DOI: 10.1002/pd.4126

ORIGINAL ARTICLE

The impact of maternal plasma DNA fetal fraction on next generation sequencing tests for common fetal aneuploidies

Jacob A. Canick*†, Glenn E. Palomaki†, Edward M. Kloza, Geralyn M. Lambert-Messerlian and James E. Haddow

Division of Medical Screening and Special Testing, Department of Pathology and Laboratory Medicine, Women & Infants Hospital and Alpert Medical School of Brown University, Providence, RI 02903, USA

*Correspondence to: Jacob A. Canick. E-mail: jcanick@wihri.org

†JAC and GEP contributed equally to this study.

ABSTRACT

Maternal plasma contains circulating cell-free DNA fragments originating from both the mother and the placenta. The proportion derived from the placenta is known as the fetal fraction. When measured between 10 and 20 gestational weeks, the average fetal fraction in the maternal plasma is 10% to 15% but can range from under 3% to over 30%. Screening performance using next-generation sequencing of circulating cell-free DNA is better with increasing fetal fraction and, generally, samples whose values are less than 3% or 4% are unsuitable. Three examples of the clinical impact of fetal fraction are discussed. First, the distribution of test results for Down syndrome pregnancies improves as fetal fraction increases, and this can be exploited in reporting patient results. Second, the strongest factor associated with fetal fraction is maternal weight; the false negative rate and rate of low fetal fractions are highest for women with high maternal weights. Third, in a mosaic, the degree of mosaicism will impact the performance of the test because it will reduce the effective fetal fraction. By understanding these aspects of the role of fetal fraction in maternal plasma DNA testing for aneuploidy, we can better appreciate the power and the limitations of this impressive new methodology. © 2013 John Wiley & Sons, Ltd.

Funding sources: None

Conflicts of interest: Canick, Palomaki, Kloza, Lambert-Messerlian, and Haddow are employees of Women and Infants Hospital of Rhode Island. Canick and Palomaki were members of the Sequenom Clinical Advisory Board between November 2007 and October 2008, and resigned when they received study funding. Palomaki and Canick were Co-PIs for a Women & Infants Hospital of Rhode Island project fully funded through a grant from Sequenom, Inc., San Diego, CA between October 2008 and February 2012. Kloza, Lambert-Messerlian, and Haddow were also part of this funded project. Palomaki and Canick have received funding to create a study design from Natera, Inc., San Carlos, CA beginning in December 2012. Kloza was a member of the Natera Clinical Advisory Board between September 2013 and January 2013.

INTRODUCTION

Circulating cell-free DNA (ccfDNA) in the plasma of pregnant women is a mixture of genomic DNA fragments of maternal and fetal (placental) origin.[1] When in the right proportion and amount, that mixture allows for the identification of fetal aneuploidy by using next generation sequencing methods that involve counting or genotyping the ccfDNA fragments. Lo and his colleagues first estimated that the fetal contribution to ccfDNA was on the order of 3% to 6%.[2] That original estimate of the fetal fraction (the percentage of ccfDNA of fetal origin) was made by calculating Y-specific contributions to the total circulating ccfDNA when a male fetus was present. Since Lo's initial estimate, the proportion of fetal ccfDNA in maternal plasma has been found to be, on average, considerably higher. More recent estimates are based on the use of epigenetic and/or genotype differences between maternal and fetal DNA fragments and estimates the average fetal fraction at 10% to 15% when sampling is carried out between 10 and 20 gestational weeks.[3–5] In fact, fetal fraction values range from below 4% to 30% or higher, with only about 1% to 3% of samples having a fetal fraction of less than 4%.[3,4] Most likely, the initial estimates of fetal fraction were lower than present day estimates because the measurement of Y-specific sites to quantify fetal contribution tend to be underestimated.[6]

The use of next generation sequencing methods to identify trisomy depends on the ability to differentiate a 50% increase in the amount of the fetal chromosome of interest (e.g., chromosome 21 for the identification of Down syndrome) in the face of a large proportion of euploid maternal ccfDNA. The proportional increase caused by the trisomy is, in fact, equal to half the fetal fraction. In testing for Down syndrome, an average fetal fraction of 10% results in an expected 5% increase in the percentage of chromosome 21 over than found in a euploid population. Next generation sequencing provides the power to statistically differentiate the modest 5% increase over expected.

In this report, we will assess the impact of fetal fraction on the performance of DNA-based maternal plasma testing for

aneuploidies, focusing for the most part on Down syndrome (trisomy 21). First, we will examine the extent of overlap and shape of the distributions of screening results for Down syndrome and euploid pregnancies in an attempt to better define screening performance and provide an avenue for laboratories to quantify the accuracy of their clinical interpretations. Second, we will describe the effect that maternal weight has on screening performance and its consequences by separately examining the maternal weight effect on the fetal and maternal components of the fetal fraction. Finally, we will contribute to the data obtained thus far on cases of trisomy mosaicism and discuss the prospects of identifying such cases. For our first and second objectives, we will extensively assess data generated during a large independent study on high risk pregnancies that we conducted.[3,7] For our examination of mosaicism, we will present, for the first time, cases found in that study, compare them with the small number of cases that have been previously reported and define an 'effective' fetal fraction and its potential impact on the detection of cases of trisomy mosaicism.

METHODS

Between November 2008 and February 2012, we conducted an independent, multicenter observational study (the InFANet Study) on the performance of a laboratory-developed test for aneuploidy by maternal plasma ccfDNA analysis.[3,6] The study was funded by Sequenom, Inc. (San Diego, CA), which also provided the testing and interpretation of blinded samples. All 4664 patients enrolled in the study were considered at increased risk of an autosomal trisomy, and all had an invasive diagnostic procedure, either by chorionic villus sampling (CVS) or amniocentesis with karyotype analysis. Thus, the DNA result could be compared with the gold standard diagnostic test result. For the nested case/control results reported here, data from the Infanet study have been further analyzed to more fully determine the role of fetal fraction in the performance of next-generation sequencing tests for chromosomal disorders. In addition, results are reported for the first time from five patients whose fetuses were confirmed by invasive testing as having mosaicism for an autosomal trisomy (four for chromosome 21 and one for trisomy 13). These samples, as well as all others, were tested over a 9-week time period without knowledge of the karyotype. Details of sample collection, storage, and shipment, as well as the sequencing and bioinformatics methods used have been provided previously.[3,6]

RESULTS AND DISCUSSION

Relationship between fetal fraction and test performance

The role of fetal fraction in correctly identifying an aneuploid or euploid fetus is dependent on the analytic method employed (e.g., counting or genotyping). When a counting algorithm is used, an increasing fetal fraction will be associated with an increasing detection rate (or sensitivity). However, that increase is unlikely to have much impact on the false positive rate (1-specificity). When genotyping is used, an increasing fetal fraction will still be associated with an increased detection rate but may also be associated with a decrease in the false positive rate.

This difference is related to the type of data generated by counting compared with genotyping. In DNA fragment alignment and counting, the maternal and fetal fragments are considered equivalent, so a euploid fetus and euploid mother are seen as making identical contributions to the chromosome counts. When the fetus is trisomic, counts for the chromosome of interest are impacted by the fetal fraction, with the higher fetal fractions generating ever higher counts. In DNA genotyping, the test is targeting highly polymorphic single nucleotide polymorphisms (SNPs) on the chromosomes of interest. Higher fetal fractions will result in more clearly defined genotypic patterns, regardless of whether the fetus is trisomic or euploid.

The results of recent studies in which counting methods were used provide clear evidence of the influence of fetal fraction on test performance.[3,4,6] In our studies,[3,6] as fetal fraction increased, the z-scores in euploid pregnancies remained constant, whereas the z-scores in Down syndrome, trisomy 18, and trisomy 13 pregnancies increased sharply (shown for Down syndrome in Figure 1). For the 212 Down syndrome cases, four (1.9%) had z-scores below the cutoff of +3, and all four occurred at low fetal fractions, between 4% and 7%. In the study of Sparks *et al.*[4], in which 36 Down syndrome, 8 trisomy 18, and 126 euploid pregnancies were examined and the same strong positive association between fetal fraction and z-score was seen. Plasma samples with less than a 5% fetal fraction were not tested. In this smaller study, z-scores for the three trisomies (two Down syndrome and one trisomy 18) with fetal fraction below 7% were elevated (6 or higher) but still considerably lower than the scores of those trisomy cases with higher fetal fractions.

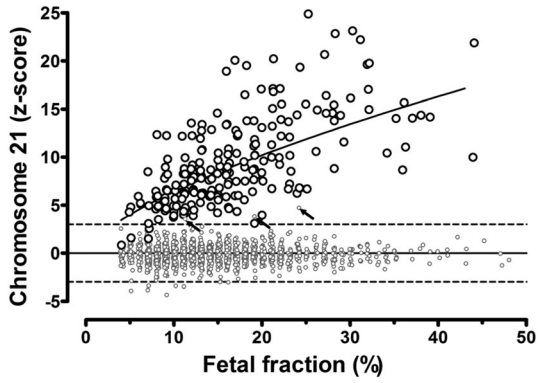

Figure 1 Relationship between fetal fraction and chromosome 21 z-score in euploid and Down syndrome pregnancies. The large open circles indicate chromosome z-scores in first and second trimester Down syndrome pregnancies. As fetal fraction increases, the average z-score also increases. The line indicates the change in the average z-score by fetal fraction. All but four of the 212 cases are above a z-score of 3, and these four occur at fetal fractions of 7% or less. Small open circles indicate chromosome 21 z-scores in 1484 euploid pregnancies. The z-scores are centered on zero and generally fall between −3 and +3. Only three euploid pregnancies fall above a z-score of 3 (arrows), and these are not associated with lower fetal fractions. This figure is similar to one previously published in reference 3

The quality of a screening test can be illustrated by examining the distribution of test results for those who are affected and those who are unaffected by a particular disorder. In prenatal screening for Down syndrome, the distribution of results for most markers (e.g., alpha-fetoprotein or NT measurements) follow a Gaussian, or bell-shaped distribution after appropriate transformation. In the example shown in Figure 2, the data are derived from the same case control sample set as shown in Figure 1. However, rather than expressing the results in z-score, the data are presented as a raw assay value, percent chromosome 21 adjusted for guanine–cytosine content and repeat masked before alignment. The percent chromosome 21 in euploid pregnancies fitted a Gaussian distribution well and was centered at 1.36%. However, the percent chromosome 21

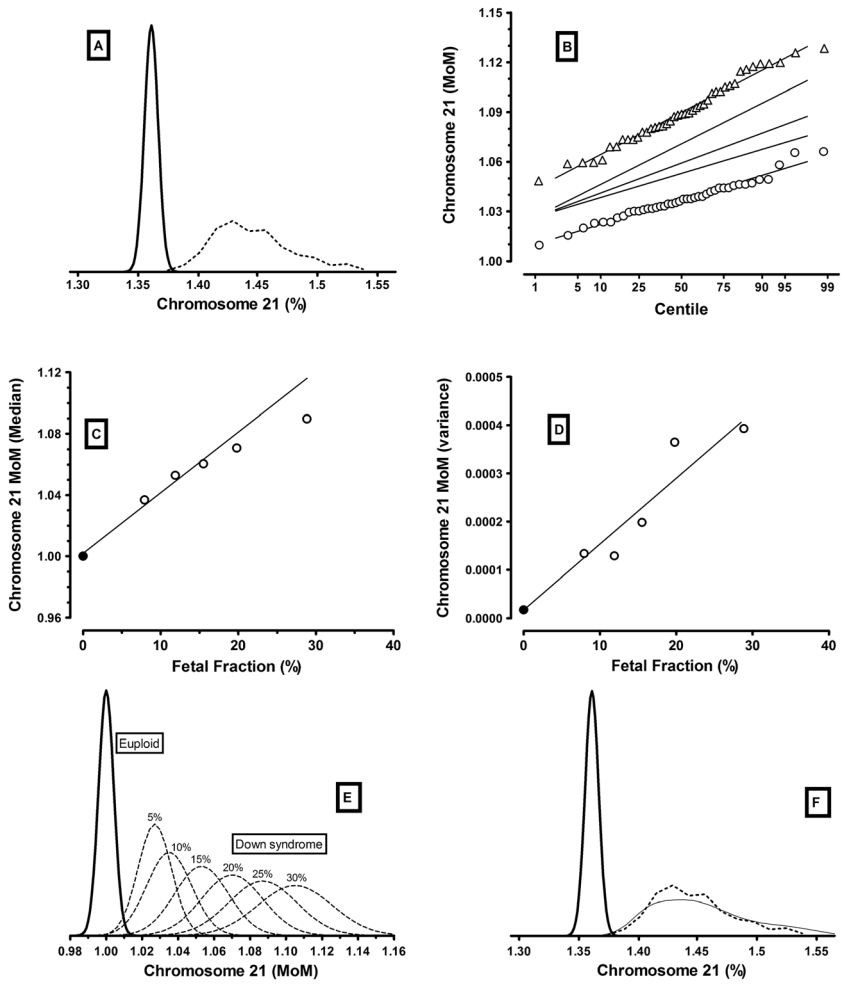

Figure 2 Modeling the relation between fetal fraction and z-score elevations in 212 Down syndrome and 1484 Euploid pregnancies. Figure 1A shows the overlapping distributions of chromosome 21% (proportion of all reads mapped to chromosome 21 divided by the sum of all reads mapped to all autosomes) in both euploid (solid curve) and Down syndrome (dashed curve). The chromosome 21% results in euploid pregnancies appear as a very tight (and thus tall) linear Gaussian distribution. In contrast, the chromosome 21% results in Down syndrome pregnancies are clearly much higher and show little overlap, confirming the high detection rate and low false positive rate. The distribution is not Gaussian and cannot be easily transformed into one. The 212 cases were then separated into quintiles on the basis of fetal fraction, and the chromosome 21% results converted to multiple of the median chromosome 21% result in all euploid pregnancies (MoM) tested within each flow cell. Figure 2B shows the chromosome 21% results, in MoM, for each of those quintiles of fetal fraction. All observations are shown for the first (open circles) and fifth centiles (open triangles), with regression lines drawn for all five quintiles. Each fitted a linear Gaussian distribution well. The slope of those lines represents the standard deviation, and the median is defined by the value found directly above the 50th centile on the horizontal axis. Figures 2C and 2D show the regression analysis for the average fetal fraction for the five quintiles (horizontal axis) versus the median chromosome 21% MoM (Figure 2C) and its variance (Figure 2D). The filled circles indicate the model constraint, that at a fetal fraction of 0%, the distribution should be equivalent to that found for euploid pregnancies. In Figure 2C, weighted regression ensured that the model fit best at low fetal fractions. These regression lines allow theoretical distributions to be drawn for chromosome 21% MoM results associated with any given fetal fraction (Figure 2E). The most overlap occurs at lower fetal fractions (e.g., 5%), whereas for those fetal fractions above 10%, few if any results would be expected to be false negatives. In the small region of overlap found for the lower fetal fractions, it would be possible to generate likelihood ratios for clinical use. The data from Figures 2C and 2D allow for the construction of a composite distribution consisting of the sum of five modeled distributions based on the average fetal fraction for each of the quintiles. This generates a theoretical distribution (thin curve) that fits the observed distribution (dashed line) in Down syndrome pregnancies (Figure 2F). Figure 2A was previously published in reference 7

distribution in the Down syndrome pregnancies was much more broad, did not appear Gaussian (Figure 2A),[8] and was not normalized by a logarithmic or other transformation to be Gaussian.

We found that the main contributor to the irregular shape of the Down syndrome distribution was the wide range of fetal fractions seen in pregnant women. When restricted to narrow ranges of fetal fractions (each of which can be thought of as approaching a single fetal fraction), the percent chromosome 21 results fitted a Gaussian distribution well for each of the quintiles of fetal fraction (Figure 2B). Furthermore, with each successively higher quintile of fetal fraction range examined, the log Gaussian distributions had higher median percent chromosome 21 and increased spread (higher logarithmic standard deviation). These increases were fitted against the average fetal fraction within each quintile to result in a linear model for these parameters, shown for both the median multiple of median (MoM) (Figure 2C) and for the standard deviation of the log MoM (Figure 2D). Both models included the constraint that at a fetal fraction of 0%, the modeled distribution of cases should be equal to that in euploid pregnancies. These parameters can be used to determine the distribution of percent chromosome 21 MoM for any given fetal fraction. Figure 2E shows selected distributions for fetal fractions ranging from 5% to 30% in 5% increments. When the modeled distributions representing the quintiles of fetal fraction observed in our study are summed and converted back to percent chromosome 21 results, the modeled results fit the original observed distribution well (Figure 2F).

The practical aspects of these findings are twofold. First, DNA-based screening is amenable to Gaussian distribution analysis to determine patient-specific risk as is now standard for the serum and ultrasound markers. However, the range over which such risks can be produced will be relatively small. Most results fall well below (consistent with a large reduction in risk) or well above (consistent with a large increase in risk) the small area of overlap (Figure 2F). In these outlying regions, modeling will not provide reliable likelihood ratios, and suitable truncation limits need to be defined. Second, the full quantitative value of the test results can be exploited in reporting results falling in the area of overlap. Test results falling in the overlap could, perhaps, be reported as patient-specific risks. Additional studies will be needed to learn how best to deal with these findings and whether it is possible or useful to include risks in final patient reports. An important caveat to this modeling is the rapidly changing nature of the test itself. Large datasets are needed to produce models such as we have described here, and as improvements to the chemistry, sequencing and bioinformatics occur, these parameters are also likely to change.

The impact of maternal weight on fetal fraction

An inverse relationship between maternal weight and levels of fetal ccfDNA in the maternal circulation was first reported in 2004 for second trimester maternal serum samples by using a Y-directed probe.[9] A possible association between the levels of total or maternal ccfDNA and maternal weight was not examined. The inverse relationship between maternal weight and fetal fraction, requiring measurement of both fetal and total ccfDNA, was reported by us in 2011[3] and was subsequently confirmed by others.[10,11] We initially attributed the maternal weight effect on fetal fraction to a fixed amount of fetal/placental DNA product being diluted by increasing maternal circulatory volume. Such an effect is routinely seen for other fetal/placental products used as maternal serum screening markers for Down syndrome.[12]

Recently, a report from Vora and colleagues[13] showed that total cell-free DNA in maternal plasma at term is directly proportional to the increase in maternal body mass index (BMI). They speculated that this is due to increased death of adipose cells in obese women that results in a higher contribution of maternal cell-free DNA to the circulation. Another possibility is the higher number of circulating white blood cells in a larger vascular system might cause the cell-free maternal DNA to be increased. These effects could act alone or in concert. A separate study in 2009 examined cell-free fetal and maternal plasma DNA in relation to maternal BMI for male fetuses and found a direct association for total ccfDNA but no association for fetal ccfDNA.[14]

We had already examined the association of fetal fraction and maternal weight in our dataset.[3] Fetal fractions were computed on the basis of differences in methylation between fetal and maternal cell-free DNA.[15] The analyses focused on 1482 singleton euploid pregnancies in which the fetal fractions and maternal weights were available.[3] We had previously shown that the expected median fetal fractions for women weighing 45, 68, 91, 113, and 136 kg were 17.8%, 13.2%, 9.8%, 7.3%, and 5.4%, respectively.[3]

Figure 3 shows the overall effect of lower fetal fractions with increasing weight (fitted solid curve, right axis) but extends the

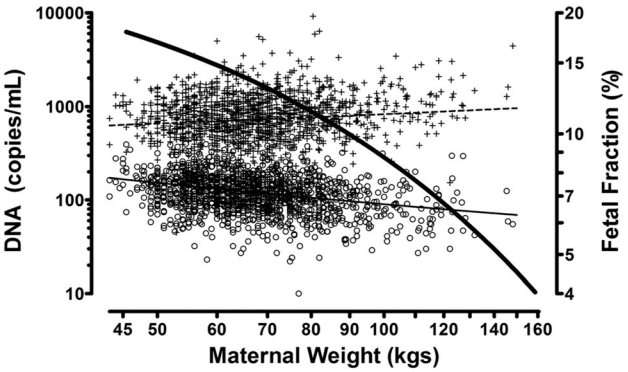

Figure 3 The relationship between maternal weight, fetal fraction, and levels of circulating cell-free (ccf) maternal and fetal DNA. The horizontal axis shows the maternal weight (in kilograms). Open circles show the fetal/placental ccfDNA levels for 1482 women, whereas the plus signs indicate the calculated maternal ccfDNA levels. Both are plotted against the left vertical axis showing DNA levels in copies per mL. Regression analysis shows the best fit to the cell-free fetal (solid lines) and maternal (dashed) DNA levels and shows that the maternal weight effect is stronger for the fetal ccfDNA. The thick black line is associated with the right vertical axis and shows how the average fetal fraction declines from a high of about 18% at 45 kg to a low of about 4% at 150 kg

findings by also showing the maternal and fetal ccfDNA levels that were used to derive the fetal fractions (left axis). Both are associated with maternal weight; the fetal contribution (open circles) decreases, while the maternal contribution (plus signs) increases with increasing weight. Significant predictors for fetal DNA levels were maternal weight (in kilograms), gestational age (in decimal weeks), and vaginal bleeding (1 = yes, 0 = no). Assuming the effect is due to dilution, a linear model of fetal copies versus the reciprocal of maternal weight was used.[12] Other potential covariates were included in the model based on stepwise linear regression. The final fitted model was fetal DNA copies/mL = $6283 \times 1/\text{weight} + 3.6 \times \text{ga\_weeks} + 12.8 \times \text{bleed} - 16.0$. The thin solid line in Figure 3 shows this fitted model at 12 weeks gestation. The reduction in fetal ccfDNA between a 45 kg and 110 kg woman is 49%.

Because dilution is unlikely to explain the effect on maternal ccfDNA, we chose a linear model that includes a second order of weight (to allow for the effect to be strongest in obese women). The final stepwise fitted model was log maternal DNA = $0.0905 \times \log(\text{weight})^2 + 0.00623 \times \text{ga\_weeks} + 2.482$. The thin dashed line in Figure 3 shows this fitted model at 12 weeks gestation. The reduction in maternal ccfDNA between a 110 kg and 45 kg woman is 26%.

In a recent publication by Ashoor and colleagues,[11] the maternal weight effect was modeled to predict when their method of testing would be considered unreliable because their fetal fractions were too low. Fetal fractions (less than 4%) were reportedly too low for <1%, 3%, and 5% of women weighing an average of 55, 73, and 102 kg, respectively. We stratified our dataset into six groups and compared the proportion of women with fetal fractions below that same cutoff level of 4.0% and at 3.5% with the predicted proportion[11] at each interval's average weight (Table 1). The cutoff of 3.5% was included because in our study fetal fractions were rounded to the nearest whole percent when results were reported. Median fetal fraction fell from 15.8% for women under 60 kg to 6.4% for women weighing 110 kg or more. At a cutoff of 4.0% (i.e., values of 3.99% or less were considered to be too low), our observed rate of low fetal fractions matched the modeled expectations.[11] At the cutoff of 3.5%, the rates were lower. Of particular clinical interest, two of the four women weighing over 130 kg had fetal fractions below 3.5%.

Our findings confirm that the inverse association between maternal weight (or BMI) and fetal fraction is likely the result of two simultaneous effects. The first is the dilution of a relatively constant amount of fetal ccfDNA into a larger maternal plasma volume. The second is an increase in the concentration of maternally derived ccfDNA as maternal weight increases. The dilution effect on fetal ccfDNA appears to be about twice as strong as the increase of maternal ccfDNA caused by weight gain. Whether the mechanism of the maternal ccfDNA increase is, in fact, an increased breakdown of adipose cells in obese women is intriguing and needs to be examined further.

The impact of fetal fraction on detection of mosaic trisomies

Identification of mosaicism for fetal autosomal trisomies using ccfDNA-based methods will be less effective than for identification of complete trisomies because the contribution from the fetal chromosome is partial. The effective (or functional) fetal fraction for a mosaic trisomy is in reality a proportion of the fetal fraction that is measured. For example, if the mosaicism for trisomy is 50% (i.e., half the placental cells are trisomic and half are euploid), and the measured fetal fraction for the maternal plasma sample is 10%, the effective fetal fraction for the mosaic trisomy will be half of 10% or 5%, making it more difficult for the test to detect the extra chromosomal material. Finally, if the maternal plasma DNA result is positive for a trisomy using a counting methodology, the finding of a mosaic or complete trisomy cannot be made until invasive diagnosis with karyotype analysis is carried out.

Predicting phenotype based on the percentage of an abnormal cell line is uncertain, and it is unclear how often fetal mosaicism for trisomy 21 would be identified by maternal plasma DNA testing and under what circumstances. A recent study included three karyotypes with relatively high levels of mosaicism; two for trisomy 21 (29%, 44% mosaicism) and one for trisomy 18 (89% mosaicism);[16] all were detected. A fourth case of trisomy 21 was mosaic for a marker chromosome, and the trisomy 21

Table 1 **Median fetal fraction and proportion considered too low for testing according to two definitions stratified by maternal weight**

Maternal Weight (kg)			Fetal Fraction[a]			
Range	Median	Number	Median (%)	<3.5% (N, %)[b]	<4.0% (N, %)[c]	Expected (%)[d]
<60	55.0	417	15.8	1 (0.2)	1 (0.3)	<1
60–69	64.5	471	14.2	2 (0.4)	3 (0.6)	1
70–79	74.0	320	12.8	4 (1.3)	6 (1.9)	2
80–89	83.6	145	10.5	4 (2.8)	6 (4.1)	3
90–109	95.5	91	9.2	1 (1.1)	3 (3.3)	10
≥110	117.7	38	6.4	4 (10.5)	9 (23.7)	20
Any	145	1482[e]	13.3	16 (1.1)	28 (1.9)	NR

[a]The value obtained on the first tested sample
[b]Values of 3.51% and higher are rounded to 4% and thus included for testing.
[c]Values of 3.99% and lower are considered low and thus not included for testing.
[d]Derived from Figure 2 in Ashoor G *et al.*, ISUOG, 2012.
[e]Among the 1484 euploid pregnancies tested, two did not have maternal weight available.

was identified. No information about fetal fraction was presented, and no low level mosaicisms were reported.

Among the pregnancies that we have examined, five mosaic karyotypes have been identified; three for chromosome 21, one for 18, and one for 13 (Table 2). Three maternal plasma samples were obtained prior to first trimester CVS and two prior to second trimester amniocentesis. The median fetal fraction from the five mosaic trisomies was 13% (range 6–23%). Because the fetal contribution to ccfDNA is from the placenta, the heterogeneity of the mosaicism within the placenta is another factor that would impact the effective fetal fraction; this could not be quantified at the time of testing. For this reason, the effective fetal fractions can only be considered crude estimates. The five cases are presented in Table 1 and described as follows:

Case 1 presented with an abnormal ultrasound finding, and the karyotype revealed 10% mosaicism for trisomy 13. The patient terminated the pregnancy on the basis of karyotype and ultrasound findings. The fetal fraction was 6% with an estimated effective fetal fraction of 0.6% (6% fetal fraction × 10% mosaicism), well below the 4% threshold needed for reliable DNA testing. Based on this, it appears reasonable that the test results were interpreted as euploid.

Case 2 presented for advanced maternal age, and the karyotype showed 10% mosaicism for trisomy 21. The patient terminated the pregnancy; no further diagnostic information was available. The fetal fraction of 17% was average, but the effective fetal fraction of 1.7% (17% × 10%) was well below the threshold of 4%. The results were interpreted as euploid, which is again reasonable based on the effective fetal fraction.

Case 3 presented for advanced maternal age, and the karyotype after CVS showed 50% mosaicism for trisomy 21. Subsequent amniocentesis showed a normal 46,XY karyotype, and an unaffected male was delivered. With a measured fetal fraction of 9%, the sample was in fact near the lower limit of acceptability (effective fetal fraction = 9% × 50% = 4.5%). The z-scores were all below 0, and the sample was interpreted as euploid. Although the DNA interpretation was consistent with the normal karyotype at amniocentesis, it is not clear why the chromosome 21 z-score was so low. The plasma sample reflects the level of mosaicism across a wider area of the placenta. Possibly, the material obtained at CVS was not representative of the placenta as a whole, which would result in a poor estimate of the effective fetal fraction.

Case 4 presented after a positive integrated test result, and the karyotype indicated 40% mosaicism for trisomy 21. The patient terminated the pregnancy; no further diagnostic information was available. The fetal fraction was 13%, and the effective fetal fraction was 5.2% (13% × 40%). The chromosome 21 z-score was 14.96 and was reported as being consistent with Down syndrome. This z-score is higher than expected for the effective fetal fraction but consistent with the expectation that it might be detectable.

Case 5 presented for advanced maternal age, and the karyotype revealed trisomy 21 in all colonies. Superimposed on this was the mosaicism for trisomy 18 in 45% of cells (11 of 20). All cells were male, and all contained a distinctive normal variant of chromosome 9, providing evidence that all cells were derived from a single fetus. The pregnancy was terminated. The fetal fraction was 23%, resulting in an effective fetal fraction of 10.3%. The chromosome 21 z-score (+12.71) was clearly elevated, but the chromosome 18 and 13 z-scores were normal. The DNA interpretation was trisomy 21. It is unclear why the mosaicism for trisomy18 was not also identified by DNA testing.

A key issue in this discussion is that 'fetal' ccfDNA fragments are really of placental cytotrophoblast origin.[16,17] One study reported on maternal plasma from a pregnancy whose placental mesenchymal core was 46,XX but whose cytotrophoblast layer was 45,X. Plasma DNA as well as karyotyping following amniocentesis revealed a single X chromosome (no Y signal), suggesting that the cytotrophoblast layer is the primary contributor of fetal DNA.[17] In another study, a pregnancy without a fetus was found to contain usual levels of 'fetal' ccfDNA in the maternal plasma, indicating that in fact the fetal DNA fragments originated in the cytotrophoblast.[17] Both results suggest that plasma ccfDNA analysis may be more consistent with results from direct analysis of CVS material than from long-term culture results.

Three of five mosaic karyotypes were in the first trimester, consistent with the higher frequency of mosaicism observed in CVS samples often attributed to confined placental

Table 2 **Demographic and pregnancy-related information for five samples in which a mosaicism for chromosome 21, 18, or 13 was reported**

Case	Age (yrs)	GA (wks)	Procedure	Karyotype	Mosaicism (cells)	Fetal Fraction (Effective FF)[b]	z-score[a]			Test interpretation
							C21	C18	C13	
1	34	14	Amnio	46,XX/47,XX,+13	10% (NR)	6% (0.6%)	−2.22	−1.95	0.08	Euploid
2	44	11	CVS	46,XY/47,XY,+21	10% (2/20)	17% (1.7%)	2.03	0.21	−0.96	Euploid
3	41	12	CVS	46,XY/47,XY,+21	50% (NR)	9% (4.5%)	−1.25	−1.12	−0.51	Euploid
4	38	20	Amnio	46,XY/47,XY,+21	40% (6/15)	13% (5.2%)	14.96	0.44	−0.72	DS
5	39	12	CVS	47,XY,+21 48,XY+thinsp;18,+21	45% (9/20)	23% (10%)	12.71	−0.22	−0.40	DS

GA, gestational age; C21, chromosome 21; NR, not reported; Amnio, amniocentesis; CVS, chorionic villus sampling; DS, Down syndrome.

[a]All results were corrected for GC content; chromosome 21 results were first aligned to the repeat masked genome

[b]Effective fetal fraction = measured fetal fraction times proportion of cells showing the mosaicism

mosaicism. Based on the effective fetal fraction, an interpretation of trisomy would not have been expected in two samples (cases 1 and 2), and these may also represent pseudomosaicism. Two cases (3 and 4) had effective fetal fractions near the 4% threshold,[18] and one of the two was correctly interpreted (case 4). Below this threshold, the test is less reliable.[3] Case 3 was found to have a euploid karyotype following a subsequent amniocentesis, raising questions about the pattern of mosaicism throughout the placenta. The last case (5) was a mosaic trisomy 18 superimposed on a trisomy 21 karyotype. It should have had elevated z-scores for both chromosomes, but only chromosome 21 was positive.

Notwithstanding the small number of mosaic cases reported here and elsewhere, it seems reasonable that a fetus mosaic for trisomy 21, 18, or 13 is more likely to be detected when both the percent mosaicism and the fetal fraction of the maternal plasma sample are high. While it is not possible to prospectively identify the effective fetal fraction when a mosaic trisomy is present, it is logical that mosaicism will attenuate the performance of maternal plasma DNA analysis. In the future, deeper sequencing might enable the identification of lower level mosaics, and studying performance using artificially generated mosaics might prove useful.

CONCLUSIONS

It is clear that the level of the fetal fraction will have an impact on the performance of maternal plasma ccfDNA methods. Many of the current clinical tests have a formal fetal fraction cutoff below which the results are considered unreliable and not reported. Because all current tests are laboratory-developed, it would be best to refer to each laboratory's current rules for no calls. In recent publications, the proportion of uninformative results due to low fetal fraction is 3% or less.[3,15] The most common factor associated with the few observed false negative results is low fetal fraction. Clinical laboratories should consider providing fetal fractions and other relevant results on the report in addition to the interpretation. Additionally, methods shown here and elsewhere in the literature[4,5] have the ability to quantify the confidence in the call or quantify the confidence in terms of a patient-specific risk estimate.

A more problematic issue is the lower screening performance in heavier women. Should women at or above a selected weight be informed of their increased chance of having a test failure due to low fetal fraction, potentially delaying diagnosis? Should they be informed that the reported result is, on average, less confident than results reported in lighter weight women? Lastly, all ccfDNA tests described here determine the number of chromosomes in the placenta, not in the fetus. Many of the studies to date have removed mosaic karyotypes from consideration, and none have performed studies to determine whether the mosaicism is confined to the placenta or is representative of the fetus.

The physical characteristics of the ccfDNA fragments from the mother and fetus are very similar. Because of this, there are no methods currently available that can effectively concentrate the fetal portion of the ccfDNA and would increase the fetal fraction. The prospects of overcoming these limitations are low.

We have described clinically relevant associations of fetal fraction to non-invasive prenatal testing for common autosomal trisomies. By being aware of and utilizing this information, test strengths and limitations can be more readily understood, test reporting could be enhanced further, and clinical correlates such as maternal weight acknowledged as part of routine clinical practice.

ACKNOWLEDGEMENTS

We acknowledge and thank our colleagues from Sequenom, Inc., in particular, Allan Bombard, Cosmin Deciu, Mathias Ehrich, and Dirk van den Boom, and the 27 enrollment sites, for their participation in the InFANet Study. The InFANet Study was funded by Sequenom, Inc.

WHAT'S ALREADY KNOWN ABOUT THIS TOPIC?

- The fetal fraction is a well described measurement of the proportion of circulating cell-free DNA in maternal plasma derived from the fetal/placental unit.
- Fetal fraction is related to several characteristics of testing for common autosomal trisomies including test failure rate and detection rate.

WHAT DOES THIS STUDY ADD?

- An existing dataset was used to create a model that fits the observed distributions of euploid and Down syndrome z-scores by stratifying results by fetal fraction and further exploring the association between increasing maternal weight and lower fetal fractions; that effect is not due solely to dilution.
- A modified estimate of 'effective' fetal fraction may be helpful in understanding test performance when mosaicism is present.

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. 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.
3. 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.
4. 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 e311–9.
5. 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.
6. Lun FM, Chiu RW, Allen Chan KC, *et al.* Microfluidics digital PCR reveals a higher than expected fraction of fetal DNA in maternal plasma. Clin Chem 2008;54:1664–72.
7. Palomaki GE, Deciu C, Kloza EM, *et al.* DNA sequencing of maternal plasma reliably identifies trisomy 18 and trisomy 13 as well as Down syndrome: An international collaborative study. Genet Med 2012;14:296–305.
8. Canick JA, Palomaki GE. Maternal plasma DNA: A major step forward in prenatal testing. J Med Screen 2012;19:57–9.

9. Wataganara T, Peter I, Messerlian GM, *et al.* Inverse correlation between maternal weight and second trimester circulating cell-free fetal DNA levels. Obstet Gynecol 2004;104:545–50.
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. Neveux LM, Palomaki GE, Larrivee DA, *et al.* Refinements in managing maternal weight adjustment for interpreting prenatal screening results. Prenat Diagn 1996;16:1115–9.
13. 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.
14. Lapaire O, Volgmann T, Grill S, *et al.* Significant correlation between maternal body mass index at delivery and in the second trimester, and second trimester circulating total cell-free DNA levels. Reprod Sci 2009;16:274–9.
15. 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.
16. Bianchi DW, Platt LD, Goldberg JD, *et al.* Genome-wide fetal aneuploidy detection by maternal plasma DNA sequencing. Obstet Gynecol 2012;119:890–901.
17. Faas BH, de Ligt J, Janssen I, *et al.* Non-invasive prenatal diagnosis of fetal aneuploidies using massively parallel sequencing-by-ligation and evidence that cell-free fetal DNA in the maternal plasma originates from cytotrophoblastic cells. Expert Opin Biol Ther 2012;12:S19–26.
18. Ehrich M, Deciu C, Zwiefelhofer T, *et al.* Noninvasive detection of fetal trisomy 21 by sequencing of DNA in maternal blood: A study in a clinical setting. Am J Obstet Gynecol 2011; 204:205 e201–11.