

Non-invasive prenatal testing for fetal aneuploidies in the first trimester of pregnancy

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KEYWORDS: early gestation; aneuploidy; fetal DNA fraction; first trimester; maternal weight; non-invasive prenatal testing

ABSTRACT

Objectives To evaluate the feasibility of non-invasive prenatal testing (NIPT) of maternal plasma samples collected from pregnant Chinese women in early gestation, between 8 + 0 and 12 + 6 weeks' gestation.

Methods In this pilot study, 212 women with high-risk pregnancies were recruited at a single Chinese Hospital. Fetal aneuploidies associated with chromosomes 21, 18, 13, X and Y were detected by massively parallel sequencing of maternal plasma DNA samples. Invasive prenatal diagnosis by either chorionic villus sampling or amniocentesis and then karyotyping was offered to all women to confirm both positive and negative NIPT results. Fetal DNA fraction was also determined in male pregnancies, by the relative percentage of Y-chromosome sequences. All confirmed NIPT-negative pregnancies were followed up to birth and neonates were clinically evaluated for any symptoms of chromosomal disease.

Results Autosomal aneuploidies trisomy 21 (n = 2), 18 (n = 1) and 13 (n = 1) were detected by NIPT and confirmed by amniocentesis and karyotyping. There were one false-positive 45,X sample and two false-negative samples associated with fetal karyotypes 47,XXY and 45,X[16]/47,XXX[14]. In the 100 male pregnancies, the median fetal DNA fraction was 8.54% and there was a trend towards an increasing fetal fraction from 8 + 0 to 12 + 6 weeks' gestation. The majority (95%) of pregnancies had a fetal DNA fraction > 4%, which is generally the limit for accurate aneuploidy detection by NIPT. Across this early gestational time period, there was a weak inverse relationship ($R^2 = 0.186$) between fetal DNA fraction and maternal weight.

Conclusions NIPT is highly reliable and accurate when applied to maternal DNA samples collected from pregnant women in the first trimester between 8 + 0 and 12 + 6 weeks. Copyright © 2014 ISUOG. Published by John Wiley & Sons Ltd.

INTRODUCTION

The practice of non-invasive prenatal testing (NIPT) using massively parallel sequencing of cell-free DNA circulating in the maternal bloodstream is becoming accepted in many countries as part of routine care for pregnant women^{1,2}. Based on the outcomes of prospective clinical studies^{3–5}, NIPT has been found to be highly reliable and accurate, with sensitivity and specificity rates exceeding 99% for detection of the three common trisomies involving chromosomes 21, 18 and 13. In the same studies, however, the false-positive and false-negative rates for sex chromosomal aneuploidies (SCAs) were significantly higher. Recent studies have identified fetoplacental mosaicism^{6–10} and maternal chromosomal abnormalities¹¹ as common causes of discordant NIPT results, while a vanishing twin, low DNA fraction or technical errors are believed to be contributing factors in some cases¹.

Currently, maternal blood sampling for NIPT is normally performed from as early as 10 weeks' gestation through to the end of the second trimester of pregnancy¹. During this gestational window, the placenta secretes significant levels of fetal DNA into the maternal circulation, with the cell-free fetal DNA fraction levelling at 10–20% between 10 and 21 weeks' gestation^{7,12,13}. In general, there is a wide range in fetal DNA fraction in the maternal plasma at each gestational age^{7,14}, indicating significant biological differences between pregnancies. Many factors have been identified that modulate the fetal DNA fraction during pregnancy, with gestational age, maternal weight and number of previous gestations being the most significant^{1,12,15}.

In a previously reported prospective study³, we demonstrated high sensitivity and specificity of NIPT for detection of fetal aneuploidies in maternal plasma samples that were collected mainly in the second trimester of pregnancy. To expand the clinical utility of NIPT, it is important to determine whether the same degree of reliability and accuracy can also be achieved earlier in gestation.

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In this pilot study of 212 pregnant women with increased risk of fetal aneuploidy, we examined the performance of NIPT in the first trimester of pregnancy, within the gestational period of 8 + 0 to 12 + 6 weeks.

METHODS

Study design

In total, 213 pregnant women presenting for NIPT between May 2012 and August 2013 were selected arbitrarily at Peking Union Medical College Hospital (PUMCH), Beijing, using the following inclusion criteria: advanced maternal age (≥ 35 years) and singleton pregnancy (Figure 1). The final study design was approved by the Hospital's Institutional Review Board and all participating patients provided informed written consent to participate. Gestational age was determined from the first day of the last menstrual period and was confirmed by ultrasound at 7–8 weeks. Detailed clinical information for each patient and sample was recorded in the study's database. Confirmatory prenatal diagnosis was performed by either chorionic villus sampling (CVS) or amniocentesis, with karyotyping. For each patient, NIPT and invasive prenatal diagnosis were conducted in a double-blind manner, at Berry Genomics, Beijing and at the PUMCH Prenatal Diagnosis Center, respectively. All pregnancies with negative prenatal test results confirmed by karyotyping were followed to birth and neonates were assessed clinically at PUMCH by the patient's pediatrician for the phenotypic expression of chromosomal diseases.

Non-invasive prenatal testing (NIPT)

For NIPT, peripheral blood samples were collected in Streck tubes between 8 + 0 and 12 + 6 weeks' gestation. Only a single blood draw was permitted. Preparation of maternal DNA, library construction and barcoding, sequencing on the Illumina HiSeq 2000 platform (Illumina Inc., San Diego, CA, USA) and data analysis were performed according to previously published methods^{3,16}. For each NIPT sample, approximately 5 million 36-bp reads were generated, of which approximately 3.5 million were uniquely mapped to the hg19 reference genome. The fetal aneuploidy status for all 24 chromosomes was determined by Z-scores (normal range, $-3 < Z < 3$). For all NIPT-positive samples, copy number variation sequencing of maternal white blood cell (WBC) DNA samples¹¹ was used to exclude any false positives resulting from maternal mosaicism. We also measured the fetal DNA fraction in the male pregnancies; in our assay, fetal DNA fraction was determined based on a standard formula which defines the percentage of Y-chromosome reads in each male plasma sample relative to that of Y-chromosome reads in the maternal plasma of euploid female pregnancies¹⁴. For aneuploid samples, the fetal DNA fraction was calculated as the difference of genome

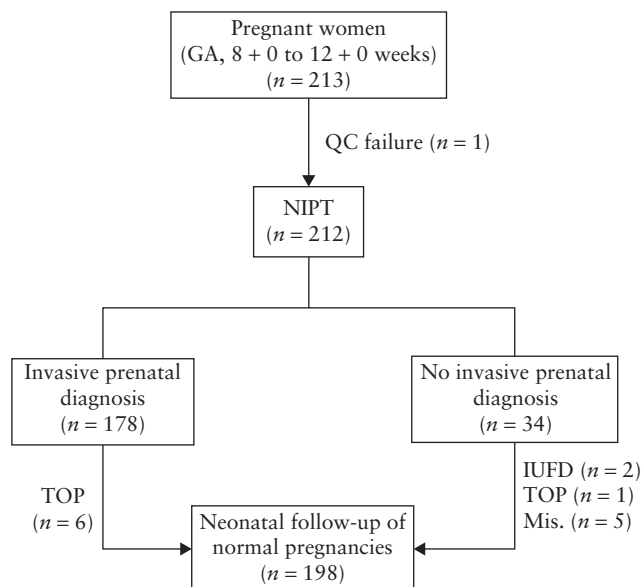


Figure 1 Study design for evaluating performance of non-invasive prenatal testing (NIPT) in early gestation. GA, gestational age; IUFD, intrauterine fetal death; Mis., miscarriage; QC, quality control; TOP, termination of pregnancy.

percentage of the abnormal chromosome between the test sample and a set of normal fetal reference samples, as previously reported¹⁷.

Invasive prenatal diagnosis

Fetal cell sampling was performed by CVS at 11 + 0 to 13 + 6 weeks or by amniocentesis at 18 + 0 to 21 + 6 weeks. Following standard metaphase conversion of cultured fetal cells, G-banding karyotyping was performed on a minimum of 20 single cells at a resolution of 320 bands.

RESULTS

A total of 213 pregnant women ≥ 35 years of age presenting for NIPT at PUMCH between May 2012 and August 2013 consented to participate in the study (Figure 1). The average maternal weight of this group was 60.02 ± 8.83 (range, 41.2–98.0) kg. Only one of the 213 blood samples taken for NIPT did not meet our quality control standard due to hemolysis and this patient was withdrawn from the study, reducing the starting cohort to 212 women. Within the selected cohort of 212 women, 42 (19.8%) had experienced a previous miscarriage and 13 (6.1%) had conceived by assisted conception. The fetus was male in 100 pregnancies and female in 112. The distributions of maternal age and gestational age at NIPT sampling for the 212 women are shown in Figure 2. The average maternal age was 37.25 (range, 35–45) years, with 182 (85.8%) women being < 40 years of age. NIPT sampling was performed exclusively within the first trimester, at a median gestational age of 9 + 6 (range, 8 + 0 to 12 + 6) weeks, with 110 of the 212 (52%) NIPT samples collected within the earlier gestational window of 8 + 0 to 9 + 6 weeks.

Following NIPT, 34 women declined to undergo confirmatory invasive prenatal diagnosis by either CVS or amniocentesis due to the small but significant risk of miscarriage and/or infection. Within this group of 34 women, eight (23.5%) had previously experienced a miscarriage and three (8.8%) had conceived by assisted conception. Thus, 178 women underwent both NIPT and invasive testing (Group A) and 34 women underwent only NIPT (Group B) (Figure 1).

Analysis of the NIPT samples from the 178 women in Group A identified five fetal aneuploidies (Table 1), including trisomy 21 ($n=2$), trisomy 18 ($n=1$), trisomy 13 ($n=1$) and 45,X ($n=1$). Invasive prenatal diagnosis confirmed the four trisomies; however, the one fetus with a 45,X NIPT result was found to have a normal 46,XX karyotype. In addition, two fetuses found normal by NIPT were confirmed by invasive testing to have abnormal karyotypes of 47,XXY and 45,X[16]/47,XXX[14]. Thus, for SCA, there were one (0.56%) false-positive and two (1.12%) false-negative samples. Sequencing of maternal WBC DNA for these three samples with NIPT results discordant for SCA identified normal maternal

46,XX karyotypes, indicating that the original NIPT result was fetal-specific. All six women with confirmed fetal aneuploidies opted for termination of pregnancy. The remaining 172 pregnancies with confirmed negative results progressed normally to full term and their babies were delivered at either the PUMCH or one of the related network hospitals. Neonatal examination by these patients' pediatricians confirmed the absence of any chromosomal disease syndromes in all 172 cases. Thus, on the basis of invasive testing and examination of neonates at birth, NIPT was highly accurate for detection of trisomies: sensitivity was 100% (95% CI, 19.79–100%) for trisomy 21, 100% (95% CI, 5.46–100%) for trisomy 18 and 100% (95% CI, 5.46–100%) for trisomy 13, and specificity was 100% (95% CI, 97.35–100%) for trisomy 21, 100% (95% CI, 97.35–100%) for trisomy 18 and 100% (95% CI, 97.35–100%) for trisomy 13. The specificity of NIPT for the detection of SCA was 99.43% (95% CI, 96.39–99.97%).

In the smaller cohort of 34 women who declined invasive prenatal diagnosis (Group B), NIPT detected three aneuploidies: one case each of trisomy 21, trisomy 2

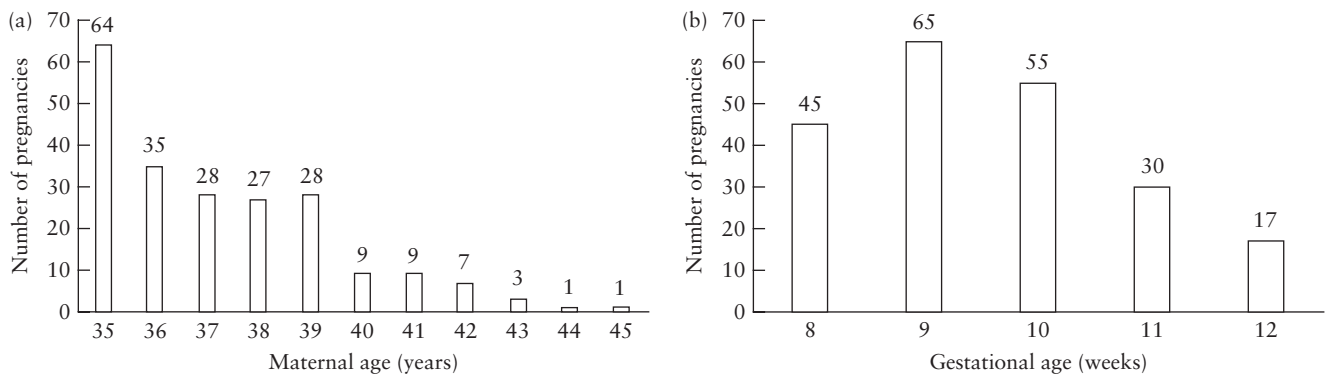


Figure 2 Demographics of study cohort of 212 pregnant women undergoing non-invasive prenatal testing (NIPT) in early pregnancy: distributions of maternal age (a) and gestational age (b) at NIPT sampling.

Table 1 Summary of patients with abnormal non-invasive prenatal testing (NIPT) results and/or adverse pregnancy outcome

Case	MA (years)	GA (weeks)	Fetal DNA fraction (%)	NIPT result	Fetal karyotype	Pregnancy outcome
Group A ($n=178$)						
1	38	8+6	8.50	T21 (male)	47,XY,+21	TOP
2	39	9+1	5.96	T21 (female)	47,XX,+21	TOP
3	41	11+5	5.45	T18 (male)	47,XY,+18	TOP
4	39	10+0	11.55	T13 (female)	47,XX,+13	TOP
5	36	9+5	5.65	45,X	46,XX	Live birth
6	42	9+5	6.06	46,XY	47,XXY	TOP
7	38	9+1	N/C	46,XX	45,X[16]/47,XXX[14]	TOP
Group B ($n=34$)						
8	36	11+3	12.53	T21 (female)	Not done	IUFD
9	42	11+3	4.80	T2 (female)	Not done	IUFD
10	39	8+5	11.85	45,X	Not done	TOP (heart malformation)
11	35	9+1	7.42	46,XY	Not done	Miscarriage
12	35	10+1	N/C	46,XX	Not done	Miscarriage
13	35	9+6	7.00	46,XY	Not done	Miscarriage
14	37	10+2	6.94	46,XY	Not done	Miscarriage
15	37	10+5	8.27	46,XY	Not done	Miscarriage

Group A, NIPT and invasive prenatal diagnosis; Group B, NIPT only. GA, gestational age at sampling; IUFD, intrauterine fetal death; MA, maternal age; N/C, not calculable; T, trisomy; TOP, termination of pregnancy.

and 45,X (Table 1). Two of these underwent intrauterine fetal death and the third was terminated due to a major heart abnormality. A further five pregnancies spontaneously miscarried within 1–2 weeks of NIPT blood sampling. Unfortunately, none of the women presented at PUMCH with signs of miscarriage and therefore tissue from the products of conception could not be recovered to confirm their normal NIPT results. The remaining 26 pregnancies with normal NIPT results progressed to term and examination of the neonates by the patients' pediatricians confirmed the absence of any chromosomal disease syndromes.

In secondary analyses, we examined the relationship between fetal DNA fraction and early gestational age (Figure 3). In the 100 male pregnancies, the median fetal DNA fraction within the 8–12 week gestational window was 8.54%, with a very wide range of 2.69–18.75%. There was an overall trend towards a slight increase in fetal DNA fraction from 8 + 0 to 12 + 6 weeks, although by one-way ANOVA, there was no significant difference in fetal DNA fraction between 8, 9, 10 and 12 weeks ($P=0.18$). Of greater clinical significance were the five (5%) plasma samples with a low fetal DNA fraction (<4%, this being the cut-off value for detection of aneuploidy), which were associated with gestational time points of 8 + 1, 10 + 0, 11 + 1, 12 + 4 and 12 + 6 weeks. Plots of fetal DNA fraction *vs* maternal weight for all 100 samples (Figure 4) showed a general trend towards an inverse relationship, with an R^2 value of 0.186 ($P<0.001$). Of the five samples with a fetal DNA fraction < 4%, four were associated with a relatively high maternal weight.

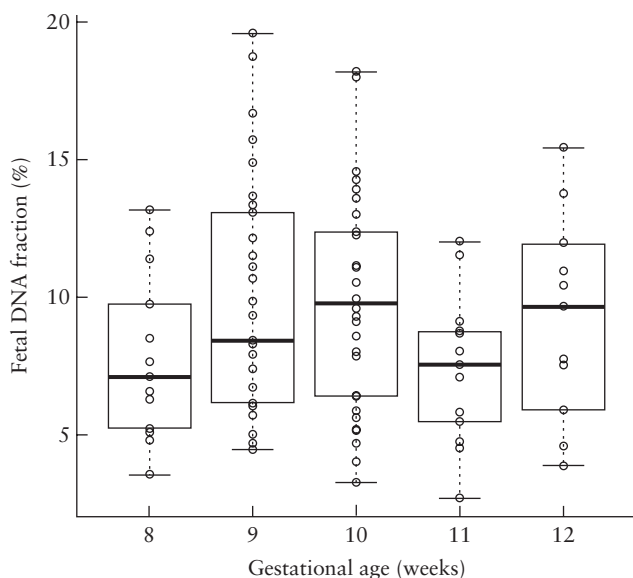


Figure 3 Box-and-whisker plots of fetal DNA fraction in 100 male pregnancies in early gestation. Boxes show median and interquartile range, whiskers show range and open circles are individual values. Median DNA fractions were 7.09% at 8 weeks, 8.41% at 9 weeks, 9.74% at 10 weeks, 7.53% at 11 weeks and 9.18% at 12 weeks. One-way ANOVA showed no significant difference in fetal DNA fraction between 8, 9, 10 and 12 weeks ($P=0.18$).

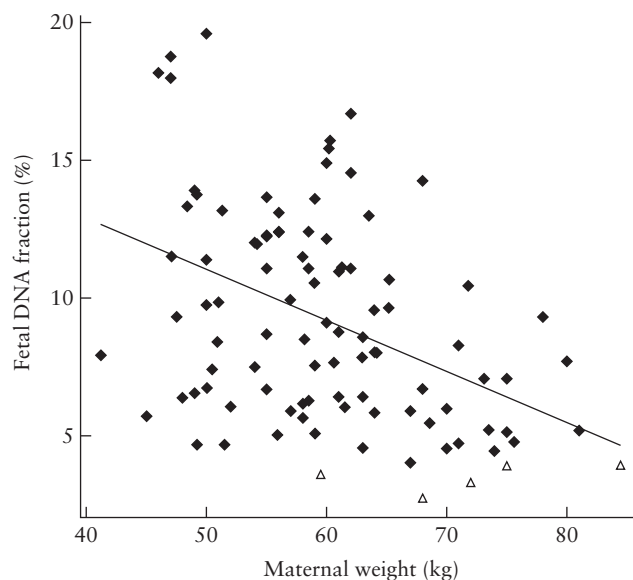


Figure 4 Correlation of fetal DNA fraction in early gestation with maternal weight in 100 male pregnancies (Pearson's correlation: $R = -0.431$; $R^2 = 0.186$; $P < 0.001$). Individual values are plotted; open triangles indicate a fetal DNA fraction below 4%.

DISCUSSION

In this pilot study of 212 pregnant women at risk for fetal aneuploidy, we assessed the clinical performance of NIPT in the first trimester, from as early as 8 + 0 weeks. Of these women, 178 had both NIPT and confirmatory invasive prenatal diagnosis, and 34 had only NIPT. Based on the diagnostic concordance of the 178 NIPT results with invasive testing and follow-up of neonates at birth, there were no false-negative or false-positive NIPT results for trisomies 21, 18 or 13. However, three other NIPT results were discordant with fetal karyotyping, including one false-positive and two false-negative SCAs, yielding false-positive and false-negative rates of 0.56% and 1.12%, respectively. The overall performance of early gestational NIPT in this small cohort of high-risk patients, particularly for fetal trisomies, was consistent with sensitivity and specificity rates reported in three recent prospective NIPT studies of 1741 Chinese women³, 1982 Cantonese women⁵ and 1914 American women with a mixed ethnic background⁴, in which blood samples were drawn primarily in the second trimester of pregnancy.

In an attempt to reconcile the three NIPT results discordant with fetal karyotyping, we reviewed all their NIPT and clinical data. For the false-positive case of 45,X the fetal DNA fraction of 5.65% was low, but still sufficient for achieving an accurate NIPT result. We speculate that this discordant NIPT result may have been due to 45,X confined placental mosaicism (CPM), a phenomenon that has been reported previously in a case of 47,XXX SCA¹⁰. Unfortunately, no fetal tissue was recovered after birth so 45,X CPM could not be confirmed. In the false-negative case of 47,XXY, the fetus was conceived by *in-vitro* fertilization following the transfer of two embryos of unknown sex. While

the fetal DNA fraction of 6.06% was relatively low, another plausible explanation for the failure of NIPT to detect 47,XXY would be an extra contribution, from a vanishing male twin, of 46,XY sequences to the fetal DNA fraction; this would effectively neutralize the expected gain in chromosome-X from the surviving 47,XXY twin. In support of this hypothesis, beta-human chorionic gonadotropin levels at 7 gestational weeks were 64 659 mIU/mL in this case, similar to levels reported for vanishing twins¹⁸. Furthermore, ultrasound examination at 7 weeks detected two fetal sacs and one heart beat, suggestive of a second non-viable fetus. In the second false-negative NIPT case, the fetus had a mosaic 45,X[16]/47,XXX[14] karyotype. Thus, the circulating fetal DNA fraction for chromosome-X should have been balanced by relatively equal contributions from 45,X and 47,XXX fetal tissues. Correspondingly, NIPT identified this sample as a normal female. This represents a rare case of true fetal mosaicism leading to a misdiagnosis by NIPT.

It is known that the vast majority of trisomy conceptions, including trisomies 21, 18 and 13, succumb very early in the first trimester of pregnancy¹⁹, resulting in spontaneous miscarriage. Within the group of 34 patients who did not have confirmatory invasive prenatal diagnosis, there were three NIPT-positive results: trisomy 21, trisomy 2 and 45,X, with corresponding fetal fractions of 12.53%, 4.80% and 11.85%, respectively. From their clinical records, both trisomy 21 and trisomy 2 fetuses had stopped growing within a few days of NIPT blood sampling, whereas the 45,X fetus was viable but had a major heart malformation. In all three cases, the fetuses were removed by curettage, but no fetal tissues were preserved to confirm the three NIPT results. In the same group of 34 patients, a further five women with negative NIPT results associated with fetal DNA fractions ranging from 6.94% to 8.27%, spontaneously miscarried within a few weeks of NIPT. Taken together, the findings in these eight patients suggest that neither fetal growth arrest nor impending miscarriage are significant risk factors associated with lowering the fetal DNA fraction below detection limits of 4%.

From the subanalysis of 100 male pregnancies, the median fetal DNA fraction between 8 + 0 and 12 + 6 gestational weeks was 8.54% in this study cohort of Chinese women, although 5% of the samples had a fetal DNA fraction below the detection limit of 4%. Interestingly, we found that there was no significant difference in fetal DNA fraction between 8, 9, 10 and 12 weeks. This suggests that there is only a relatively small incremental increase in fetal DNA fraction during this 4-week gestational time frame. In comparison, in a cohort of Cantonese women¹⁴ in whom NIPT was conducted between 11 and 14 gestational weeks, the median fetal DNA fraction was considerably higher, at 14.5%. It is therefore possible that the fetal DNA fraction may start to rise more steadily at the end of the first trimester. This would be consistent with previous studies that report fetal DNA fractions of 10–21% in the second trimester and an approximate increase of 1% per gestational week^{7,13}.

During early gestation, we also found a weak association between lower fetal DNA fraction and increased maternal weight ($R^2 = 0.186$, $P < 0.001$). A similar trend from 11 to 14 weeks' gestation was reported in another study of 195 male pregnancies ($R^2 = 0.16$, $P < 0.01$)¹⁴. Overall, these data suggest that, in a clinical setting, increased maternal weight cannot be used to stratify women and identify those more likely to have a fetal DNA fraction > 4% to ensure the validity of NIPT in early gestation.

In conclusion, based on our findings, NIPT is feasible in Chinese women in the first trimester of pregnancy from as early as 8 + 0 weeks to as late as 12 + 6 weeks, although further studies are warranted in other populations, particularly in those in which the average maternal weights are generally much higher. The major concern raised from this study was that, in approximately 5% of pregnancies, the DNA fraction was below the sensitivity limit of 4%, thus rendering the NIPT assay invalid. Before we can even contemplate introduction of early gestational NIPT into clinical practice, the sensitivity and specificity of the assay must be further increased to provide a valid assay for all samples. In a recent study²⁰, deeper sequencing to generate approximately 20 million reads was able to detect fetal trisomies at a fetal DNA fraction of 3%. If this finding proves to be reproducible at even lower fetal DNA fractions, it may become practical to offer early gestational NIPT with deeper sequencing, since it is expected that the cost of sequencing will continue to fall over time.

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