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Fetal Fraction in Maternal Plasma Cell-Free DNA at 11–13 Weeks' Gestation: Effect of Maternal and Fetal Factors

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Key Words
Non-invasive prenatal diagnosis · Trisomy 21 · Trisomy 18 · First trimester

Abstract
Objective: It was the aim of this study to examine the possible effects of maternal and fetal characteristics on the fetal fraction in maternal plasma cell-free DNA (cfDNA) at 11–13 weeks' gestation. ***Methods:*** In a nested case-control study, cfDNA was extracted from maternal plasma obtained before chorionic villous sampling from 300 euploid, 50 trisomy 21 and 50 trisomy 18 pregnancies at 11–13 weeks' gestation. Chromosome-selective sequencing of maternal cfDNA non-polymorphic and polymorphic loci, where fetal alleles differ from maternal alleles, was used to determine the proportion of DNA which is of fetal origin. Multivariate regression analysis was used to determine which of the factors amongst maternal weight, racial origin, smoking status, plasma storage time, serum pregnancy-associated plasma protein (PAPP)-A and free β-subunit of human chorionic gonadotropin (β-hCG), fetal crown-rump length, nuchal translucency thickness, gender and karyotype were significant predictors of the fetal fraction. ***Results:*** Significant independent prediction of fetal fraction was provided by maternal weight, serum PAPP-A and serum free β-hCG multiples of the median, but not by other maternal characteristics, fetal karyotype, crown-rump length or nuchal translucency thickness. Fetal fraction increased with serum metabolite levels and decreased with maternal weight. ***Conclusions:*** The fetal fraction in maternal plasma cfDNA increases with serum PAPP-A and free β-hCG and decreases with maternal weight.

Introduction

Non-invasive prenatal detection of fetal trisomies 21, 18 and 13 has been made possible by examining cell-free DNA (cfDNA) in maternal plasma [1–11]. Essentially, maternal plasma cfDNA molecules are sequenced and the chromosomal origin of each molecule is identified by comparison to the human genome. In trisomic pregnancies, the number of molecules derived from the extra chromosome, as a proportion of all sequenced molecules, is higher than in euploid pregnancies. Studies in pregnancies at high risk for trisomy 21 have demonstrated that analysis of maternal plasma cfDNA can detect >99% of affected cases at a false-positive rate <1% [4–9].

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The ability to detect the small increase in the amount of a given chromosome in maternal plasma in a trisomic compared to a disomic pregnancy is directly related to the relative proportion of the fetal to the maternal origin of the cfDNA in maternal plasma. For example, if the fetal fraction is 20% in 100 units of maternal cfDNA there would be 20 units coming from the fetus and 80 from the mother. In the presence of a fetus with trisomy 21, the maternal plasma cfDNA would contain 30 units of chromosome 21 coming from the fetus and 80 from the mother. Thus, there would be 110 units of chromosome 21 compared to 100 units of any other chromosome. This relative increase in chromosome 21 in maternal plasma cfDNA (110 vs. 100) is easier to detect than in a case with a fetal fraction of 4% where the relative increase would be 102 vs. 100. Consequently, in the studies examining the performance of non-invasive detection of fetal aneuploidies, the samples with a fetal fraction <3–4% were considered inappropriate for further analysis [5, 6, 8].

The objective of this study is to examine the possible effects of maternal and fetal characteristics on the fetal fraction in maternal plasma cfDNA at 11–13 weeks' gestation.

Methods

This was a nested case-control study of stored maternal plasma from 400 singleton pregnancies at 11–13 weeks' gestation between March 2006 and July 2011, including 300 with euploid fetuses, 50 with trisomy 21 and 50 with trisomy 18. In all cases, fetal karyotyping was carried out by chorionic villous sampling because screening by the combined test suggested that the risk for aneuploidies was >1 in 300 [12]. Maternal characteristics recorded were age, racial origin (Caucasian, African, South Asian, East Asian and mixed), smoking status during pregnancy (yes or no) and weight, which was measured at the time of screening. Gestational age was determined from the measurement of the fetal crown-rump length (CRL) [13]. The measured nuchal translucency (NT) thickness was transformed into the likelihood ratio for each trisomy using the mixture model of NT distributions [14]. The measured free β-subunit of human chorionic gonadotropin (β-hCG) and pregnancy-associated plasma protein (PAPP)-A were converted into multiples of the median (MoM) for gestational age adjusted for maternal weight, racial origin, smoking status, method of conception, parity and machine for the assays [15].

Maternal venous blood collected before chorionic villous sampling in ethylene diamine tetraacetic acid BD vacutainer™ tubes (Becton Dickinson UK limited, Oxfordshire, UK) was processed within 15 min of collection and centrifuged at 2,000 *g* for 10 min and subsequently at 16,000 *g* for 10 min. Plasma samples were stored at –80°C for a median of 879 days (range 38–1,970). Written informed consent was obtained from the women agreeing to participate in the study, which was approved by King's College Hospital Ethics Committee.

Plasma samples (2 ml) from 400 cases that fulfilled the acceptance criteria of the laboratory were analyzed for risk of trisomies 21 and 18 by Aria Diagnostics (San Jose, Calif., USA), as previously described [8, 11]. In the case of the 300 euploid controls, we selected normal pregnancies without hypertensive disorders or diabetes resulting in live births after 37 weeks of neonates with a birth weight above the 5th percentile of the normal range for gestational age [16].

Chromosome-selective sequencing, referred to as digital analysis of selected regions, and fetal fraction optimized risk of trisomy evaluation were used to assay non-polymorphic and polymorphic loci, where fetal alleles differ from maternal alleles, enabling simultaneous determination of chromosome proportion and fetal fraction [8]. The laboratory personnel who were unaware of the fetal karyotype provided risk scores for trisomy 21 and trisomy 18 for 297 of the euploid and for all 100 aneuploid pregnancies. The estimated risk for trisomy 21 was ≥99% in all cases of trisomy 21 and ≤0.01% in all euploid and trisomy 18 pregnancies. The estimated risk for trisomy 18 was >88% in 49 cases of trisomy 18 and <0.03% in 1 case of trisomy 18 and in all euploid and trisomy 21 pregnancies. In 3 (1%) of the 300 euploid pregnancies, no risk score was provided because of failed amplification and sequencing. These results have been published previously [9]. In this study, we report the estimates of the fetal fraction in the 397 cases where a result was provided.

Statistical Analyses

Descriptive data were presented as medians and interquartile ranges for continuous variables and as numbers and percentages for categorical variables. Comparison between the outcome groups was done by χ^2 or Fisher's exact test for categorical variables and the Mann-Whitney U test for continuous variables.

The measured fetal fraction was square root ($\sqrt{}$) transformed to make the distribution Gaussian. Normality was assessed by the Kolmogorov-Smirnov Z test ($p = 0.646$). The measured free β-hCG and PAPP-A were converted into MoM values [15]. The measured NT was expressed as a difference from the expected normal mean for gestation (delta value) [14]. Regression analysis was used to determine which of the factors amongst maternal weight, racial origin, smoking status, plasma storage time, $\log_{10}$ PAPP-A MoM, $\log_{10}$ β-hCG MoM, fetal CRL, delta NT, gender and karyotype were significant predictors of $\sqrt{}$fetal fraction.

Results

Maternal characteristics, results of the combined test and fetal fraction of maternal plasma cfDNA of the study population are presented in table 1. In trisomies 21 and 18, compared to the euploid pregnancies, the median maternal age and delta NT were significantly higher. In trisomy 21, serum free β-hCG was higher and PAPP-A was lower, and in trisomy 18, both free β-hCG and PAPP-A were lower. In trisomy 18, fetal CRL was lower. The median fetal fraction in the total population was 11.4% (interquartile range 8.5–14.6) and there was no significant difference between euploid and trisomy 21 pregnancies.

Table 1. Characteristics of cases and controls

	Total (n = 397)	Euploid (n = 297)	Trisomy 21 (n = 50)	Trisomy 18 (n = 50)
Maternal age, years	35.9 (30.9–39.1)	35.4 (29.7–38.5)	38.8 (34.7–41.2)*	38.0 (33.4–40.7)*
Maternal weight, kg	66.3 (60.0–76.3)	66.7 (60.0–76.3)	63.3 (58.3–68.2)	69.3 (60.5–78.0)
Racial origin				
Caucasian	352 [88.7]	265 [89.2]	45 [90.0]	42 [84.0]
African	20 [5.0]	15 [5.1]	0	5 [10.0]
South Asian	16 [4.0]	9 [3.0]	4 [8.0]	3 [60.0]
East Asian	7 [1.8]	6 [2.0]	1 [2.0]	0
Mixed	2 [0.5]	2 [0.7]	0	0
Smoking	43 [10.8]	138 [8.2]	6 [12.0]*	2 [4.0]*
Fetal CRL, mm	70.9 (63.2–76.7)	72.6 (64.9–77.6)	71.2 (65.7–76.9)	58.5 (55.0–63.5)*
PAPP-A MoM	0.593 (0.378–0.916)	0.688 (0.459–1.013)	0.538 (0.405–0.769)*	0.193 (0.111–0.264)*
Free β-hCG MoM	1.433 (0.752–2.455)	1.521 (0.888–2.392)	2.516 (1.751–4.214)*	0.213 (0.118–0.380)*
Delta NT	0.953 (0.287–2.019)	0.671 (0.221–1.378)	1.574 (1.134–2.984)*	4.764 (2.202–6.144)*
Fetal fraction, %	11.4 (8.5–14.6)	11.8 (9.0–15.1)	13.0 (9.4–15.5)	7.8 (6.1–10.9)*

Data are medians with interquartile ranges in parentheses, or number of cases with percentages in brackets. Comparisons between aneuploid and euploid pregnancies are done by the Mann-Whitney U test for continuous variables and by the χ^2 or Fisher's exact test for categorical variables, with Bonferroni correction. * Adjusted p value <0.025.

In trisomy 18, the fetal fraction was significantly lower than in euploid pregnancies.

The frequency distribution of √fetal fraction is demonstrated in figure 1. Univariate regression analysis demonstrated that √fetal fraction was significantly associated with maternal weight, fetal CRL, trisomy 18 karyotype, delta NT, $\log_{10}$ PAPP-A MoM and $\log_{10}$ β-hCG MoM but not with maternal age, racial origin, smoking status, fetal gender, trisomy 21 karyotype or plasma storage time (table 2). Multivariate regression analysis demonstrated that significant independent prediction of √fetal fraction was provided by maternal weight (r = –0.371), $\log_{10}$ PAPP-A MoM (r = 0.291) and $\log_{10}$ β-hCG MoM (r = 0.341), but not by fetal CRL, delta NT or karyotype (table 2; fig. 2–4). Similarly, in the euploid pregnancies alone, multivariate regression analysis demonstrated that the only significant independent predictors for √fetal fraction were maternal weight (r = –0.424), $\log_{10}$ PAPP-A MoM (r = 0.176) and $\log_{10}$ β-hCG MoM (r = 0.200).

In the estimation of MoM values for free β-hCG and PAPP-A, the measured values are corrected for maternal characteristics including weight. Therefore, we conducted a separate analysis for the prediction of √fetal fraction in which the measured rather than the MoM values of free β-hCG and PAPP-A were considered. Multivariate regression analysis demonstrated that significant independent predictors for √fetal fraction, both in the total

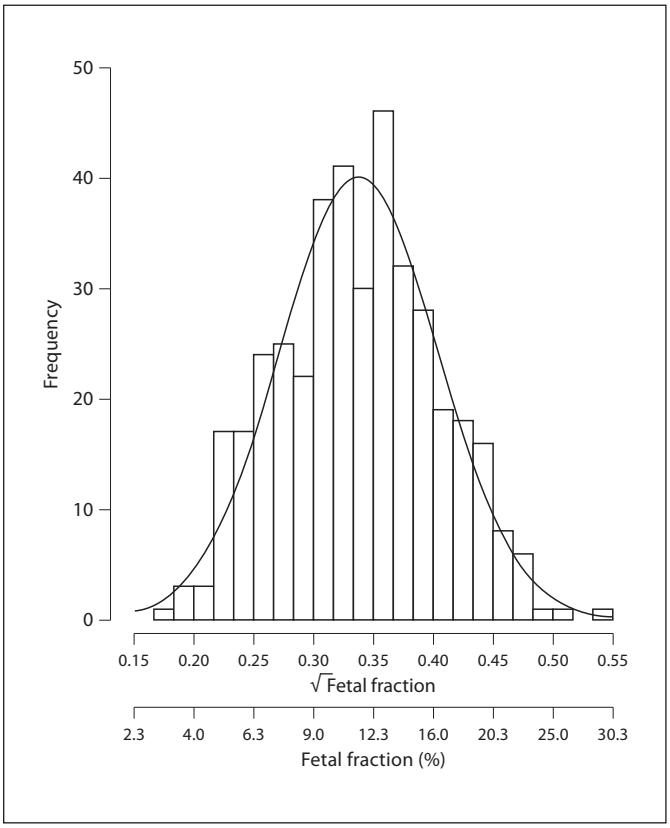

Fig. 1. Frequency distribution of √fetal fraction in maternal plasma cfDNA.

Table 2. Regression analysis for the prediction of √fetal fraction

Independent variable	Univariate		Multivariate	
	regression coefficient	p	regression coefficient	p
Maternal age	–3.5E-05 (–0.001 to 0.001)	0.952	–	–
Maternal weight	–0.002 (–0.002 to –0.001)	<0.0001	–0.002 (–0.002 to –0.001)	<0.0001
Racial origin				
Caucasian	1		–	
African	–0.029 (–0.058 to 0.001)	0.057	–	–
South Asian	0.019 (–0.014 to 0.052)	0.268	–	–
East Asian	0.039 (–0.010 to 0.089)	0.116	–	–
Mixed	0.006 (–0.086 to 0.097)	0.906	–	–
Smoking	0.009 (–0.011 to 0.030)	0.374	–	–
Fetal CRL	0.001 (0.001 to 0.002)	<0.0001	0.0003 (–0.0003 to 0.0010)	0.287
Fetal gender				
Male	1		–	–
Female	0.004 (–0.009 to 0.017)	0.549	–	–
Karyotype				
Euploid	1		1	
Trisomy 21	0.007 (–0.012 to 0.027)	0.445	–	–
Trisomy 18	–0.051 (–0.071 to –0.032)	<0.0001	0.021 (–0.009 to 0.050)	0.164
Delta NT	–0.005 (–0.008 to –0.001)	0.006	–0.001 (–0.005 to 0.002)	0.453
$\log_{10}$ PAPP-A MoM	0.058 (0.039 to 0.077)	<0.0001	0.039 (0.020 to 0.058)	<0.0001
$\log_{10}$ β-hCG MoM	0.053 (0.039 to 0.068)	<0.0001	0.038 (0.023 to 0.053)	<0.0001
Plasma storage time	8.36E-06 (–5.7E-06 to 2.24E-05)	0.242	–	–

Figures in parentheses are 95% confidence intervals.

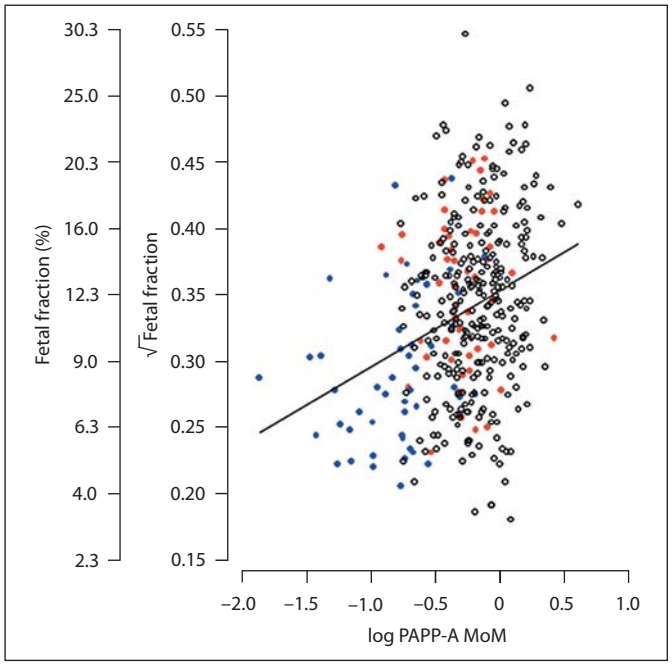

Fig. 2. Relationship of √fetal fraction with $\log_{10}$ PAPP-A MoM: euploid pregnancy (black), trisomy 21 (red) and trisomy 18 (blue). Colors refer to the online version only.

Color version available online

population and in the euploid pregnancies alone, were maternal weight (r = –0.371 and r = –0.424), $\log_{10}$ PAPP-A (r = 0.381 and r = 0.309) and $\log_{10}$ β-hCG (r = 0.378 and r = 0.265).

Discussion

This nested case-control study has shown that in singleton pregnancies at high risk for aneuploidies at 11–13 weeks' gestation, about 10% of cfDNA in maternal plasma is fetal in origin. The fetal fraction increased with the maternal serum level of free β-hCG and PAPP-A and decreased with maternal weight. There was no significant independent contribution to fetal fraction from plasma storage time, maternal age, racial origin or smoking status and fetal CRL, NT thickness, gender or karyotype.

In this study, compared to previous publications on cfDNA, we have exclusively used samples obtained in the first trimester. This is important because in the last decade, there has been a major shift from second- to first-trimester screening and diagnosis of aneuploidies. Additionally, assessment at 11–13 weeks' gestation is emerging

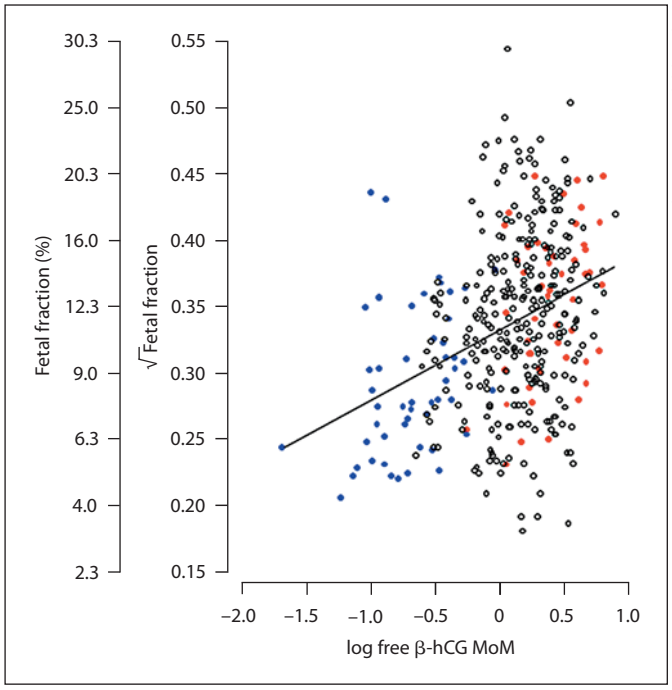

Fig. 3. Relationship of √fetal fraction with $\log_{10}$ β-hCG MoM: euploid pregnancy (black), trisomy 21 (red) and trisomy 18 (blue). Colors refer to the online version only.

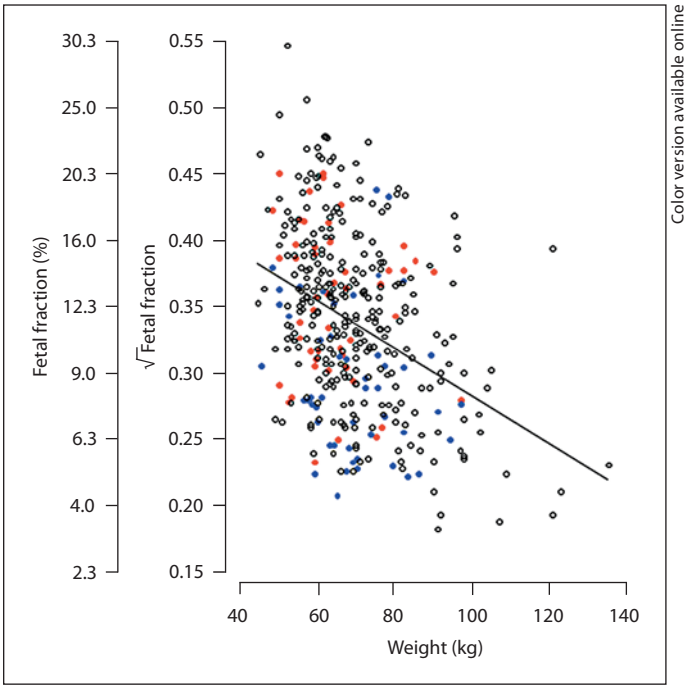

Fig. 4. Relationship of √fetal fraction with maternal weight: euploid pregnancy (black), trisomy 21 (red) and trisomy 18 (blue). Colors refer to the online version only.

as a vital visit not only in screening for aneuploidies but also in the prediction of many pregnancy complications, including preterm birth, preeclampsia, gestational diabetes mellitus, stillbirth, fetal growth restriction and macrosomia [17].

The fetal cfDNA fraction decreased with maternal weight from a mean of about 12% for a maternal weight of 60 kg to 6% for a weight of 120 kg. This inverse association between fetal fraction and maternal weight, which could be attributed to a dilutional effect, is compatible with the results of a previous study [6] and has also been reported for other fetoplacental products in the maternal circulation [15, 18, 19].

Free β-hCG and PAPP-A are produced by the placenta, and the serum concentration of these metabolites presumably provides an indirect measure of placental mass. A 3-dimensional ultrasound study reported a correlation between placental volume in early pregnancy and the levels of serum free β-hCG and PAPP-A [20]. This is the most likely explanation for the linear association between fetal fraction and serum levels of free β-hCG and PAPP-A since the source of fetal cfDNA in maternal plasma is also the placenta. However, these findings are apparently contradictory to the results of previous studies which reported that the maternal serum concentration of fetal cfDNA in maternal blood is higher in pregnancies with impaired rather than with normal placentation. These studies used Y chromosome-specific markers to quantify fetal cfDNA in maternal blood in pregnancies with male fetuses. They reported that in patients with preeclampsia and fetal growth restriction, the concentration of fetal cfDNA is substantially higher than in normotensive controls [21–24]. The increase in fetal cfDNA precedes the clinical onset of the disease being evident from 11 to 13 weeks of gestation and is particularly marked in severe early-onset preeclampsia [25]. In such cases, impaired trophoblastic invasion of the maternal spiral arteries leads to placental ischemia and damage. Increased fetal cfDNA could be released into the maternal circulation either from dying cells in the placenta and/or fetal cells and microvilli whose escape into the maternal circulation is increased in preeclampsia [26–30].

In our euploid group, we selected cases with no preeclampsia and normal pregnancy outcome. If in such cases the source of fetal cfDNA in maternal blood is dying cells in the placenta, then the number of apoptotic cells would be proportional to the placental mass, reflected in the level of serum free β-hCG and PAPP-A. In severe ear-

ly-onset preeclampsia, which is associated with low serum PAPP-A at 11–13 weeks' gestation [31], the physiological relationship between fetal fraction and serum PAPP-A would be disturbed by the ischemia-related accelerated placental cell death. In trisomy 21 pregnancies, although serum PAPP-A is reduced to a similar degree as in euploid pregnancies developing early-onset preeclampsia, Doppler assessment of the uterine arteries has shown no evidence of impaired placental perfusion [32]. Consequently, in trisomy 21 pregnancies, the physiological relationship between fetal fraction and the serum level of placental metabolites is not disturbed and the fetal fraction was not significantly different from our euploid pregnancies. A 3-dimensional ultrasound study reported that in trisomy 21 pregnancies, placental volume at 11–13 weeks' gestation was not significantly different from euploid pregnancies [33]. In trisomy 18, the fetal fraction was significantly lower than in euploid pregnancies. This is likely to be the mere consequence of the small placental volume [33], reflected in the low serum levels of both free β-hCG and PAPP-A, because in the multivariate regression analysis, there was no significant independent effect of trisomy 18 on fetal fraction.

Successful non-invasive prenatal detection of fetal trisomies from maternal plasma cfDNA necessitates that the minimum fetal fraction is 3–4% because if the fraction is lower, the small differences in circulating cfDNA between trisomic and disomic pregnancies may not be detectable [5, 6, 8]. On the basis of the results of this study, a very low fetal fraction, with consequent failure of non-invasive testing, may be observed in very obese women, in pregnancies with a small placenta because of very early gestational age or a combination of these two factors.

Recent studies in high-risk pregnancies have reported successful non-invasive detection from maternal cfDNA of about 99% of fetuses with trisomy 21, at a false-positive rate of <1% [4–9]. The ability to detect trisomy 21 with cfDNA is dependent upon assay precision and fetal fraction in the sample rather than upon the prevalence of the disease in the study population. Consequently, our results that fetal fraction is not dependent on fetal karyotype suggest that cfDNA testing may be applicable not only in high-risk but also in low-risk pregnancies. However, this requires further investigation.

Acknowledgments

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