ORIGINAL ARTICLE

Factors affecting levels of circulating cell-free fetal DNA in maternal plasma and their implications for noninvasive prenatal testing

Sarah L. Kinnings[1], Jennifer A. Geis[1], Eyad Almasri[1], Huiquan Wang[1], Xiaojun Guan[1], Ron M. McCullough[1], Allan T. Bombard[2], Juan-Sebastian Saldivar[1], Paul Oeth[1] and Cosmin Deciu[1]*

[1]Sequenom Laboratories, San Diego, CA, USA
[2]Sequenom, Inc., San Diego, CA, USA
*Correspondence to: Cosmin Deciu. E-mail: cdeciu@sequenom.com

ABSTRACT

Objective Sufficient fetal DNA in a maternal plasma sample is required for accurate aneuploidy detection via noninvasive prenatal testing, thus highlighting a need to understand the factors affecting fetal fraction.

Method The MaterniT21™ PLUS test uses massively parallel sequencing to analyze cell-free fetal DNA in maternal plasma and detect chromosomal abnormalities. We assess the impact of a variety of factors, both maternal and fetal, on the fetal fraction across a large number of samples processed by Sequenom Laboratories.

Results The rate of increase in fetal fraction with increasing gestational age varies across the duration of the testing period and is also influenced by fetal aneuploidy status. Maternal weight trends inversely with fetal fraction, and we find no added benefit from analyzing body mass index or blood volume instead of weight. Strong correlations exist between fetal fractions from aliquots taken from the same patient at the same blood draw and also at different blood draws.

Conclusion While a number of factors trend with fetal fraction across the cohort as a whole, they are not the sole determinants of fetal fraction. In this study, the variability for any one patient does not appear large enough to justify postponing testing to a later gestational age. © 2015 John Wiley & Sons, Ltd.

Funding sources: Sequenom, Inc. and Sequenom Laboratories. The sponsors participated in the design of the study, data collection, data management, data analysis, decision to submit the report for publication, manuscript preparation, and review.
Conflicts of interest: Kinnings, Geis, Almasri, Wang, Guan, McCullough, Saldivar, Oeth, and Deciu are employees of Sequenom Laboratories and Sequenom shareholders. Bombard is an employee of Sequenom, Inc. and a Sequenom shareholder.

INTRODUCTION

Following the discovery that the plasma of pregnant women contains circulating cell-free DNA (ccfDNA) of fetal origin,[1] various clinical tests have been developed to detect the presence of fetal aneuploidies noninvasively from maternal blood samples.[2–9] Because ccfDNA is a mixture of both maternal and fetal ccfDNA, the ability to detect fetal chromosomal aneuploidies is directly related to the fetal-to-total DNA fraction of a sample, that is, the fetal fraction. If the fetal fraction is too small, then any abnormalities in the fetal ccfDNA will be masked by the overwhelming proportion of euploid maternal ccfDNA, thereby making their detection impractical. Recent studies have consistently shown that fetal fractions tend to average around 10% to 15%, but can range up to 30% or more.[4,7,8,10–13] Approximately 1% to 3% of samples are thought to have fetal fractions less than 4%, which is a common minimum threshold employed for accurate fetal trisomy classification.[3,6] Overestimates of fetal fraction may lead to false negative results, while underestimates of fetal fraction may cause suitable samples to be rejected.

To date, the majority of methods to estimate fetal fraction have relied on differential methylation[14] or have exploited polymorphic differences between the maternal and fetal ccfDNA based on paternally inherited fetal alleles.[8,9,15,16] However, such methods require additional enrichment and/or quantification steps, providing greater opportunity for errors in fetal fraction estimation, particularly at lower fetal fractions.[17] While other methods have estimated the fetal fraction directly from massively parallel sequencing tag-count data, such analysis has been limited to samples from male and aneuploid pregnancies.[13,17,18] Indeed, because of the lack of maternal chromosome Y, the fetal fraction of a male fetus may be estimated simply from the chromosome Y representation in a sample. Similarly, because the maternal ccfDNA is presumed to be euploid, the fetal fraction may be estimated from the overrepresentation of an aneuploid

chromosome in a sample. To date, the accurate prediction of fetal fraction in euploid female pregnancies from whole genome sequencing data has remained a challenge. However, through the use of advanced statistical modeling techniques, we are able to accurately predict fetal fraction from the behavior of autosomal chromosomes, therefore providing an accurate whole genome sequencing-based fetal fraction estimate for all clinical samples, regardless of fetal gender or aneuploidy status.[19]

The MaterniT21™ PLUS test uses massively parallel sequencing to analyze cell-free fetal DNA in maternal plasma, measuring a relative increase or decrease in chromosomal representation in order to detect chromosomal abnormalities.[5,20] Since its launch in October 2011, Sequenom Laboratories have processed more than 400 000 clinical samples with the MaterniT21™ PLUS test as described by Jensen *et al.*[5] All samples tested in our laboratories are subject to multiple levels of quality control, including the measurement of fetal fraction to ensure the presence of sufficient fetal DNA for accurate classification. This is the largest known study size to date from which to assess the impact of a variety of maternal and fetal factors on the fetal fraction. First, we examine the effect of factors such as gestational age, maternal weight, body mass index (BMI) and blood volume, and fetal sex and aneuploidy status on the fetal fraction. We draw comparisons with previous studies and provide further insight into these complex relationships. Then, for the first time, we compare fetal fractions between aliquots of blood drawn from the same patient at the same time, and from the same patient at a different blood draw, before discussing the overall clinical implications of our findings.

METHODS

All samples processed by Sequenom Laboratories according to the methods described by Jensen *et al.*[5] were selected for this study. A novel approach for fetal fraction estimation by Sequenom Laboratories was used to derive an estimate for all samples. This statistical approach uses a priori knowledge of the fragment length of circulating cell-free (ccf) maternal and fetal DNA in an optimal fashion by utilizing whole genome sequencing patterns and exploiting their known underlying distribution structures.[19] This concept is well-documented by Lo *et al.*[21] and is further augmented by statistical data-adaptive methodology to provide a fetal fraction estimate that is referred to as the sequencing-based fetal fraction estimate. This method has been validated extensively using established methods including the Fetal Quantifier Assay[14] and targeted SNP-based approaches.[8,9,16,19]

Samples that failed to meet any of the quality control criteria employed by the clinical laboratory (due to insufficient library concentration or insufficient number of aligned sequencing reads, etc.), except for insufficient fetal fraction, were filtered out. The median read depth per sample for this study was 16 million reads, as previously reported.[5] Where referenced, 'sufficient' fetal fraction refers to samples with an estimated fetal fraction of greater than or equal to 3.7%. The 3.7% cutoff was chosen using statistical modeling, based on the requirement for sufficient sequencing read depth as a function of fetal fraction in order to detect fetal aneuploidies in cell-free plasma.[5,10] Samples from pregnancies determined to be multi-fetal by the physician were excluded from all analyses except for the analysis of reproducibility between aliquots. Because the physicians' observations of maternal height, weight, and gestational age do not always follow a standardized format, these metrics were converted into a common set of units. Some samples were either missing annotations entirely, or missing units in their annotations, resulting in these samples being excluded only from the analyses involving maternal metrics. Even after data curation, some values displayed strong outlier behavior, suggesting that they were erroneous. Therefore, additional filtering was performed to remove any samples with maternal metrics outside of acceptable ranges (the values for which are denoted by the minima and maxima in Table 1). Note that a patient's blood volume and BMI were calculated from the height and weight information supplied by the physician.

This study was a retrospective study performed on previously existing data that was de-identified and thereby qualifies for exemption from the regulatory requirements in 45 CFR 46, which includes an Institutional Review Board review.

RESULTS

Dataset summary

Across all 140 377 samples in this study, the median fetal fraction was 9.47%, and the median gestational age at which a sample was taken was 13.10 weeks (91.7 days). The median reported weight of a patient was 151 lbs (68.5 kg), with a BMI of 25.66 kg/m^2, and a blood volume of 4.05 L (Table 1). In agreement with Wang *et al.*,[22] the distribution of gestational age is bimodal, with two peaks, one at approximately 10 to 12 weeks' gestation and a second at around 18 to 20 weeks' gestation (Figure 1). Overall, fetal fractions appear to be higher for samples classified as euploid versus those classified as positive for a trisomy (in particular trisomy 18 and 13) (Figure S1 and Table S1, Supporting Information). However, the relationship between fetal fraction and fetal aneuploidy status is complex and is examined further in the next section.

Fetal fraction increases with gestational age

In agreement with previous studies,[7,10,12,17,23] our results reveal that gestational age is positively correlated with fetal fraction

Table 1 Summary of fetal fraction and maternal metrics for the final dataset of all 140 377 samples used in this study

	Median	MAD[a]	Min.	Max.
Fetal fraction (%)	9.47	3.73	0.00	65.49
Gestational age (weeks)	13.10	3.33	10.00	40.00
Maternal weight (lbs)	151.00	34.10	81.00	493.83
Maternal BMI (kg/m^2)	25.66	5.64	15.00	95.58
Maternal blood volume (L)	4.05	0.64	2.52	9.00

BMI, body mass index.
[a]MAD is the median absolute deviation multiplied by a factor of 1.4826 to ensure consistency with the normal distribution.

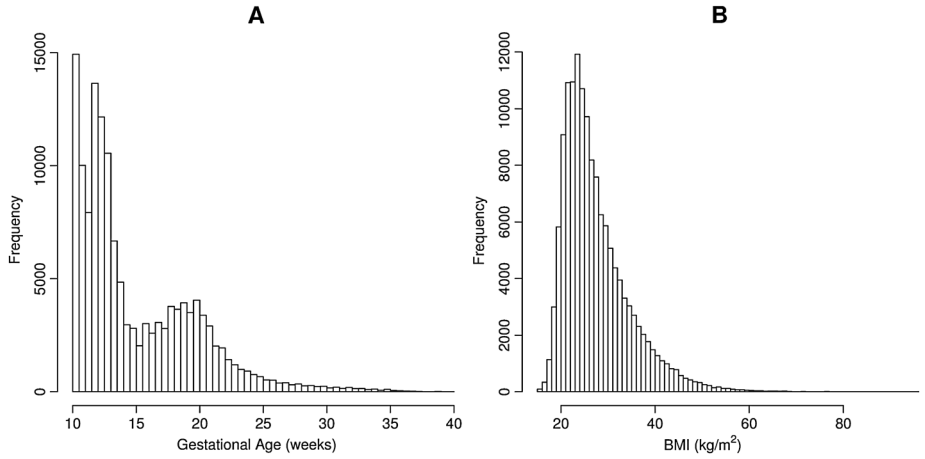

Figure 1 Distribution of (A) gestational age and (B) maternal body mass index (BMI) across all 140 377 samples used in this study

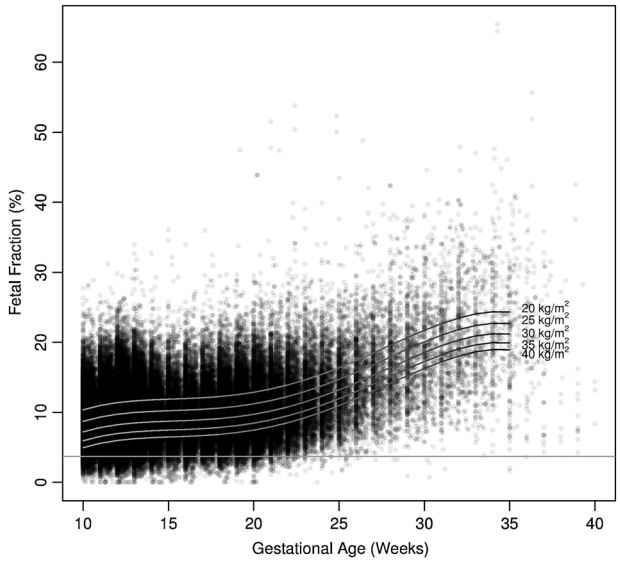

Figure 2 Relationship between gestational age and fetal fraction. A polynomial model of fetal fraction versus gestational age and maternal body mass index (BMI) was fitted, and the resulting regression lines are shown for various different BMI values

(Spearman's $\rho = 0.2141$). Further examination using polynomial quantile regression reveals that overall the rate of increase in fetal fraction is not constant across different gestational ages (Figure 2). From 10 to 12.5 weeks' gestation, the fetal fraction increases at a rate of 0.44% per week, before leveling off to a rate of 0.083% per week between 12.5 and 20 weeks' gestation. The rate of increase becomes noticeably higher by around 20 weeks' gestation and remains constant from then onwards. Over this period, the fetal fraction is increasing at its maximal rate of 0.821% per week. On average, the fetal fraction at around 30 weeks' gestation onwards (approximately 20%) is more than twice that observed at up to 20 weeks' gestation (approximately 9%) (Table 2). However, only around 4% of tests are actually performed after 25 weeks' gestation, with the majority of patients (approximately 60%) opting to take the test at 10 to 15 weeks' gestation. Even at 10 to 15 weeks' gestation though, more than 96% of samples have sufficient fetal fraction for classification.

The gestational age versus fetal fraction relationship may be further influenced by factors such as fetal sex and aneuploidy status. While no significant difference in fetal fraction for male versus female fetuses is observable at any gestational age (Figure S2, Supporting Information), aneuploidy status was shown to influence the relationship between fetal fraction and gestational age (Figure 3). Overall, the samples classified as T21-positive exhibit similar fetal fractions to samples classified as euploid until approximately 16 weeks' gestation. At that time and beyond, the T21-positive samples show considerably higher fetal fractions throughout the remainder of the pregnancy [$p < 0.0001$ from a two-sample, one-tailed Kolmogorov–Smirnov (KS)-test]. The T18-positive samples have much lower fetal fractions than the euploid samples until approximately 21 weeks' gestation ($p < 0.0001$ from a

Table 2 Summary of fetal fractions for samples grouped by gestational age

Gestational age range (weeks)	Median fetal fraction	MAD fetal fraction	% sufficient fetal fraction (≥3.7%)	*n*	% of samples
10–15	9.02	3.50	96.32 (96.20–96.45)	84 758	60.38
15–20	9.45	3.62	97.20 (97.02–97.39)	31 785	22.64
20–25	10.62	3.91	98.82 (98.66–98.98)	18 360	13.08
25–30	14.75	5.63	99.60 (99.39–99.82)	3757	2.68
30–40	19.58	7.29	99.59 (99.26–99.92)	1717	1.22

MAD, median absolute deviation.
95% confidence intervals are given in parentheses.

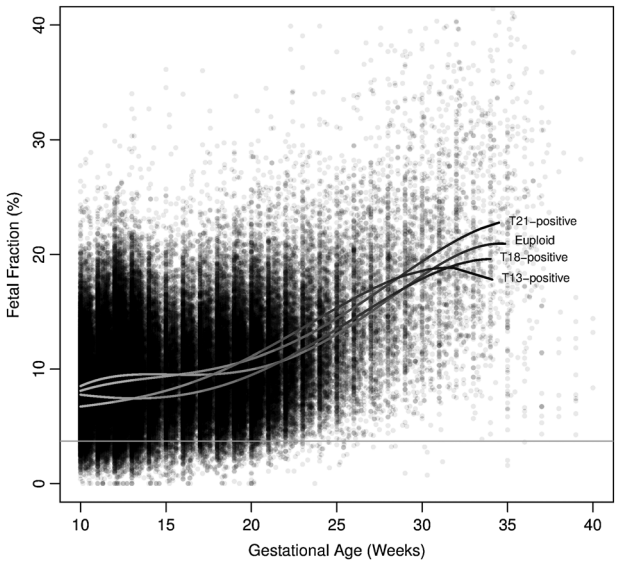

Figure 3 Relationship between gestational age and fetal fraction. The median polynomial quantile regression lines are shown for samples classified as euploid, T21-positive, T18-positive, or T13-positive

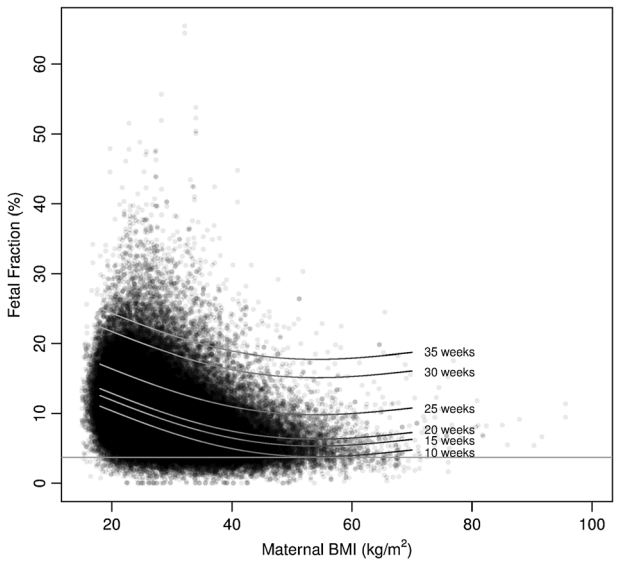

Figure 4 Relationship between maternal body mass index (BMI) and fetal fraction. A polynomial model of fetal fraction versus maternal BMI and gestational age was fitted, and the resulting regression lines are shown for various different gestational age values

two-sample, one-tailed KS-test) (with differences as high as 3% fetal fraction), after which, they show a very similar trend. The T13-positive samples start with the lowest fetal fraction across all sample types, but by 18 weeks' gestation, they have overtaken the euploid samples, and by 20 weeks' gestation, they have surpassed even the T21-positive samples to become the sample type with the highest fetal fraction from this point until approximately 28 weeks' gestation ($p < 0.0211$ from a two-sample, one-tailed KS-test).

Fetal fraction decreases with maternal BMI

Although fetal fraction was shown to trend positively with gestational age (Spearman's $\rho = 0.2141$), its relationship is confounded by maternal BMI, which also increases with gestational age (Spearman's $\rho = 0.1970$) (Figure S3, Supporting Information). Figure 4 shows a clear decrease in fetal fraction with increasing maternal BMI (Spearman's $\rho = -0.3934$). It is worth noting that the correlations between fetal fraction and maternal weight, BMI, and blood volume are virtually identical (Table 3 and Figure S4**,** Supporting Information). Furthermore, height alone shows a weak inverse correlation with fetal fraction (Spearman's $\rho = -0.0980$) (Figure S5, Supporting Information). Table 4 shows that around 86% of samples fall within the maternal BMI range of 20 to 40 kg/m^2. Within this BMI range, linear regression reveals that there is a decrease of 1.17% in fetal fraction for every increase in BMI of 5 kg/m^2. Above a BMI of 40 kg/m^2, the decrease in fetal fraction levels off, and at a BMI of approximately 50 kg/m^2, the fetal fraction remains constant. Even in the BMI range of 60 kg/m^2 and above, classification results are provided for 74.7% of samples (Table 4).

The effect of patient-specific redraws on fetal fraction

Within the dataset used for this study, a number of patients had redraws requested either due to insufficient fetal fraction or technical issues, generally after processing two aliquots from the original draw. Some of the redraws themselves had two aliquots processed. Figure 5 compares fetal fraction values between first and second draws for all possible aliquots from the same patient. Strong correlation between fetal fractions obtained from the same patient at different blood draws is seen, although much more variability in fetal fraction is observed between different draws than between different aliquots from the same draw (Figure S6, Supporting Information), especially at higher fetal fractions.

In comparison with the entire cohort, samples that had a redraw due to insufficient fetal fraction from both aliquots on the first draw had an earlier median gestational age (12.0 weeks vs 13.1 weeks, $p < 0.0001$ from a one-tailed, two-sample KS-test) and a much higher median BMI (38.5 kg/m^2 vs 25.7 kg/m^2, $p < 0.0001$ from a one-tailed, two-sample KS-test) (Tables 1 and 5). As expected, when compared with the original draw, the redraws of these samples had a higher median fetal fraction (3.50% vs 2.70%, $p < 0.0001$ from a one-tailed, two-sample KS-test) (Table 5). The median interval time between the order dates of these draws was 14 days, and the median

Table 3 Strength of the relationship between various different maternal metrics and fetal fraction, ordered by strongest to weakest correlation

Metric 1	Metric 2	Spearman's rho (ρ)
Maternal weight	Fetal fraction	−0.4072
Maternal BMI	Fetal fraction	−0.3934
Maternal blood volume	Fetal fraction	−0.3780
Gestational age	Fetal fraction	0.2141
Gestational age	Maternal BMI	0.1970
Maternal height	Fetal fraction	−0.0980

BMI, body mass index.

Table 4 **Summary of fetal fractions for samples grouped by maternal BMI**

Maternal BMI range (kg/m^2)	Median fetal fraction	MAD fetal fraction	% sufficient fetal fraction (≥3.7%)	*n*	% of samples
<20	11.62	3.55	99.49 (99.35–99.63)	10 399	7.41
20–30	10.09	3.47	98.77 (98.70–98.85)	91 196	64.97
30–40	7.67	3.11	94.36 (94.10–94.63)	30 069	21.42
40–50	5.99	2.56	85.40 (84.57–86.23)	7102	5.06
50–60	5.40	2.50	79.88 (77.67–82.09)	1307	0.93
>60	5.27	2.59	74.67 (69.62–79.72)	304	0.22

BMI, body mass index; MAD, median absolute deviation.
95% confidence intervals are given in parentheses.

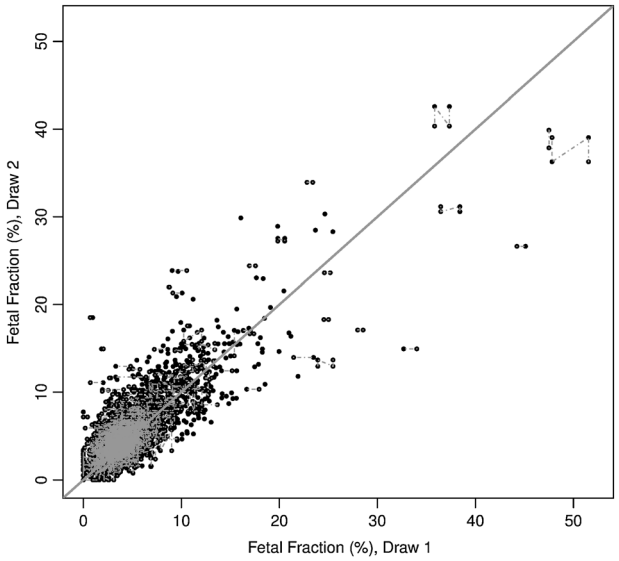

Figure 5 **Comparison of fetal fraction between the original draw and redraw for all patients with more than one blood draw. There are up to two aliquots per draw, and all aliquots are shown on this plot. Data points from the same patient are joined by gray dashed lines. The thick gray line indicates the ideal line (x = y)**

Table 5 **Summary statistics by draw for those samples with insufficient fetal fraction (<3.7%) at the first draw**

	Draw 1 (original)			Draw 2 (redraw)		
	Median	MAD	*n*	Median	MAD	*n*
Fetal fraction (%)	2.70	0.90	684	3.50	1.71	534
Gestational age (weeks)	12.00	2.08	381	14.14	2.29	381
Maternal weight (lbs)	233.00	66.72	381	234.00	65.23	381
Maternal BMI (kg/m^2)	38.54	9.53	381	38.53	9.29	381
Maternal blood volume (L)	5.25	1.07	381	5.28	1.09	381

BMI, body mass index; MAD, median absolute deviation.

increase in fetal fraction between these draws was 1.08%. This rate of increase of 0.54% fetal fraction per week roughly corresponds with the rate of increase observed across the cohort as a whole prior to 12.5 weeks' gestation (0.44% per week) (Figure 2), therefore suggesting that it may be attributed to the later gestational age of the second draw.

Table 6 **Rescue rate of samples with insufficient fetal fraction (<3.7%) at the first draw, grouped by time interval between draws**

Interval between draws (days)	No. with insufficient fetal fraction (<3.7%) at Draw 1	% rescued by Draw 2
1–10	35	71.43 (55.03–87.82)
11–20	247	53.85 (47.43–60.27)
21–30	73	53.42 (41.30–65.55)
31–40	16	50.00 (22.38–77.63)
41–60	10	80.00 (50.21–100.00)

95% confidence intervals are given in parentheses.

For those patients with redraws due to insufficient fetal fraction on the first draw, 56.2% had sufficient fetal fraction on the second draw. As expected, this rescue rate is higher than that achieved by simply processing a second aliquot from the same draw (38.9%). Table 6 shows the rescue rates corresponding to different lengths of time between draws. Even with a minimum interval time between draws (1–10 days), the rescue rate is higher than average (71.43%); thus, based on this analysis, there appears to be no merit in postponing the second draw to a later date. In fact, the rescue rate remains fairly consistent at just above 50% when the redraw is taken anywhere from 11 to 40 days after the original draw. While the rescue rate does appear to increase as interval times extend beyond 40 days, the sample size is very small by this stage. Interestingly, while a higher BMI increases the chances of insufficient fetal fraction on the first draw, it does not appear to affect the rescue rate at the second draw. Table 7 shows that while the rescue rate is the highest (60.47%), albeit marginally, for the lowest BMI range of 15 to 20 kg/m^2, it actually remains at a fairly consistent 50% to 60% across all BMI ranges.

DISCUSSION

Our results indicate that the lowest rate of increase in fetal fraction with gestational age occurs between 12.5 and 20 weeks' gestation (0.083% per week), before increasing tenfold to a rate of 0.821% per week from 20 weeks' gestation

Table 7 Rescue rate of samples with insufficient fetal fraction (<3.7%) at the first draw, grouped by maternal body mass index (BMI) at the first draw

Maternal BMI (kg/m^2)	No. with insufficient fetal fraction (<3.7%) at Draw 1	% rescued by Draw 2
15–20	43	60.47 (44.69–76.24)
21–30	105	56.19 (46.22–66.16)
31–40	150	58.00 (49.77–66.23)
41–50	128	54.69 (45.67–63.70)
51–60	70	51.43 (39.01–63.85)
61–70	45	57.78 (42.24–73.32)
71–80	39	56.41 (39.57–73.26)
81–85	39	53.85 (36.92–70.77)

95% confidence intervals are given in parentheses.

onwards. These findings are in general agreement with Wang *et al.*[22] who observed that the fetal fraction increased at a rate of approximately 1% per week starting at 21 weeks' gestation.

Previous studies[11,17] have observed an overall increase in fetal fraction in T21-positive samples and a decrease in fetal fraction in T18-positive and T13-positive samples, when compared with euploid samples. However, we have shown for the first time that the effect of aneuploidy status on fetal fraction varies with gestational age. Indeed, when compared with euploid samples, T21-positive samples only showed a relative increase in fetal fraction from 16 weeks' gestation onwards. Similarly, T18-positive and T13-positive samples only showed a relative decrease in fetal fraction up until 21 and 18 weeks' gestation, respectively.

Of all of the factors impacting the fetal fraction, maternal weight, BMI, and blood volume are the most significant, showing strong inverse trends. Within the most common maternal BMI range of 20 to 40 kg/m^2, we found a decrease of 1.17% fetal fraction for every BMI increase of 5 kg/m^2. However, because nearly three quarters of all samples from patients with a BMI in the range of 60 kg/m^2 and above still contain sufficient fetal fraction for classification, testing remains a reasonable option for patients with a greater than average BMI. Previous studies have described the inverse relationship between maternal weight or BMI and fetal fraction, which has been attributed to the dilution of a fixed amount of fetal DNA within increasing amounts of maternal DNA in the circulation.[10,12,17,22] Because circulatory volume is a direct consequence of both weight and height, Wang *et al.*[22] suggested that the use of BMI or blood volume, which incorporate both of these measures, may be more insightful than weight alone. However, we compared weight with BMI and blood volume and found that they trend with fetal fraction in a similar manner. These findings suggest that additional factors associated with obesity, such as body fat percentage (not observable in this study) may provide further insights into this complex relationship. Indeed, increased death of adipose cells and increased white blood cell count in obese women has been shown to lead to higher concentrations of circulating maternal DNA.[24,25]

Finally, we show that there is a strong correlation between the fetal fractions of samples taken from the same patient at different gestational age draws. As expected, samples with redraws requested due to insufficient fetal fraction on the first draw tend to be from patients with higher BMIs and slightly earlier gestational ages. These redraws result in a rescue rate of 56.2% (compared with 38.9% for second aliquots from the same draw), and the rescue rate does not appear to be affected by the number of days between the draws or the BMI at the first draw. Thus, it appears, based on this analysis, that there is no merit in postponing such redraws to a later date in order to increase the chance of achieving sufficient fetal fraction.

CONCLUSION

This is the largest known study size to date from which to assess the impact of a variety of maternal and fetal factors on the amount of circulating cell-free fetal DNA. Our results indicate that the likelihood of a sample containing sufficient fetal fraction to accurately classify chromosomal aneuploidies generally increases with gestational age. However, overall, the rate of increase in fetal fraction varies across different gestational ages, and the vast majority of our samples are collected in the gestational age range at which the rate of increase of fetal fraction with gestational age is at its lowest. In addition, the relationship between gestational age and fetal fraction is confounded by a number of other factors, such as maternal weight or BMI and fetal aneuploidy status. While these factors may trend with fetal fraction in general, the variability for any one patient appears to be so small that we see no merit in postponing testing to a later gestational age to increase the likelihood of being able to produce a classification result. However, it is worth noting that the minimum gestational age included in this study is 10 weeks. Our comparisons of fetal fractions between different aliquots from the same patient at the same time of phlebotomy and from the same patient at different times support these conclusions.

WHAT'S ALREADY KNOWN ABOUT THIS TOPIC?

- Sufficient fetal-to-total DNA fraction ('fetal fraction') of a maternal plasma sample is required for accurate aneuploidy detection via noninvasive prenatal testing.
- Fetal fraction increases with gestational age and decreases with maternal weight, which also increases with gestational age.
- When compared with euploid samples, an overall increase in fetal fraction in T21-positive samples and a decrease in fetal fraction in T18-positive and T13-positive samples are observed.

WHAT DOES THIS STUDY ADD?

- This is the largest known study size from which to assess the effects of maternal and fetal factors on the fetal fraction.
- The effects of maternal weight, body mass index, and blood volume on fetal fraction are compared.
- The effect of fetal aneuploidy status on the fetal fraction is shown across different gestational ages.
- Multiple measurements of fetal fraction per patient are compared both from the same and different blood draws.

REFERENCES

1. Lo YM, Corbetta N, Chamberlain PF, *et al.* Presence of fetal DNA in maternal plasma and serum. Lancet 1997;350:485–7.
2. Bianchi DW, Platt LD, Goldberg JD, *et al.* Genome-wide fetal aneuploidy detection by maternal plasma DNA sequencing. Obstet Gynecol 2012; 119:890–901.
3. Canick JA, Kloza EM, Lambert-Messerlian GM, *et al.* DNA sequencing of maternal plasma to identify Down syndrome and other trisomies in multiple gestations. Prenat Diagn 2012;32:730–4.
4. Chiu RW, Akolekar R, Zheng YW, *et al.* Non-invasive prenatal assessment of trisomy 21 by multiplexed maternal plasma DNA sequencing: large scale validity study. BMJ 2011;342:c7401.
5. Jensen TJ, Zwiefelhofer T, Tim RC, *et al.* High-throughput massively parallel sequencing for fetal aneuploidy detection from maternal plasma. PLoS One 2013;8:e57381.
6. Norton ME, Brar H, Weiss J, *et al.* Non-Invasive Chromosomal Evaluation (NICE) Study: results of a multicenter prospective cohort study for detection of fetal trisomy 21 and trisomy 18. Am J Obstet Gynecol 2012;207:137.e1–8.
7. Palomaki GE, Kloza EM, Lambert-Messerlian GM, *et al.* DNA sequencing of maternal plasma to detect Down syndrome: an international clinical validation study. Genet Med 2011;13:913–20.
8. Sparks AB, Struble CA, Wang ET, *et al.* Noninvasive prenatal detection and selective analysis of cell-free DNA obtained from maternal blood: evaluation for trisomy 21 and trisomy 18. Am J Obstet Gynecol 2012; 206:319.e1–9.
9. Zimmermann B, Hill M, Gemelos G, *et al.* Noninvasive prenatal aneuploidy testing of chromosomes 13, 18, 21, X, and Y, using targeted sequencing of polymorphic loci. Prenat Diagn 2012;32:1233–41.
10. Ashoor G, Poon L, Syngelaki A, *et al.* Fetal fraction in maternal plasma cell-free DNA at 11–13 weeks' gestation: effect of maternal and fetal factors. Fetal Diagn Ther 2012;31:237–43.
11. Ashoor G, Syngelaki A, Poon LC, *et al.* Fetal fraction in maternal plasma cell-free DNA at 11–13 weeks' gestation: relation to maternal and fetal characteristics. Ultrasound Obstet Gynecol 2013;41:26–32.
12. Canick JA, Palomaki GE, Kloza EM, *et al.* The impact of maternal plasma DNA fetal fraction on next generation sequencing tests for common fetal aneuploidies. Prenat Diagn 2013;33:667–74.
13. Hudecova I, Sahota D, Heung MMS, *et al.* Maternal plasma fetal DNA fractions in pregnancies with low and high risks for fetal chromosomal aneuploidies. PLoS One 2014; 9:e88484.
14. Nygren AO, Dean J, Jensen TJ, *et al.* Quantification of fetal DNA by use of methylation-based DNA discrimination. Clin Chem 2010;56:1627–35.
15. Birch L, English CA, O'Donoghue K, *et al.* Accurate and robust quantification of circulating fetal and total DNA in maternal plasma from 5 to 41 weeks of gestation. Clin Chem 2005;51:312–20.
16. Jiang P, Chan KC, Liao GJ, *et al.* FetalQuant: deducing fractional fetal DNA concentration from massively parallel sequencing of DNA in maternal plasma. Bioinformatics 2012;28:2883–90.
17. Rava RP, Srinivasan A, Sehnert AJ, Bianchi DW. Circulating fetal cell-free DNA fractions differ in autosomal aneuploidies and monosomy X. Clin Chem 2014;60:243–50.
18. Fan HC, Blumenfeld YJ, Chitkara U, *et al.* Noninvasive diagnosis of fetal aneuploidy by shotgun sequencing DNA from maternal blood. Proc Natl Acad Sci U S A 2008;105:16266–71.
19. Kim S, Hannum G, Geis J, *et al.* Determination of fetal DNA fraction from the plasma of pregnant women using sequence read counts. Prenat Diagn 2015. DOI:10.1002/pd.4615.
20. Mazloom AR, Dzakula Z, Oeth P, *et al.* Noninvasive prenatal detection of sex chromosomal aneuploidies by sequencing circulating cell-free DNA from maternal plasma. Prenat Diagn 2013;33:591–7.
21. Lo YM, Tein MS, Lau TK, *et al.* Quantitative analysis of fetal DNA in maternal plasma and serum: implications for noninvasive prenatal diagnosis. Am J Hum Genet 1998;62:768–75.
22. Wang E, Batey A, Struble C, *et al.* Gestational age and maternal weight effects on fetal cell-free DNA in maternal plasma. Prenat Diagn 2013; 33: 662–6.
23. Brar H, Wang E, Struble C, *et al.* The fetal fraction of cell-free DNA in maternal plasma is not affected by a priori risk of fetal trisomy. J Matern Fetal Neonatal Med 2013;26:143–5.
24. Haghiac M, Vora NL, Basu S, *et al.* Increased death of adipose cells, a path to release cell-free DNA into systemic circulation of obese women. Obesity (Silver Spring) 2012;20:2213–9.
25. Vora NL, Johnson KL, Basu S, *et al.* A multifactorial relationship exists between total circulating cell-free DNA levels and maternal BMI. Prenat Diagn 2012;32:912–4.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web site.