



Non-invasive Prenatal Testing: A Unique Approach with Single Nucleotide Polymorphism

Rupin Dhamankar¹ · Elizabeth Valenti¹ · Herman L. Hedriana²

Received: 13 March 2018 / Accepted: 29 March 2018 / Published online: 10 April 2018
© Society of Fetal Medicine 2018

Abstract The purpose of this review is to demonstrate the unique properties of a single nucleotide polymorphism (SNP)-based approach in non-invasive prenatal testing (NIPT). The identification of cell free fetal DNA in the plasma of pregnant women led to the development of NIPT. This can be performed with either a quantitative approach (massively parallel shotgun sequencing, chromosome selective sequencing) or a qualitative approach (SNP-based). NIPT tests have been shown to have superior performance as a screen for common fetal chromosome abnormalities compared with maternal serum screening. At low fetal fractions, NIPT sensitivity falls, particularly when quantitative methods are used. A SNP-based approach allows both accurate assessment of fetal fraction, and a robust test performance at lower fetal fractions. The ability of the SNP-based approach to screen for vanishing twins and maternal copy number variants reduces false positives; and the ability to make high confidence calls at lower fetal fraction, minimizes discordance between the NIPT result and the true fetal status.

Keywords cffDNA · NIPT · SNP-based NIPT · Fetal fraction · NIPS · Prenatal diagnosis

Introduction

Historically, maternal serum screening and ultrasound imaging are two of the most common approaches used to screen for chromosome aneuploidy and possible fetal anomalies [1]. While both are a part of routine prenatal care, their biggest limitation is a high false positive rate approaching 5% [2]. To offset the false positive results, patients are offered invasive diagnostic procedures such as chorionic villus sampling (CVS) or amniocentesis. However, these invasive tests carry a 0.13–0.27% procedure risk of miscarriage [3] and therefore, are not very popular first tier tests among most patients. Recently, cell-free fetal DNA in maternal plasma has been the choice for non-invasive prenatal testing over the historical approaches with false positive rates below 1% [2].

The presence of cell free fetal DNA (cffDNA) in the plasma of pregnant mothers was first reported by Lo et al. [4] and led to the development of non-invasive prenatal testing (NIPT). NIPT involves an analytical quantification of cffDNA from maternal plasma to evaluate for common fetal aneuploidies, particularly trisomy 21, which became commercially available in 2011. Overtime, NIPT became an intermediate step between serum screening and invasive testing (CVS and amniocentesis) given its improved sensitivity and specificity of fetal aneuploidy screening, very low false positive rate and without the pregnancy risks of invasive testing [5].

Approximately 10% of fragmented cffDNA found in the plasma of a pregnant woman is placental (fetal) in origin [6], thus making it a suitable surrogate for pregnancy testing targeting the fetus. Commercially, the two common NIPT approaches to analyze cffDNA to detect the presence of aneuploidy are the quantitative comparison of the relative number of sequence reads from a chromosome of

✉ Herman L. Hedriana
hlhedriana@ucdavis.edu

¹ Natera, Inc, San Carlos, CA, USA

² Division of Maternal-Fetal Medicine, Department of Obstetrics and Gynecology, University of California Davis Health System, Sacramento, CA, USA

interest to a reference chromosome, and the qualitative use of single nucleotide polymorphisms (SNPs) to determine copy number. A SNP is a variation in the DNA sequence that occurs when a single nucleotide in the DNA sequence is altered.

Quantitative methods of assessing aneuploidy using NIPT include massively parallel shotgun sequencing (MPSS) or chromosome selective sequencing (CSS) [7, 8]. MPSS involves sequencing all 23 pairs of chromosomes while CSS involves sequencing loci only from chromosomes of interest. Both approaches have shown sensitivities and specificities of over 99% for detection of trisomies 21 and 18 while the sensitivities for trisomy 13 and sex chromosome aneuploidies have been lower. This lowered sensitivity could be attributed to the variability on the quality of sequencing data due to high content of guanine and cytosine on these chromosomes [9]. An inability to differentiate between maternal and fetal genotypes is a limitation of the quantitative approach, which can lead to both false positive and false negative results in cases where mosaicism is present in either the mother or the fetus [10].

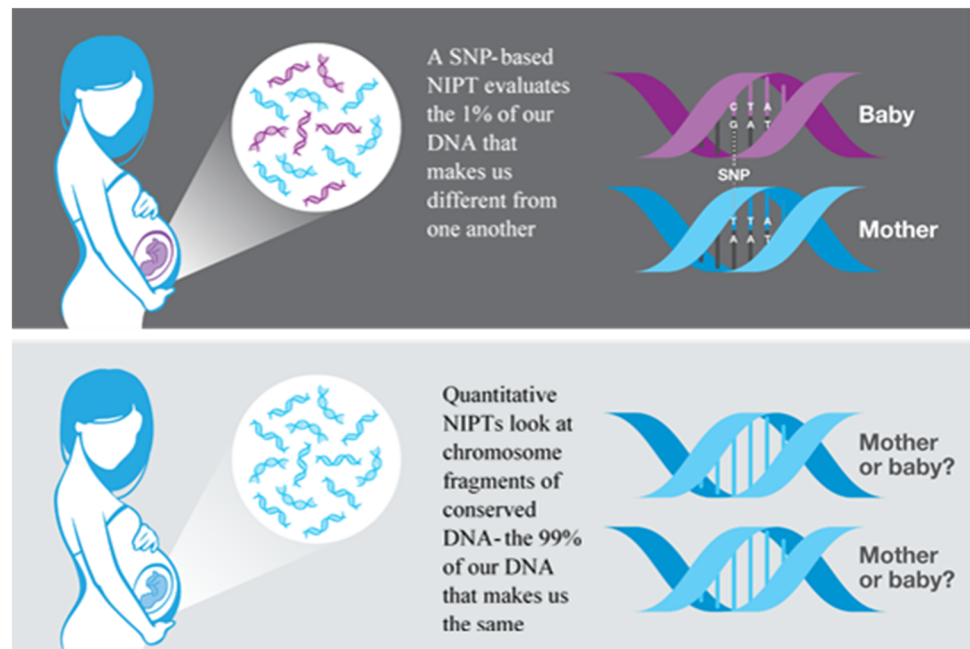
The qualitative approach uses over 13,000 highly polymorphic SNPs, in addition to cross over frequency during meiosis, to evaluate the likelihood of a fetal aneuploidy. Accurate evaluation of fetal fraction allows allele ratios to be determined across all chromosomes of interest and, allow the fetal ploidy status to be evaluated without the need for a reference chromosome, eliminating a source of error recognized with the quantitative approach. The differentiation is highlighted in Fig. 1.

Clinical Performance

At its inception, NIPT offered aneuploidy risk assessment for trisomies 21, 18 and 13. Over time, the number of aneuploidies (abnormal number of chromosomes) screened for has increased to include monosomy X, sex chromosome trisomies, and aneuploidies for chromosomes 16 and 22 [11]. In addition to screening for fetal aneuploidies, NIPT also includes analysis of sub-chromosomal abnormalities typically referred to as microdeletions on chromosomes 1p, 4p, 5p, 8q, 11q, 15q and 22q.

Two recent meta-analyses reviewed the performance of NIPT for the detection of common chromosome aneuploidies [12, 13]. Gil et al. [13] reported their findings based on analysis of data from 37 studies. The weighted detection rates (DR) and false positive rates (FPR) they reported in singleton pregnancies were 99.2% (95% CI 98.5–99.6%) and 0.09% (95% CI 0.05–0.14%) for trisomy 21, 96.3% (95% CI 94.3–97.9%) and 0.13% (95% CI 0.07–0.20%) for trisomy 18, 91% (95% CI 85–95.6%) and 0.13% (95% CI 0.05–0.26%) for trisomy 13, 90.3% (95% CI 85.7–94.2%) and 0.23% (95% CI 0.14–0.34%) for monosomy X and 93% (95% CI 85.8–97.8%) and 0.14% (95% CI 0.06–0.24%) for sex chromosome aneuploidies other than monosomy X, respectively. The second meta-analysis by Iwarsson et al. [12] reported a data analysis from 32 studies. This meta-analysis was specifically aimed to review the performance of NIPT for detection of trisomies 21, 18 and 13 in the general pregnant population and to update the data on high-risk pregnancies. Iwarsson and colleagues reported that in the general population there

Fig. 1 Illustration of the differences in the SNP-based NIPT approach (top) compared to the quantitative NIPT approach (bottom)



is moderate evidence that the pooled sensitivity is 99.3% (95% CI 95.5–99.9%) and specificity is 99.9% (95% CI 99.8–99.9%) for the analysis of trisomy 21. They were unable to calculate pooled sensitivities and specificities for trisomies 18 and 13 in the general population citing low number of studies. While analyzing the high-risk pregnant population, they reported that there is moderate evidence that the pooled sensitivities for trisomies 21 and 18 are 99.8% (95% CI 98.1–99.9%) and 97.7% (95% CI 95.8–98.7%) respectively. For trisomy 13, they reported low evidence that the pooled sensitivity is 97.5% (95% CI 81.9–99.7%). In the high-risk population, they reported pooled specificity for all three trisomies to be 99.9% (95% CI 99.8–99.9%).

These meta-analyses conclude that NIPT is a superior screen for trisomy 21 in both low-risk and high-risk populations. At least one of these suggests that the performance of NIPT screening for trisomies 18, 13 and sex chromosome aneuploidies does not seem to match the performance of NIPT screening for trisomy 21 [12]. Other retrospective studies have reported clinical experiences from different commercial laboratories offering NIPT [2, 14]. In general, these studies have reported positive predictive values (PPV) for trisomies 21, 18, 13 to be much higher, as compared to serum screening (PPVs ranging from 38 to 100% for NIPT compared to 3.4% for serum screening) [2, 14, 15]. While the cohorts in some of these studies did have a higher percentage of high-risk women thereby creating a bias, two of these studies did have a significant percentage of low risk women as well [2, 14, 15]. Both studies that had a large percentage of low risk women also displayed that test performance in the low risk group was significantly improved compared to serum screening (combined PPV of 87.2% and 76% for NIPT compared to 3.4% for serum screening) [2, 14].

There is less data available regarding use of NIPT to screen for microdeletions, when compared to common chromosome aneuploidies. Zhao et al. [16] used whole-genome sequencing data to detect genome-wide fetal microdeletions/microduplications (MDs). While this study showed that the sensitivity in determining genome-wide MDs was 94.4%, it was also observed that the sensitivity was greatly affected by fetal fraction, with sensitivity for the 22q11.2 deletion syndrome dropping to under 20% when the fetal fraction was below 12%. In a different study, Wapner et al. [17] investigated the performance of a SNP-based NIPT for detection of 5 microdeletion syndromes. This study reported a combined sensitivity of 97.8% that was unchanged with varying fetal fractions at or over 3.8%. Since the general prevalence of MDs is lower compared to that of fetal aneuploidy, one limitation of both studies was the use of artificially generated mixture samples rather than pregnancy plasmas. Keeping this limitation

in mind, Martin et al. [18] reported the screening performance of a SNP-based NIPT for the same 5 MDs on > 80,000 true pregnancy plasmas. They modified the Wapner protocol [17] by reflexively sequencing all MD high risk results to a higher depth of read. Using this new protocol for SNP-based MD screening, Martin et al. [18] report PPVs of 44.2% for the 22q11.2 deletion syndrome and a combined PPV of 31.7% for MDs on chromosomes 1p, 5p, and 15q; without compromising the false positive rates which were lower than the Wapner cohort.

Fetal Fraction

Fetal fraction is the amount of DNA circulating in a pregnant woman's plasma that is fetal (placental) in origin. While the median fetal fraction in maternal plasma between 11 and 13 weeks' gestation is 10% [6], there is considerable variation observed. Certain laboratories that offer NIPT have established a threshold for fetal fraction below which assessment for fetal aneuploidy is not provided, while other laboratories do not have such a threshold [6, 19, 20]. For NIPTs that use a quantitative approach for analysis of cfDNA, test performance is dependent on the fetal fraction as well as depth of sequencing or the total number of unique DNA fragments that are counted. A study published by Canick et al. [21] demonstrated this relationship by showing that pregnancies affected by trisomy 21 had z score values that were overlapping with z score values for euploid pregnancies when fetal fractions were below 8%. Another case report by Allen et al. [22], highlighted a trisomy 21 false negative result on NIPT, that on further investigation with the performing laboratory could be attributed to low fetal fraction.

Some factors that are associated with a lower fetal fraction include: gestational age under 10 weeks, high maternal body mass index, fetal aneuploidy and placental health [23]. A few anecdotal case reports have indicated that patients on anticoagulation may have lower fetal fractions as well [24]. Laboratories that analyze and report fetal fraction have proven an association of aneuploidy among the low fetal fraction cases [2, 25]. If a confident high or low risk call cannot be made, a “no call” is issued, low fetal fraction cited and association between low fetal fraction and aneuploidy included in the report. Laboratories that do not consider fetal fraction as a quality metric, and do not report this metric; may be at higher chance of issuing a low risk result, or merely indicating “no call” due to a ‘technical failure’ rather than alerting the provider to an increased risk for fetal aneuploidy [26].

Pregnancies that receive a ‘no call’ due to low fetal fraction have the option of repeating the test or proceeding with diagnostic testing. Professional societies including the

American College of Obstetricians and Gynecologists (ACOG) as well as the American College of Medical Genetics and Genomics (ACMG) have both issued statements acknowledging the association of aneuploidy with ‘no call’ results. Both societies have made recommendations stating that pregnancies receiving a ‘no call’ due to low fetal fraction on NIPT should be offered the option of genetic counseling, a detailed ultrasound evaluation and prenatal diagnosis [20, 23].

Vanishing Twin and Fetal Triploidy

As described earlier, a SNP-based NIPT works by determining copy number at 13,392 SNPs [27]. Since the quantitative approach compares the relative number of sequence reads from a chromosome of interest to a reference chromosome, it is unable to determine the source of the DNA (fetal or maternal) [10]. Further, the cfDNA fragments may have similar sizes as the maternal cell-free DNA confounding the differentiation of cfDNA from the latter by the quantification method (Fig. 1). If fetal triploidy is present, the ratio between the chromosome of interest and reference chromosome is preserved, leading to a false negative result. In comparison, due to extensive probability of allele distribution, the SNP-based approach relies on analytical bioinformatics to differentiate two distinct haplotypes (mother and fetus) and is also able to differentiate an additional haplotype when fetal triploidy is present. These extra haplotypes are also observed when there is a vanishing or ongoing twin pregnancy.

A study by Curnow et al. [10] and a case report by Niles et al. [28] demonstrate the persistence of cfDNA from the demised twin in maternal circulation for as long as 8–15 weeks post demise. While the incidence of identifying a vanishing twin case on NIPT is 0.42–0.6% [10], vanishing twins are responsible for up to 42.1% of confirmed false positive NIPT results [28]. Hence, practitioners need to be aware of the potential for a false positive NIPT result that can arise due to the demise of a co-twin. The SNP-based NIPT is unique in its ability to identify the presence of additional haplotypes, minimizing the likelihood of a false positive and negative results.

Maternal Copy Number Variant (CNV)

Wang et al. [29] reported maternal findings in 187 NIPT tests with discordant results for sex chromosome abnormalities. Maternal chromosome analysis revealed that in approximately 8.56%, the pregnant women themselves were mosaic for an X chromosome aneuploidy [29]. Russell et al. [30] in a previous study showed that there is an

increase in the rate of somatic mosaicism for X chromosome abnormalities as women increase in age. As the quantitative approach is unable to determine the source of cell-free DNA (maternal or fetal), there is a high possibility of a false positive result if the pregnant mother herself has a CNV. The strength of the SNP-based approach is its ability to determine the source of cell-free DNA and thus minimizes the risk for a false positive result that could arise secondary to a maternal CNV.

Implications of Low Fetal Fraction

Two large prospective studies [2, 25] that analyzed over 16,000 pregnancies reported an association between a low fetal fraction noted in maternal circulation with maternal aneuploidy. Pergament et al. [25] indicated that samples in the low fetal fraction range, as low as the 1.5th percentile, were six times more likely to be aneuploid compared to samples that had higher fetal fractions. In another study, Rava et al. [31] showed that nearly 5% of euploid cases with fetal fractions under 4%, are seen in pregnancies with trisomy 18 and 13 as well as 45, X and digynic triploidy. Thus, in recognizing the importance of this association, ACMG suggested that in case of a ‘no call’, laboratories performing NIPT should report the reason for the no call and report if a low fetal fraction was identified [20].

There are two approaches that laboratories could take to overcome this challenge posed by low fetal fraction. A laboratory can increase the number of sequence reads so that there are more reads per chromosome analyzed. The disadvantage of this approach is that there are only a limited number of reads that can be added to the testing platform beyond which the test is economically unsustainable. Additionally, the lower the fetal fraction, the less diverse the library of cfDNA fragments and the lower the quality of data after sequencing [32]. Fan et al. [32] report that the sensitivity of detecting an aneuploidy depends on the fetal fraction as well as the number of molecules counted and is higher at higher fetal fractions. On the other hand, laboratories can establish a lower bound threshold for fetal fraction beyond which the performance of the test (sensitivity and specificity) becomes unreliable. While the latter approach is easier to implement, it will potentially increase the number of samples that receive a ‘no call’. Therefore, no matter how many times you read the DNA sequence, the laboratory will need to decide between accepting a higher false negative rate or a threshold and accept a higher no call rate.

NIPT in Twins

There are limited data regarding the performance of NIPT in twins and higher order multiples compared to singleton pregnancies. A recent meta-analysis by Liao et al. [33] evaluated the performance for trisomies 21, 18, and 13 in ten studies of NIPT in twin pregnancies. NIPT was found to have high sensitivity and specificity for trisomy 21 screening in twin pregnancies. In comparison, the trisomy 18 screening performance was less satisfactory because of lower pooled sensitivity. The numbers were insufficient to draw any conclusions for trisomy 13. Overall, the conclusion also states that NIPT has a much superior performance than serum screening. One of the distinctive features of a SNP-based approach in twin gestations is the ability to identify the zygosity of the pregnancy with > 99% accuracy (publication in process). This in turn can allow practitioners to ensure that the chorionicity is reliably assigned as early as possible in pregnancy so that monochorionic pregnancies can receive increased surveillance. Another potential benefit of a SNP-based approach is the ability to modify the analytical bioinformatics to distinguish and report two fetal fractions for dizygotic twins, as opposed to a single or lowest fetal fraction. This allows additional confidence that two fetuses have received evaluation.

Limitations and Conclusions

NIPT offers a much more efficient way of screening for common chromosome aneuploidy with higher sensitivities and specificities, and lower false positive rates than serum screening. Additionally, NIPT does not have the risk associated with invasive diagnostic procedures [11]. NIPT has made significant, typically positive impact on the way prenatal testing has been received by the patient population [5]. Patients appear to prefer NIPT as a first line for aneuploidy screening as shown in one prospective population study showing a 35% increase in utilization over no screening [32]. Since its advent, there has been a significant decline in the uptake of invasive prenatal diagnosis and combined first trimester screening despite the birth rate remaining steady [5].

Although methodologies used for NIPT incorporate sophisticated bioinformatics, one study has shown that pre-test counseling can be accomplished by the general obstetrical provider [34]. However, additional studies and more data are required to reaffirm this. In the interim, genetic counselors are uniquely trained in medical genetics as well as psychological counseling and are well equipped to discuss NIPT with patients. However, there are not nearly enough trained genetic counselors to meet the needs

of the patients and most patients do not receive the services of a genetic counselor [35]. It is important that the health care professionals and the professional societies recognize the need for increased genetic education at all levels of the health care system, and support the development of tools that can assist practitioners in counseling patients about NIPT.

Another limitation of these tests is that in their current form they do not replace diagnostic tests, because of the potential for confined placental mosaicism, in addition to the smaller scope of testing compared to what is available using chorionic villi or amniotic fluid. A low risk result does not eliminate the possibility of a chromosome abnormality. Hence, when a structural fetal malformation is suspected, invasive prenatal diagnosis provides the most information about the presence or absence of an associated genetic abnormality. There may also be false positive or negative results due to maternal CNVs, vanishing twins, and rarely even maternal cancers. In a small percentage of pregnancies, NIPT is unable to yield a result due to low fetal fraction. Many of these can be affected by the choice of a SNP-based NIPT.

In conclusion, NIPT is a test that satisfies two important metrics of a good screening test, high sensitivity/specificity and low false positive and negative rates. A SNP-based approach has additional benefits: an ability to screen for vanishing twins and fetal triploidy; is less compromised by maternal CNVs; and can make high confidence calls at lower fetal fractions. All these factors contribute immensely in minimizing false positive and false negative rates compared to the quantitative approach [10, 21, 29]. Providers can develop the necessary skills, supplemented by ancillary materials and genetic counselors, to obtain informed consent or refusal for NIPT, which has the potential to provide families with valuable information as they plan for their family and aid the practitioner in pregnancy management.

Compliance with Ethical Standards

Disclosures Rupin Dhamankar and Elizabeth Valenti are Natera Inc employees; Herman Hedriana was a former Natera employee.

References

1. Russo ML, Blakemore KJ. A historical and practical review of first trimester aneuploidy screening. *Sem Fetal Neonatal Med.* 2014;19(3):183–7. <https://doi.org/10.1016/j.siny.2013.11.013>.
2. Norton ME, Jacobsson B, Swamy GK, Laurent LC, Ranzini AC, Brar H, et al. Cell-free DNA analysis for noninvasive examination of trisomy. *N Engl J Med.* 2015;372(17):1589–97. <https://doi.org/10.1056/NEJMoa1407349> **PubMed PMID: 25830321**.
3. American College of Obstetricians and Gynecologists' Committee on Practice Bulletins—Obstetrics, Committee on Genetics, Society for Maternal-Fetal Medicine. Practice bulletin no. 162:

- prenatal diagnostic testing for genetic disorders. *Obstet Gynecol.* 2016;127(5):e108–22. <https://doi.org/10.1097/aog.0000000000001405>.
4. Lo YM, Corbetta N, Chamberlain PF, Rai V, Sargent IL, Redman CW, et al. Presence of fetal DNA in maternal plasma and serum. *Lancet (London, England)*. 1997;350(9076):485–7. [https://doi.org/10.1016/s0140-6736\(97\)02174-0](https://doi.org/10.1016/s0140-6736(97)02174-0).
 5. Hui L, Hutchinson B, Poulton A, Halliday J. Population-based impact of noninvasive prenatal screening on screening and diagnostic testing for fetal aneuploidy. *Genet Med.* 2017;19:1338. <https://doi.org/10.1038/gim.2017.55>.
 6. Ashoor G, Syngelaki A, Poon LC, Rezende JC, Nicolaides KH. Fetal fraction in maternal plasma cell-free DNA at 11–13 weeks' gestation: relation to maternal and fetal characteristics. *Ultrasound Obstet Gynecol.* 2013;41(1):26–32. <https://doi.org/10.1002/uog.12331>.
 7. Boon EMJ, Faas BHW. Benefits and limitations of whole genome versus targeted approaches for noninvasive prenatal testing for fetal aneuploidies. *Prenat Diagn.* 2013;33(6):563–8. <https://doi.org/10.1002/pd.4111>.
 8. Sayres LC, Cho MK. Cell-free fetal nucleic acid testing: a review of the technology and its applications. *Obstet Gynecol Surv.* 2011;66(7):431–42. <https://doi.org/10.1097/OGX.0b013e31822dfbe2>.
 9. Dohm JC, Lottaz C, Borodina T, Himmelbauer H. Substantial biases in ultra-short read data sets from high-throughput DNA sequencing. *Nucleic Acids Res.* 2008;36(16):e105. <https://doi.org/10.1093/nar/gkn425>.
 10. Curnow KJ, Wilkins-Haug L, Ryan A, Kirkizlar E, Stosic M, Hall MP, et al. Detection of triploid, molar, and vanishing twin pregnancies by a single-nucleotide polymorphism-based noninvasive prenatal test. *Am J Obstet Gynecol.* 2015;212(1):79.e1–9. <https://doi.org/10.1016/j.ajog.2014.10.012>.
 11. Allyse M, Minear MA, Berson E, Sridhar S, Rote M, Hung A, et al. Non-invasive prenatal testing: a review of international implementation and challenges. *Int J Women's Health.* 2015;7:113–26. <https://doi.org/10.2147/ijwh.S67124>.
 12. Iwarsson E, Jacobsson B, Dagerhamn J, Davidon T, Bernabé E, Heibert Arnlind M. Analysis of cell-free fetal DNA in maternal blood for detection of trisomy 21, 18 and 13 in a general pregnant population and in a high risk population—a systematic review and meta-analysis. *Acta Obstet Gynecol Scand.* 2017;96(1):7–18. <https://doi.org/10.1111/aogs.13047>.
 13. Gil MM, Quezada MS, Revello R, Akolekar R, Nicolaides KH. Analysis of cell-free DNA in maternal blood in screening for fetal aneuploidies: updated meta-analysis. *Ultrasound Obstet Gynecol.* 2015;45(3):249–66. <https://doi.org/10.1002/uog.14791>.
 14. Dar P, Curnow KJ, Gross SJ, Hall MP, Stosic M, Demko Z, et al. Clinical experience and follow-up with large scale single-nucleotide polymorphism-based noninvasive prenatal aneuploidy testing. *Am J Obstet Gynecol.* 2014;211(5):527.e1–17. <https://doi.org/10.1016/j.ajog.2014.08.006>.
 15. Porreco RP, Garite TJ, Maurel K, Marusiak B, Ehrich M, van den Boom D, et al. Noninvasive prenatal screening for fetal trisomies 21, 18, 13 and the common sex chromosome aneuploidies from maternal blood using massively parallel genomic sequencing of DNA. *Am J Obstet Gynecol.* 2014;211(4):365.e1–12. <https://doi.org/10.1016/j.ajog.2014.03.042>.
 16. Zhao C, Tynan J, Ehrich M, Hannum G, McCullough R, Saldivar J-S, et al. Detection of fetal subchromosomal abnormalities by sequencing circulating cell-free DNA from maternal plasma. *Clin Chem.* 2015;61(4):608–16. <https://doi.org/10.1373/clinchem.2014.233312>.
 17. Wapner RJ, Babiarez JE, Levy B, Stosic M, Zimmermann B, Sigurjonsson S, et al. Expanding the scope of noninvasive prenatal testing: detection of fetal microdeletion syndromes. *Am J Obstet Gynecol.* 2015;212(3):332.e1–9. <https://doi.org/10.1016/j.ajog.2014.11.041>.
 18. Martin K, Iyengar S, Kalyan A, Lan C, Simon AL, Stosic M, et al. Clinical experience with a single-nucleotide polymorphism-based non-invasive prenatal test for five clinically significant microdeletions. *Clin Genet.* 2018;93(2):293–300. <https://doi.org/10.1111/cge.13098>.
 19. Palomaki GE, Kloza EM, Lambert-Messerlian GM, Haddow JE, Neveux LM, Ehrich M, et al. DNA sequencing of maternal plasma to detect Down syndrome: an international clinical validation study. *Genet Med.* 2011;13:913. <https://doi.org/10.1097/GIM.0b013e3182368a0e>.
 20. Gregg AR, Skotko BG, Benkendorf JL, Monaghan KG, Bajaj K, Best RG, et al. Noninvasive prenatal screening for fetal aneuploidy, 2016 update: a position statement of the American College of Medical Genetics and Genomics. *Genet Med.* 2016;18(10):1056–65. <https://doi.org/10.1038/gim.2016.97>.
 21. Canick JA, Palomaki GE, Kloza EM, Lambert-Messerlian GM, Haddow JE. The impact of maternal plasma DNA fetal fraction on next generation sequencing tests for common fetal aneuploidies. *Prenat Diagn.* 2013;33(7):667–74. <https://doi.org/10.1002/pd.4126>.
 22. Allen R, Kezmarsky P, Lescale L. False negative NIPT and potential implications for genetic counseling. ACMG annual clinical genetics meeting 2013, Abstract 47; Phoenix2013.
 23. Committee on Practice Bulletins—Obstetrics, Committee on Genetics, and the Society for Maternal-Fetal Medicine. Practice bulletin no. 163: screening for fetal aneuploidy. *Obstet Gynecol.* 2016;127(5):e123–37. <https://doi.org/10.1097/aog.0000000000001406>.
 24. Hui L, Bethune M, Weeks A, Kelley J, Hayes L. Repeated failed non-invasive prenatal testing owing to low cell-free fetal DNA fraction and increased variance in a woman with severe autoimmune disease. *Ultrasound Obstet Gynecol.* 2014;44(2):242–3. <https://doi.org/10.1002/uog.13418>.
 25. Pergament E, Cuckle H, Zimmermann B, Banjevic M, Sigurjonsson S, Ryan A, et al. Single-nucleotide polymorphism-based noninvasive prenatal screening in a high-risk and low-risk cohort. *Obstet Gynecol.* 2014;124(2 Pt 1):210–8. <https://doi.org/10.1097/aog.0000000000000363>.
 26. Taneja PA, Snyder HL, de Feo E, Kruglyak KM, Halks-Miller M, Curnow KJ, et al. Noninvasive prenatal testing in the general obstetric population: clinical performance and counseling considerations in over 85 000 cases. *Prenat Diagn.* 2016;36(3):237–43. <https://doi.org/10.1002/pd.4766>.
 27. Ryan A, Hunkapiller N, Banjevic M, Vankayalapati N, Fong N, Jinnett KN, et al. Validation of an enhanced version of a single-nucleotide polymorphism-based noninvasive prenatal test for detection of fetal aneuploidies. *Fetal Diagn Ther.* 2016;40(3):219–23. <https://doi.org/10.1159/000442931>.
 28. Niles KM, Murji A, Chitayat D. Prolonged duration of persistent cell free fetal DNA from a vanishing twin. *Ultrasound Obstet Gynecol.* 2018. <https://doi.org/10.1002/uog.19004>.
 29. Wang Y, Chen Y, Tian F, Zhang J, Song Z, Wu Y, et al. Maternal mosaicism is a significant contributor to discordant sex chromosomal aneuploidies associated with noninvasive prenatal testing. *Clin Chem.* 2014;60(1):251–9. <https://doi.org/10.1373/clinchem.2013.215145>.
 30. Russell LM, Strike P, Browne CE, Jacobs PA. X chromosome loss and ageing. *Cytogenet Genome Res.* 2007;116(3):181–5. <https://doi.org/10.1159/000098184>.
 31. 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(1):243–50. <https://doi.org/10.1373/clinchem.2013.207951>.

32. Fan HC, Blumenfeld YJ, Chitkara U, Hudgins L, Quake SR. Analysis of the size distributions of fetal and maternal cell-free DNA by paired-end sequencing. *Clin Chem*. 2010;56(8):1279–86. <https://doi.org/10.1373/clinchem.2010.144188>.
33. Liao H, Liu S, Wang H. Performance of non-invasive prenatal screening for fetal aneuploidy in twin pregnancies: a meta-analysis. *Prenat Diagn*. 2017;37(9):874–82. <https://doi.org/10.1002/pd.5118>.
34. Palomaki GE, Kloza EM, O'Brien BM, Eklund EE, Lambert-Messerlian GM. The clinical utility of DNA-based screening for fetal aneuploidy by primary obstetrical care providers in the general pregnancy population. *Genet Med*. 2017;19:778. <https://doi.org/10.1038/gim.2016.194>.
35. Hoskovec JM, Bennett RL, Carey ME, DaVanzo JE, Dougherty M, Hahn SE, et al. Projecting the supply and demand for certified genetic counselors: a workforce study. *J Genet Couns*. 2018;27(1):16–20. <https://doi.org/10.1007/s10897-017-0158-8>.