

Do fetal extravillous trophoblasts circulate in maternal blood postpartum?

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Abstract

Introduction: Circulating fetal extravillous trophoblasts may offer a superior alternative to cell-free fetal DNA for noninvasive prenatal testing. Cells of fetal origin are a pure source of fetal genome; hence, unlike the cell-free noninvasive prenatal test, the fetal cell-based noninvasive prenatal test is not expected to be affected by maternal DNA. However, circulating fetal cells from previous pregnancies may lead to confounding results.

Material and methods: To study whether fetal trophoblast cells persist in maternal circulation postpartum, blood samples were collected from 11 women who had given birth to a boy, with blood sampling at 1-3 days (W0), 4-5 weeks (W4-5), around 8 weeks (W8) and around 12 weeks (W12) postpartum. The existence of fetal extravillous trophoblasts was verified either by X and Y chromosome fluorescence in situ hybridization analysis or by short tandem repeat analysis. To exclude technological bias in isolating fetal cells, blood samples were also collected from 10 pregnant women between a gestational age of 10 and 14 weeks, the optimal time frame for cell-based noninvasive prenatal test sampling. All the samples were processed according to protocols established by ARCEDI Biotech for fetal extravillous trophoblast enrichment and isolation.

Results: Fetal extravillous trophoblasts were found in all the 10 samples from pregnant women between a gestational age of 10 and 14 weeks. However, only 4 of 11 blood samples taken from women at 1-3 days postpartum rendered fetal extravillous trophoblasts, and only 2 of 11 samples rendered fetal extravillous trophoblasts at 4 weeks postpartum.

Conclusions: In this preliminary dataset on few pregnancies, none of the samples rendered any fetal cells at or after 8 weeks postpartum, showing that cell-based noninvasive prenatal testing based on fetal extravillous trophoblasts is unlikely to be influenced by circulating cells from previous pregnancies.

KEYWORDS

chorionic villi sampling, fetal cells, fetal extravillous trophoblasts, noninvasive prenatal testing, prenatal diagnosis

Abbreviations: cffDNA, cell-free fetal DNA; fEVTs, fetal extravillous trophoblasts; STR, short tandem repeat.

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1 | INTRODUCTION

During the last 50 years, extensive research has been carried out to assess whether noninvasive prenatal diagnosis on circulating fetal cells could possibly replace the use of invasive forms of prenatal diagnosis such as chorionic villus sampling and amniocentesis.¹ Both these invasive methods are associated with a risk of miscarriage of approximately 0.20% and 0.30%, respectively.² A noninvasive alternative to these invasive procedures is based on cell-free fetal DNA (cffDNA), which is released into the maternal blood circulation by apoptotic fetal extravillous trophoblasts (fEVTs).³⁻⁵ The cell-free noninvasive prenatal test based on cffDNA has been introduced to the national guidelines for prenatal diagnosis in Denmark in 2017, and is offered as an alternative to chorionic villus sampling to all women identified as “high-risk” (>1:300) based on a combined first trimester screening. Although the cell-free noninvasive prenatal test has the advantage of being noninvasive, it has certain limitations and can only be used as a screening method and not as a diagnostic tool. As it needs to be accessed in order to detect fetal conditions, cffDNA is contaminated with maternal DNA. In such a case, the interpretation of cell-free noninvasive prenatal test results is prone to bias from maternal conditions, such as body mass index, and malignancies.⁶ Furthermore, the cffDNA might not be present in sufficient quantities, leading to false-negatives. On the other hand, fetal cells circulating in maternal blood during pregnancy are very rare, and require specific markers for their isolation. Also, because the half-life of these cells in maternal circulation is not known, their enrichment from maternal blood might be hampered because of the cells undergoing apoptosis. However, using circulating fetal cells for prenatal diagnosis compared with cffDNA allows for whole genome analysis on DNA from pure fetal cells without maternal contamination.⁷⁻⁹ Several different types of circulating fetal cells have been identified and targeted for prenatal diagnosis, including lymphoid progenitor cells, nucleated red blood cells and fEVTs.¹⁰⁻¹⁴

It has been conclusively established that fetal cells are transferred to the maternal circulation during pregnancy and can be found in maternal peripheral blood and tissue.¹⁵⁻¹⁷ Some of these cells persist in maternal tissues postpartum, a phenomenon, known as fetomaternal microchimerism.^{10,18} The microchimeric state is believed to be possible due to the maternal immune adaptation during pregnancy. It has been shown that some of these cells persisting in the mother after delivery can develop stem cell-like properties, and they have been found in patients with autoimmune diseases of the thyroid gland, systemic sclerosis, rheumatoid arthritis and cervical cancer, among other diseases.¹⁹ Bianchi et al¹⁸ detected lymphoid male progenitor cells in a blood sample from a mother who had given birth to a boy 27 years earlier. Such discoveries can have far-reaching implications for the use of fetal cells for cell-based noninvasive prenatal testing.

Currently, the persistence of fEVTs in the maternal circulation postpartum is unknown. The potential persistence of fetal cells from previous pregnancies would make it difficult to distinguish between cells from a current fetus and cells from previous fetuses. If fEVTs from previous pregnancies should persist in maternal circulation

Key message

From 8 weeks postpartum onwards, a small amount of data from 11 consecutive pregnancies show that fetal extravillous trophoblasts cease to circulate in maternal blood, making them unlikely to influence noninvasive prenatal testing of subsequent pregnancies.

after delivery of the child, sampling of previous children would be necessary to determine whether the circulating fEVTs during pregnancy belong to the current pregnancy or the previous one. This determination would be a prerequisite to any prenatal testing on the circulating fetal cells.

The aim of this study is to investigate whether fEVTs circulate in maternal blood postpartum.

2 | MATERIAL AND METHODS

Blood samples were collected from 13 women who had delivered a boy and were admitted to the Birth Section at the Department of Women's Disease and Birth, Aarhus University Hospital, Denmark, between 23 April and 14 May 2019. Except for one woman (sample II) who gave birth to male twins, the rest of the women had singleton pregnancies. Two of the 13 women who had earlier agreed to participate, left the study after first blood sampling and were therefore excluded from the final tally. Four women (III, VI, VII and IX) were not available for sampling in week 8 postpartum. Women who had severe blood loss, infections or serious complications in relation to birth were not recruited for the study. The participants were recruited by a nurse and the recruitment team at the Department of Women's Disease and Birth, Aarhus University Hospital. All the women who agreed to participate in the study signed a written consent form before blood samples were taken. Blood samples were obtained 1-3 days, 4-5 weeks, around 8 weeks and around 12 weeks after birth. Additionally, blood samples were collected from 10 pregnant women between a gestational age of 10 and 14 weeks from 12 February to 14 March 2019. As the dates of collection of the two sample sets did not overlap, the samples were not blinded. However, the methods of blood processing, fetal cell enrichment, staining and isolation were the same for both these sample sets. An exception was in cases I and II of postpartum women, in whom after isolation, the cells were verified by X and Y fluorescence in situ hybridization. In the rest of the samples, the isolated cells were verified by short tandem repeat (STR) analysis.

2.1 | Sample processing

Thirty milliliters of peripheral blood was collected in three cell-free DNA BCT tubes (Streck Laboratories). The blood was processed

and fEVTs enriched and isolated using protocols described previously with minor modifications.^{15,20} In brief, whole blood was fixed with formaldehyde, red blood cells lysed, and white blood cells pelleted. The cell pellet was treated with fetal cell-specific antibodies and fEVTs enriched using Magnetic Activated Cell Sorting (Miltenyi Biotec). After enrichment, fEVTs were immunofluorescently stained with a cocktail of cytokeratin antibodies. The potential fetal cell candidates (if any) were isolated individually, and their fetal origin confirmed either by X and Y chromosome fluorescence in situ hybridization analysis (only I and II) or by STR (all the remaining samples).

2.2 | X and Y chromosome fluorescence in situ hybridization

In the case of women I and II, the cell pellets after fetal cell enrichment and staining were smeared on the FLEX IHC glass slides (DAKO). The slides were air-dried, fixed in formaldehyde, washed with phosphate-buffered saline, and mounted with Vectashield containing 0.6 µg/mL DAPI (Vector Laboratories). The slides were scanned using a Zeiss microscope (Axioplan-2 Imaging) integrated with a MetaSystems scanner. During scanning of the slides, an in-house-developed “classifier,” piece of software was used to give a list of “positive events”. These “positive events” from every sample were then manually validated, and true fetal cells, if any, were marked.⁷ X and Y fluorescence in situ hybridization was performed using a previously described protocol.⁴ X-chromosome-specific probe, DXZ1 (CEP X alpha satellite) (Abbott Molecular) labeled with spectrum green, and two Y-chromosome-specific probes, DYZ1 (CEP Y satellite III) (Abbott Molecular), labeled with spectrum orange and spectrum aqua, respectively, were used. Following X and Y fluorescence in situ hybridization, the fetal cells were visually inspected for validation.

2.3 | Short tandem repeat

For cases III-XI of postpartum women, and cases 1-10 of the pregnant women, potential fetal cell candidates were isolated with another proprietary method which uses a fixed set of criteria for cell size, fluorescence staining intensity and cell morphology to differentiate potential fetal cells from maternal cells. STR analyses were performed on these potential fetal cell candidates to confirm their true identity. This method of isolating cells differs from the previous method in that it gives a smaller number of putative cells that need to be verified using STR. A patent application for this unique method of isolating fetal cells has been filed.

STR analysis was performed using a GlobalFiler PCR amplification kit (Thermo Fisher Scientific) to confirm their fetal origin. Prior to STR analysis, cells were lysed using prepGEM Universal (ZyGEM) according to the manufacturer's instructions. No whole genome amplification step on the fetal DNA was performed before STR.

STR analysis was done by fragment size capillary electrophoresis using an ABI 3500 genetic analyzer. Data analysis was performed in GENEMAPPER ID-X software for genotyping (Thermo Fisher Scientific). For comparison, STR was also performed on maternal DNA extracted from whole blood.

2.4 | Statistical analyses

The Mann-Whitney *U* test was performed to show whether there was any significant difference in the number of fetal cells between W0 and W4-5, as these were the only two groups of postpartum women who rendered fetal cells. Statistical analyses were done in Microsoft EXCEL (Microsoft Corp.).

2.5 | Ethical approval

The project was approved by the Danish Science Ethics Committee—Region of Southern Denmark (approval number S-20070045, 1 April 2019).

3 | RESULTS

The number of fEVTs in 40 samples from 11 postpartum women is presented in Table 1. In cases I and II, on average 66, 24 and 10 “positive events” were found by the “classifier” at W0, W4-5 and W12, respectively, that had to be manually inspected, and fetal cells marked and verified by X and Y fluorescence in situ hybridization.

In cases III-XI, on average 6.7, 2.9, 1.2 and 2.2 potential fetal cell candidates were isolated at W0, W4-5, W8 and W12, respectively, that had to be verified by STR analysis.

In comparison, on average 19.2 potential fetal cell candidates were isolated from 10 consecutive cases of pregnant women (gestational age 10-14 weeks) and verified by STR. The cases rendered 3-36 fetal cells, with an average of 10.4 cells and a median of 8 (Table 2).

In Figure 1, STR read-out from one of the five fetal cells isolated from sample X is compared with the STR read-out from maternal genomic DNA. Three loci—Y indel, AMEL and DYS391—were present on sex chromosomes and were informative in verifying the fetal cell. Twenty-one loci were present on autosomal chromosomes; 15 of these were heterozygous in the fetal DNA, of which 13 were informative in verifying the fetal cell because they contained distinct maternal and paternal alleles. Six loci were homozygous in the fetal DNA and, in the absence of paternal STR profile, could not be used to differentiate between maternal and fetal cells.

The STR read-outs from all the fetal cells from samples III, VI and X are compared with the respective STR profiles from maternal DNA, and are presented in Figures S1, S2 and S3, respectively.

TABLE 1 The number of fetal cells (FC) found at 1-3 d (W0), weeks 4-5 (W4-5), week 8 (W8) and week 12 (W12) postpartum are presented

Number ^a	W0	W4-5	W8	W12
I	5	4	0	0
II	0	1	0	0
III	2	0	–	0
IV	0	0	0	0
V	0	0	0	0
VI	1	0	–	0
VII	0	0	–	0
VIII	0	0	0	0
IX	0	0	–	0
X	5	0	0	0
XI	0	0	0	0
Total FC	13	5	0	0
Average FC	1.2	0.5	0	0

Total and average number of fetal cells between four different time points postpartum are also presented. No fetal cells were present at or after W8. Fetal cells were present in 4/11 samples in W0 and 2/11 samples in W4. For sample II, no cells were present in W0 even though one cell was present in W4-5.

^aSample numbers are arbitrary.

TABLE 2 Fetal cell (FC) number, average and median from 10 samples from pregnancies at gestation age of 10-14 wk

Number ^a	Fetal cells identified
1	6
2	3
3	4
4	36
5	8
6	12
7	8
8	4
9	10
10	11
Total FC	104
Average FC	10.4
Median FC	8

^aSample numbers are arbitrary.

Only sample I rendered fetal cells in two consecutive samplings at W0 and W4-5 postpartum. Four (36%) samples rendered fetal cells in W0 (mean = 1.18), two (18%) in W4-5 (mean = 0.45) and none in W8 and W12. As fetal cells were found only in W0 and W4-5, the Mann-Whitney *U* test did not show a significant difference in fetal cells between these two groups (*P* value of .43; *U*-value of 48).

4 | DISCUSSION

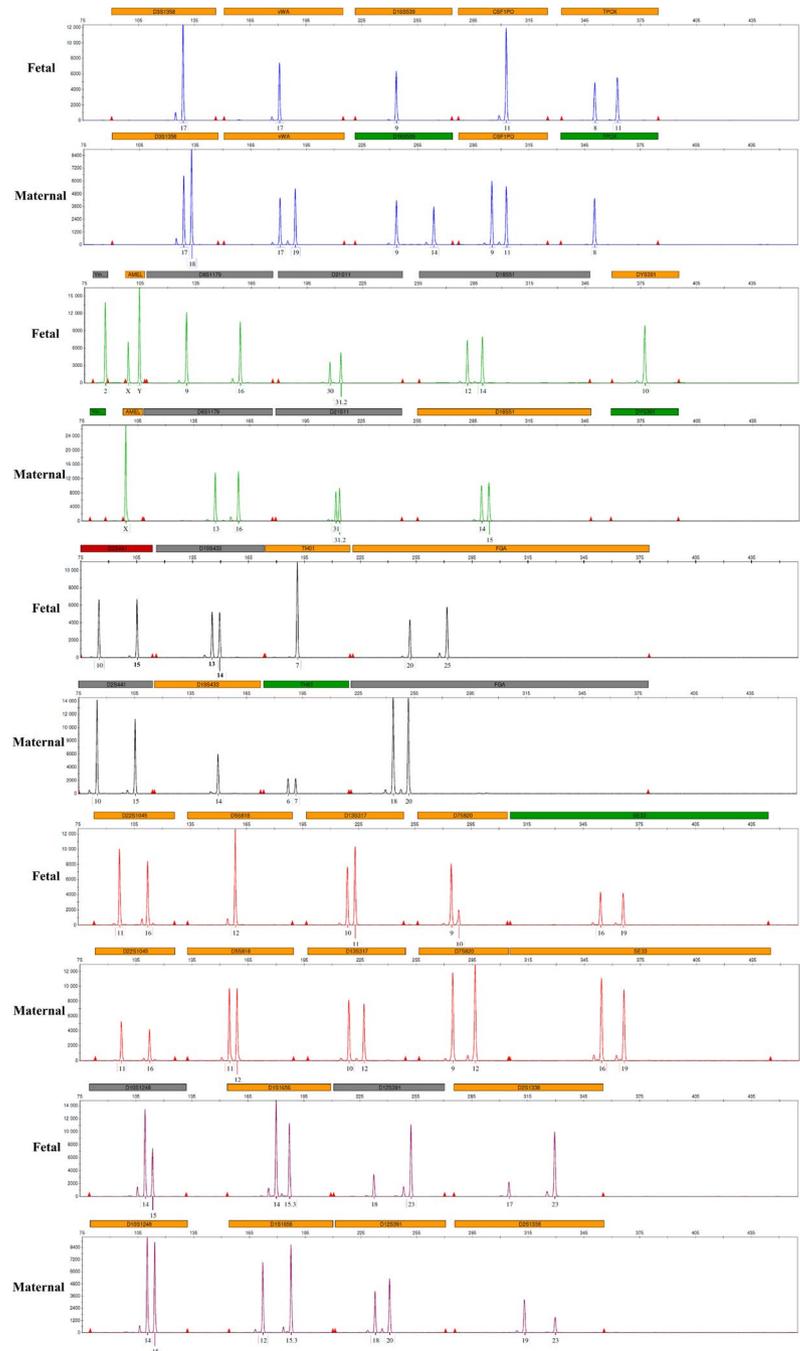
Fetal trophoblast cells circulating in maternal blood have long been proposed to be superior alternatives to cfDNA for noninvasive prenatal testing. Most of the studies looking for fetal cells in maternal circulation have focused on pregnancies at the end of the first trimester and at the beginning of the second trimester—a window in the gestational age where cell-based noninvasive prenatal testing can be offered as a safer alternative to chorionic villus sampling. However, very little is known about the half-life of the fetal trophoblast cells in maternal circulation. Also, an important question that relates to the applicability of cells for prenatal diagnosis is whether cells from previous pregnancies still persist in maternal circulation. Until now, only a few studies have addressed this question. This could be due to the fact that finding fetal cells consistently in early gestational age (weeks 10-14) has been a challenge because of the rarity of these cells, and because of a lack of a robust method to isolate these cells from maternal circulation. The most widely referred study on the persistence of fetal cells in maternal circulation is Bianchi et al's paper exploring whether fetal progenitor cells were present in women's blood postpartum.¹⁸ They studied this by performing Y-PCR on mononuclear cells sorted using a pool of CD antibodies. Of the eight samples from non-pregnant women, male DNA was detected in six samples from the women, one of whom gave birth to a boy 27 years ago. Interestingly, among the 19 ongoing pregnancies expecting male fetuses, six did not show any male DNA.

The present study is the first to look at the persistence of fEVTs in maternal blood postpartum. There is a common consensus that the circulating cfDNA in maternal plasma originates from apoptotic fetal trophoblastic cells.^{3,5} Moreover, all the current cell-based noninvasive prenatal testing technologies target the trophoblastic cells in maternal circulation. It is therefore of importance to explore whether these cells persist in the maternal blood postpartum and, if they do, whether this can influence interpretation of cell-based noninvasive prenatal testing results in subsequent pregnancies. In our current dataset, barring two samples, none of the other 11 samples rendered any fetal cell at weeks 4-5 postpartum. There were no fetal cells in any of the samples at week 8 postpartum. To exclude any technological biases leading to low fetal cell numbers in this cohort, we also looked at fetal cells in weeks 10-14 of gestation in a group of 10 pregnant women. All the samples from ongoing pregnancies rendered 3-36 fetal cells.

The number of fetal cells during pregnancy is not static. The numbers increase from week 7 of gestation to week 12 and then drop again in week 19 (ongoing study, data not shown). The fEVTs invade the maternal tissue during the first trimester. Some of these cells line the spiral arteries, ensuring adequate blood flow from the mother to the developing fetus.²¹ This is also the time in the gestation when there is a release of the EVTts that block the lumen of the spiral arteries. We hypothesize that the increase in the number of fetal cells from week 7 to week 12 is due to this phenomenon.

In this study, we focused only on the fetal trophoblasts and their persistence in the maternal blood. These data do not imply that the

FIGURE 1 Short tandem repeat profile from one of the five fetal cells isolated from sample “X” is compared with maternal DNA from white blood cells. Alleles from 24 loci (presented on x-axis), each represented with a colored bar, are compared between maternal and fetal DNA [Color figure can be viewed at wileyonlinelibrary.com]



other types of fetal cells do not circulate in maternal circulation postpartum. Moreover, this pilot study was performed on a relatively small number of pregnancies, which were without any recorded complications during gestation. Hence, it is difficult to conclude whether the fetal cell numbers postpartum shown in this dataset reflect the numbers from pregnancies with complications, such as preeclampsia, preterm birth and intrauterine growth retardation. As in this study we did not sample pregnancies in the second or third trimester, we do not know the correlation between circulating fEVTs and gestational age, leading up to birth.

However, we can conclude in this initial study that none of the samples in the present data show fetal trophoblasts in week 8 postpartum, hence the interpretation of noninvasive prenatal testing

results from technologies targeting fetal trophoblasts is not expected to be affected by persisting fetal cells from previous pregnancies.

5 | CONCLUSION

Fetal extravillous trophoblasts are not likely to persist in maternal blood circulation postpartum.

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CONFLICT OF INTEREST

Ripudaman Singh, Lotte Hatt, Katarina Ravn, Line Dahl Jeppesen, Bolette Hestbek Nicolaisen, Mathias Kølvrå and Palle Schelde are employed by ARCEDI Biotech ApS (Vejle, Denmark). Ida Vogel is collaborating with ARCEDI in the clinical validation of its technology.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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