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The effect of maternal body mass index and gestational age on circulating trophoblast yield in cell-based noninvasive prenatal testing

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Abstract

Objective: To examine the effect of maternal body mass index (BMI) and gestational age (GA) on the number of single circulating trophoblasts (SCT).

Methods: Maternal blood was collected in 20–40 mL. All singleton pregnant women at any gestation were recruited. Trophoblasts were recovered by immunomagnetic enrichment and stained for cytokeratin and CD45. Candidate trophoblasts were identified by fluorescence microscopy.

Results: Blood samples were collected from 425 singleton pregnancies; April 2018 to December 2019. At least one candidate cell was identified in 88% (373/425). There was an inverse correlation between trophoblasts yield and increasing BMI ($r = -0.19$, $p < 0.001$). The mean $\pm$ SD number of trophoblasts/mL was 0.12 ± 0.22 in the underweight group ($n = 5$), 0.23 ± 0.25 in the normal weight ($n = 169$), 0.18 ± 0.19 in the overweight ($n = 114$) and 0.13 ± 0.15 in the obese ($n = 109$).

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Conflict of interest statement

Baylor Genetics (BG) is a diagnostic laboratory which is partially owned by Baylor College of Medicine. A.L.B. and I.B.V. had or have, respectively, consulting or committee roles at BG. A.L.B. is a founder of Luna Genetics.

Data availability statement

Data available on request from the authors

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Significantly more cells were identified in the normal weight compared to those in the obese ($p=0.001$). In addition, the mean $\pm$ SD number of cells/mL was 0.21 ± 0.21 at GA 10-14 weeks ($n=260$), 0.14 ± 0.23 at GA 15 ($n=102$) and 0.12 ± 0.12 at GA <10 ($n=63$); $p<0.001$.

Conclusion: The lower number of SCT were identified from samples of women with a high BMI. Cell recovery for SCT testing seems optimal at GA 10-14 weeks, but earlier and later testing are still possible.

Keywords

Body mass index; cell-based noninvasive prenatal testing (NIPT); gestational age; prenatal diagnosis; single circulating trophoblast

Introduction

At present, when pregnancies are at increased risk for fetal chromosomal abnormalities because of structural anomalies in the fetus, other familial risk factors, or after aneuploidy screening, the recommended prenatal genetic testing strategy is to offer a diagnostic procedure, chorionic villous sampling (CVS) or amniocentesis, followed by a chromosomal microarray analysis (CMA) and in some case a karyotype on the retrieved CVS or amniotic fluid sample.⁽¹⁻⁴⁾ Although the risk associated with these procedures is very low when performed by adequately trained experienced providers, many women decline the procedures because of concern for pregnancy loss or other harm to their fetus.^(5, 6) The increasing use of noninvasive prenatal screening methods has led to a recent sharp decline in the number of CVS and amniocentesis procedures.⁽⁷⁾ These screening tests include analysis of conventional biomarkers in maternal serum in the first or second trimester, along with prenatal ultrasound, and more recently cell-free DNA (cf-DNA)-based noninvasive prenatal testing (cell-free NIPT). However none of these methods are diagnostic tests and have various rates of efficacy for detecting the most common aneuploidies.^(8, 9) Screening for common fetal aneuploidies by cell-free NIPT has been widely clinically implemented in recent years. In several studies, the detection rates for Down syndrome-trisomy 21 (T21), Edward syndrome-T18 and Patau syndrome-T13, 99%, 96% and 91% respectively, were found to be superior to the conventional first-trimester screening, but positive predictive values are more variable and cell-free NIPT cannot replace diagnostic testing.^(8, 10, 11) Cell-free NIPT for subchromosomal fetal copy number variants (CNVs) is now also offered, but, it is more limited in its ability to detect smaller genetic aberrations.⁽¹²⁾

Multiple studies have attempted to demonstrate the reliable detection of smaller copy number variants (CNVs) in cell-free NIPT, but the respective positive predictive values are much lower compared to those for aneuploidies. One of the most important factors influencing test performance of cell-free NIPT is the fetal fraction (FF), a value for the proportion of all cell-free DNA in maternal plasma that originates from the fetal genotype. Several factors, such as maternal weight or body mass index (BMI) and gestational age (GA) can have an impact on the FF and consequently the test performance of cell-free NIPT.^(13, 14) It has been demonstrated that the FF is inversely proportional to maternal BMI. One study showed that the average FF in women with a BMI of 35–39.9 kg/m² was 11.57%, significantly lower compared to a FF of 14.54% in women with a BMI <18.5 kg/m².⁽¹⁵⁾

Although the fetal DNA can be observed in the maternal plasma as early as 4 weeks of gestation, the FF is usually too low for reliable testing at early gestational ages and professional organizations recommend that cell-free NIPT should only be offered after 10 weeks of gestation.^(8, 11)

Fetal cells present in the maternal circulation have been studied for many years. Our laboratory has worked on the validation of single circulating trophoblast (SCT) testing for over 8 years, with the aim to use this method as a form of noninvasive prenatal diagnosis.⁽¹⁶⁻¹⁸⁾ Our workflow includes the processing of maternal peripheral blood samples by positive trophoblast enrichment, and subsequent CNV analysis by whole genome sequencing of all isolated putative trophoblasts, yielding a result very similar to that obtained after invasive prenatal sampling.

Similar to cell-free NIPT, various factors might influence the number of fetal cells in the maternal circulation including gestational age, fetal genetic abnormalities, pregnancy-related complications, maternal physical activity before blood draw, maternal/fetal/placental health and interindividual variation. A few studies have reported on the association between the number of fetal cells and gestational age. However, those studies have reported variable results and it is difficult to conclude if there is a definite association between recovered fetal cell numbers and gestational age.⁽¹⁸⁻²¹⁾ Furthermore, there are only two previously reported studies in which the influence of BMI on the number of fetal cells was explored in a small number of samples (91 and 85 samples).^(18, 22) Here we present the results of our study evaluating how the number of fetal cells recovered varies with maternal BMI and gestational age of the pregnancy on a larger number of samples than previously reported.

Material and Methods

This study was approved by the institutional review board of Baylor College of Medicine, Houston and Columbia University, NYC; protocol number H-24326. All pregnant women presenting for prenatal care or in Maternal-Fetal-Medicine clinics were eligible according to the inclusion criteria, but we preferentially enrolled women in the first trimester of pregnancy. Some who will undergo prenatal diagnosis including ultrasound and amniocentesis or CVS were enrolled. Some subjects have already had available genetic results on their fetus. Some subjects who have twins or multiple pregnancies were recruited in our validation project in cell-based NIPT but excluded from this study. Exclusion criteria were subjects who diagnosed with embryonic death or dead fetus in utero at the time of blood collection. There were 333 samples collected in Houston and 92 samples were collected in NYC. Maternal blood was collected in two to four 10 mL EDTA tubes after informed consent was obtained. Blood samples were processed within 24 hours. Maternal characteristics and obstetrical history including BMI and gestational age at the time of blood collection were recorded. The protocol used in this study was validated and published by Vossaert et al. in 2019.⁽¹⁸⁾ Briefly, after an initial red blood cell lysis step, trophoblast cells were obtained by immunomagnetic enrichment with one in-house developed and three commercial antibodies and using magnetic-activated cell sorting (MACS). After enrichment, immunostaining was performed with two anticytokeratin antibodies (both pan- CK reactive, BioLegend and eBioScience) and one antibody against the white blood cell (WBC) marker

CD45 (BioLegend): DAPI nuclear stain was added. Enriched samples were evaluated by fluorescence microscopy to identify candidate trophoblast cells based on a set of criteria including nuclear shape, absence of CD45 staining, and positive cytokeratin staining and staining pattern. All candidate cells were counted towards the number of trophoblasts for each sample and normalized to cells/mL. Typically, about 60% of cells scored as fetal by this method will yield useful clinical data, but some will be apoptotic and some will be in S phase. Cells were genotyped and scored for copy number variants as in Vossaert et al.⁽¹⁸⁾ All putative cells were processed and sequenced at Baylor College of Medicine and Baylor Genetics. Importantly, for all subjects, any result in this study was not used in clinical care or reported to attending clinicians or patients.

Statistical analysis

Categorical variables were described as numbers and percentages, while continuous variables were expressed as a mean and a median, with standard deviation (SD), and min-max ranges. The Kolmogorov-Smirnov test was used for testing the normality of distribution. Due to non-normality distribution, the data were analyzed by using chi-square and Mann-Whitney U test. Kruskal-Wallis one-way analysis of variance (ANOVA) was used to compare the different BMI and gestational groups. The number of trophoblast cells/mL was analyzed in function of maternal BMI by using Spearman's correlation coefficient. A p-value less than 0.05 was considered statistically significant. All statistical analyses were done using SPSS version 26 (SPSS Inc., Chicago, IL, USA).

Results

We collected maternal blood samples from 425 singleton pregnancies, between April 2018 and December 2019, that were processed and analyzed using the described protocol for isolation and analysis of circulating trophoblast cells. Among these samples, there were 13 redraw samples. The number of trophoblast cells for each sample was normalized to number of cells per milliliter of maternal blood (cells/mL). Candidate fetal cells were identified in 373/425 blood samples. Maternal BMI data were available for 397/425 samples and the mean maternal BMI was 27.33 kg/m². An overview of the different BMI groups with the respective yield of trophoblast cells per group is shown in Table 1. By using Spearman's correlation coefficient (r), trophoblast cells yield shows an inverse correlation with increasing BMI (r = -0.19, p-value < 0.001).

All women from whom samples were collected were categorized into four BMI subgroups according to the World Health Organization (WHO) classification; underweight if BMI is <18.5 kg/m² (5 samples), normal if BMI is 18.5-24.9 kg/m² (169 samples), overweight if BMI is 25.0-29.9 kg/m² (114 samples) and obese if BMI is ≥ 30.0 kg/m² (109 samples). Trophoblasts numbers/mL in each BMI subgroups were demonstrated by the scatter plot in Figure 1. The mean ±SD of identified trophoblast cells/mL of maternal blood in each group was 0.12 ±0.22, 0.23 ±0.25, 0.18 ±0.19 and 0.13 ±0.15, respectively. The numbers of cells/mL were statistically different among BMI groups (p=0.001; by using Kruskal-Wallis one-way ANOVA). Significantly more cells were identified in blood of women in the normal weight group compared to those in the obese group (p=0.001), but this difference was not

found between the normal weight or obese group and each other group. The number of samples from which no cells were obtained was significantly increased with increasing BMI. No trophoblast cells were identified in the blood of the 5.9% (10/169) of women in the normal BMI group while for the overweight and obese groups, no cells were found for 14.9% (17/114) and 15.6% (17/109), respectively as illustrated in Figure 2; $p < 0.001$. The number of samples for which no cells were found also compared between the BMI ≥ 40 kg/m² and all BMI < 40 kg/m², which was 6/20 (30.0%) and 41/377 (10.8%), respectively ($p = 0.01$).

Gestational age data were available for all 425 cases. The mean gestational age at which samples were collected was 13.5 weeks. The samples were split up into three subgroups according to GA: GA < 10 weeks (63 samples), GA 10-14 weeks (260 samples) and GA ≥ 15 weeks (102 samples). The mean $\pm$ SD number of cells/mL was 0.12 ± 0.12 at GA < 10 weeks, 0.21 ± 0.21 at GA 10-14 weeks and 0.14 ± 0.23 at GA ≥ 15 weeks as shown in Figure 3. The number of identified cells/mL from samples at GA 10-14 weeks was statistically higher than those at GA < 10 weeks and GA ≥ 15 weeks; $p < 0.001$. According to subgroup analysis from the samples at GA 8-10 weeks (8w0d to 9w6d; 44 samples), the mean $\pm$ SD (median) number of cells/mL was 0.15 ± 0.13 (0.13) and there was no significant difference from those samples in the later gestation at GA > 10 weeks ($p > 0.05$), while the number of cells/mL from the samples at GA < 8 weeks (19 samples) was 0.08 ± 0.10 (0.06) and this was statistically lower than those samples at GA 10-14 weeks ($p < 0.001$). In addition, the number of samples which no cells at GA 10-14 weeks was 8.8% (23/260), which was significantly different from 17.4% (11/63) at GA < 10 weeks and 17.6% (18/102) at GA ≥ 15 weeks; p -value = 0.03 as demonstrated in Figure 4.

Discussion

Our group has developed a reproducible method in the great majority of samples to isolate intact trophoblast cells from maternal blood that are usable for the detection and analysis of fetal CNVs. Several factors might affect the number of trophoblast cells in the circulation and the ability to identify them. Similar to cell-free NIPT, maternal BMI and GA appear to have an effect on trophoblast recovery. The results of the current study suggest that there was a significant difference in trophoblast cell yield among the different BMI groups. Specifically, the number of trophoblast cells in the normal weight group is significantly higher than in the obese group. In our previous publication, a downward trend for cell recovery with increasing BMI was seen but was not found to be statistically significant. This difference between previous data and current data could be explained by the fact that the considerably larger number of maternal samples (425 samples in the current study vs 85 samples in the previous study).⁽¹⁸⁾

Importantly, the number of samples from which no cells could be recovered from women with an extremely high BMI (≥ 40 kg/m²) is significantly higher compared to all with BMI < 40 kg/m². Regardless, even though maternal BMI has an effect on the number of identifiable trophoblasts, this effect is less prominent than for cell-free NIPT. The no-call or failure to report results due to lower fetal fraction on cell-free NIPT is about 50% for women with a BMI ≥ 40 kg/m².⁽²³⁾ In contrast, in our study, we can recover cells from 70% of women in

this BMI group. There are two possible mechanisms to explain how higher maternal BMI may affect cell-free NIPT. Obesity increases cell-free DNA concentration of maternal DNA, due to increased necrosis and apoptosis of adipose tissue,⁽²⁴⁾ this accounts in part for lower fetal fraction in cell-free NIPT, but should not affect cell-based NIPT. Maternal blood volume and perhaps extravascular space may reduce the absolute concentration of cf-DNA in cell-free NIPT, but this effect should not affect the fetal fraction. For cell-based NIPT, the maternal blood volume is one potential variable for frequency of trophoblasts, but there may be others. In addition, one previous study reported that number of recovered trophoblasts increased modestly after 30-minute of moderate physical activity when compared in the same participants.⁽²⁵⁾ More routine daily activity in the normal weight pregnant women may increase the number of recovered cells. On the other hand, the overweight and obese pregnant women usually have less activities than in the normal weight group, and it would be helpful to have additional data on the effect of exercise. It is important to keep in mind that if one or two trophoblast cells can be successfully isolated, those cells can still be analyzed for diagnostic genetic testing in chromosomal and submicroscopic genetic aberrations.

Although the average of BMI in our study was in overweight range, we also recruited the adequate samples in each BMI group (except, only 5 cases in underweight group). Because BMI is affected by normal weight gain during pregnancy, the baseline BMI at the start of the pregnancy will differ from that at various stages of gestation. For purposes of this discussion, the BMI at the time blood is drawn is presumably the most relevant. For certain other medical risks, the baseline BMI may be more relevant. If there was a routine option of cell-free NIPT or cell-based NIPT in the future, obesity might favor the choice of cell-based, but this is not an option at this time.

Placentation is a long-term developing process. Our study showed higher number of trophoblasts recovered from pregnant women during 10-14 weeks of gestation when compared to samples collected before 10 weeks and after 15 weeks. This can be explained by the fact that the timing of cytotrophoblast invasion into maternal circulation begins at 8 weeks, peaks at 12 weeks, slows down after 15 weeks and is completed by 18-20 weeks.^(26, 27) Despite fewer trophoblast cells being recovered at very early gestation during <10 weeks, at least one or two trophoblast cells can be identified from over 80% of samples and those cells can be used for fetal genetic analysis. In contrast, cell-free NIPT before 10 weeks of gestation is not performed due to the FF being lower than after 10 weeks.^(8, 11) It could be argued that in the very early gestation, cell-based NIPT is feasible and likely to be superior to cell-free NIPT for prenatal testing before 10 weeks of gestation. However, it is important to note that fetal genetic testing at this very early gestation could potentially be complicated by remaining cells from a vanishing twin and that this needs to be taken into account for interpretation of the results. Similarly, cell-free NIPT studies showed that the presence of DNA from a vanishing fetus can be detected in maternal blood for at least 8 weeks after fetal demise, although cells with usable DNA quality may persist for less time.⁽²⁸⁾

There are some limitations in our study. First, there were only 5 samples in underweight group and for 3 of the 5 samples which no cells were recovered. Therefore, the results could not be applied in this specific BMI group. Second, because trophoblasts represent placental

cells, placental-related conditions might affect the number of isolated trophoblast cells, but our study did not take any of these factors into account; such as pre-existing hypertension or smoking which may influence the fetal fraction in cell-free NIPT.⁽²⁹⁾ Nevertheless, we evaluated a larger number of samples than reported so far to demonstrate the effect of BMI and GA on the number of trophoblast cells in maternal circulation. Among pregnant women in our study, the prevalence of obesity is similar to the national average, so our data likely to apply for the general population.⁽³⁰⁾ In studies of very early gestation, we have sufficient numbers of samples suggesting that very early testing may be useful, especially at 8-10 weeks. Furthermore, the current protocol in our study is a highly specific technique to isolate the trophoblast cells from maternal cells.

Although, cell-based NIPT is still under development, it is likely that this method will be used for clinical prenatal genetic testing soon. The optimal cell-based NIPT testing window appears to be at GA 10-14 weeks, which is similar to chorionic villous sampling. Cell-based NIPT may have advantages over cell-free NIPT at 8-10 weeks of gestation and in cases of obesity.

Conclusion

It is not surprising that the number of recovered trophoblasts in maternal circulation decreases with increasing BMI, particularly in obese group. The effect of high BMI on testing may be less prominent than for cell-free NIPT with recovery of cells in 70% of cell-based cases with BMI $< 40 \text{ kg/m}^2$. In addition, cell recovery for SCT testing is optimal 10-14 weeks of gestation, but earlier and later testing is possible. Based on our report, this current protocol has potential for use as a prenatal genetic diagnosis.

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What is already known about this topic?

- Cell-free DNA-based noninvasive prenatal testing (cell-free NIPT) is now very widely utilized for aneuploidy screening, and this has led to a dramatic reduction in the number of amniocentesis and CVS procedures performed in many countries.
- Cell-based NIPT is not yet available for routine clinical testing, but its feasibility and potential advantages over cell-free NIPT have been demonstrated.

What does this study add?

- The effects of gestational age and maternal body mass index (BMI) on cell-based NIPT are not well delineated.
- Using the single circulating trophoblast (SCT) form of testing, there is an inverse correlation of trophoblasts yield with BMI, although not as prominent as for cell-free NIPT.
- The optimal timing for identifying fetal cells using SCT testing is 10-14 weeks (10w0d to 14w6d), although testing can be performed at earlier and later gestational times.

Figure 1: Trophoblasts cells yield in different maternal body mass index (BMI) groups
This scatter plot shows the distribution of trophoblast cells (cells/mL) in 397 maternal blood samples according to maternal BMI.

Figure 2. Frequency of samples with no cells among maternal body mass index (BMI) groups. There were 3/5 samples which no enriched cell in underweight group. Only 5.9% of samples with no cells were in the normal weight group; $p < 0.001$.

Figure 3: Trophoblasts cells yield in different gestation age (GA) groups.
The scatter plot shows the distribution of trophoblast cells (cells/mL) in 425 maternal blood samples according to gestational age.

Figure 4: Frequency of samples with no cells among gestational age (GA) groups
There were 8.8% of samples with no cells at GA 10-14 weeks; $p=0.03$.

Table 1:

Trophoblast cells yield according to maternal body mass index (BMI) and gestational age (GA)

	Number of samples	Trophoblast (cells/mL)		
		Mean ±SD	Median	Range
Maternal BMI (kg/m ²) [*]				
• Underweight (<18.5)	5	0.12 ±0.22	0.0	0.0-0.50
• Normal weight (18.5-24.9)	169	0.23 ±0.25	0.15	0.0-1.83
• Overweight (25.0-29.9)	114	0.18 ±0.19	0.13	0.0-1.03
• Obese (>30.0)	109	0.13 ±0.15	0.10	0.0-1.00
Total	397	0.18 ±0.21	0.13	0.0-1.83
GA (weeks) ^{**}				
• GA < 10	92	0.17 ±0.17	0.13	0.0-0.91
• GA 10 - 14	231	0.20 ±0.20	0.14	0.0-1.08
• GA 15	102	0.14 ±0.23	0.09	0.0-1.83
Total	425	0.18 ±0.20	0.13	0.0-1.83

SD = standard deviation

* p-value =0.001; significantly more cells were identified from samples of women in the normal weight group compared to those in the obese group, but this difference was not found between the normal weight or obese group and each other group

** p-value <0.001; the number of identified cells/mL from samples at GA 10-14 weeks was statistically higher than those in the other gestational age group.