-914. https://doi.org/10.1002/gcc.22399
9. Liang Y, Yeligar SM, Brown LAS. Exhaled breath condensate: a promising source for biomarkers of lung disease. *Sci World J*. 2012;2012:1-7. https://doi.org/10.1100/2012/217518
10. Horváth I, Hunt J, Barnes PJ. Exhaled breath condensate: methodological recommendations and unresolved questions. *Eur Respir J*. 2005;26(3):523-548. https://doi.org/10.1183/09031936.05.00029705
11. Minárik G, Nagyová E, Repiská G, et al. Detection of contamination in noninvasive prenatal fetal gender test. *Science*. 2015;357:181-184.
12. Repiská G, Sedláčková T, Szemes T, Celec P, Minarik G. Selection of the optimal manual method of cell free fetal DNA isolation from maternal plasma. *Clin Chem Lab Med*. 2013;51(6):1185-1189. https://doi.org/10.1515/cclm-2012-0418
13. Rose NC, Kaimal AJ, Dugoff L, Norton ME. Screening for fetal chromosomal abnormalities: ACOG practice bulletin, number 226. *Obstet Gynecol*. 2020;136(4):e48-e69. https://doi.org/10.1097/aog.0000000000004084
14. Markar SR, Wiggins T, Antonowicz S, et al. Assessment of a noninvasive exhaled breath test for the diagnosis of oesophagogastric cancer. *JAMA Oncol*. 2018;4(7):970. https://doi.org/10.1001/jamaoncol.2018.0991
15. Wan Q-S, Zhang K-H. Noninvasive detection of gastric cancer. *Tumor Biol*. 2016;37(9):11633-11643. https://doi.org/10.1007/s13277-016-5129-4
16. Mehta A, Cordero J, Dobersch S, et al. Non-invasive lung cancer diagnosis by detection of GATA 6 and NKX 2-1 isoforms in exhaled breath condensate. *EMBO Mol Med*. 2016;8(12):1380-1389. https://doi.org/10.15252/emmm.201606382
17. Soyer T, Soyer ÖU, Birben E, Kisa U, Kalayci O, Cakmak M. Pepsin levels and oxidative stress markers in exhaled breath condensate of patients with gastroesophageal reflux disease. *J Pediatr Surg*. 2013;48(11):2247-2250. https://doi.org/10.1016/j.jpedsurg.2013.02.100
18. Rahimpour E, Khoubnasabjafari M, Jouyban-Gharamaleki V, Jouyban A. Non-volatile compounds in exhaled breath condensate: review of methodological aspects. *Anal Bioanal Chem*. 2018;410(25):6411-6440. https://doi.org/10.1007/s00216-018-1259-4
19. Ignatiadis M, Sledge GW, Jeffrey SS. Liquid biopsy enters the clinic—implementation issues and future challenges. *Nat Rev Clin Oncol*. 2021;18(5):297-312. https://doi.org/10.1038/s41571-020-00457-x
20. Davis MD, Montpetit AJ. Exhaled breath condensate. *Immunol Allergy Clin*. 2018;38(4):667-678. https://doi.org/10.1016/j.iac.2018.06.002
21. Tang H, Cai Q, Li H, Hu P. Comparison of droplet digital PCR to real-time PCR for quantification of hepatitis B virus DNA. *Biosci Biotechnol Biochem*. 2016;80(11):2159-2164. https://doi.org/10.1080/09168451.2016.1196576
22. Karakas B, Qubbaj W, Al-Hassan S, Coskun S. Noninvasive digital detection of fetal DNA in plasma of 4-week-pregnant women following in vitro fertilization and embryo transfer. *PLoS One*. 2015;10(5):e0126501. https://doi.org/10.1371/journal.pone.0126501
23. O'Leary B, Hrebien S, Beaney M, et al. Comparison of BEAMing and droplet digital PCR for circulating tumor DNA analysis. *Clin Chem*. 2019;65(11):1405-1413. https://doi.org/10.1373/clinchem.2019.305805
24. Warton K, Graham L-J, Yuwono N, Samimi G. Comparison of 4 commercial kits for the extraction of circulating DNA from plasma. *Cancer Genet*. 2018;228-229:143-150. published online March. https://doi.org/10.1016/j.cancergen.2018.02.004
25. Jain M, Balatsky AV, Revina DB, Samokhodskaya LM. Direct comparison of QIAamp DSP Virus Kit and QIAamp Circulating Nucleic Acid Kit regarding cell-free fetal DNA isolation from maternal peripheral blood. *Mol Cell Probes*. 2019;43:13-19. https://doi.org/10.1016/j.mcp.2018.12.006
26. Zhou Y, Zhu Z, Gao Y, et al. Effects of maternal and fetal characteristics on cell-free fetal DNA fraction in maternal plasma. *Reprod Sci*. 2015;22(11):1429-1435. https://doi.org/10.1177/1933719115584445
27. Schlütter JM, Kirkegaard I, Petersen OB, et al. Fetal gender and several cytokines are associated with the number of fetal cells in maternal blood – an observational study. *PLoS One*. 2014;9:e106934. https://doi.org/10.1371/journal.pone.0106934
28. Vogel I, Goepfert AR, Thorsen P, et al. Early second-trimester inflammatory markers and short cervical length and the risk of recurrent preterm birth. *J Reprod Immunol*. 2007;75(2):133-140. https://doi.org/10.1016/j.jri.2007.02.008
29. Beksac MS, Fadiloglu E, Cakar AN, et al. Fetal cell microchimerism; normal and immunocompromised gestations in mice. *Fetal Pediatr Pathol*. 2020;39(4):277-287. https://doi.org/10.1080/15513815.2019.1651803
30. Carbone L, Cariati F, Sarno L, et al. Non-invasive prenatal testing: current perspectives and future challenges. *Genes*. 2020;12(1):15. https://doi.org/10.3390/genes12010015

How to cite this article: Tıplamaz S, Eyüboğlu İP, Ünal C, Soyer Ö, Beksaç MS, Akkiprik M. Presence of fetal DNA in maternal exhaled breath condensate. *Prenat Diagn*. 2022;1-8. https://doi.org/10.1002/pd.6277