

# Have we done our last amniocentesis? Updates on cell-free DNA for Down syndrome screening

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**Abstract** Prenatal aneuploidy screening changed significantly in 2012 when cell-free fetal deoxyribonucleic acid (DNA) was introduced as a noninvasive prenatal test. A noninvasive prenatal test detects cell free fragments of fetal DNA from the placenta circulating in maternal blood that coexist with cell-free DNA (cfDNA) of maternal origin. Using next-generation sequencing, the noninvasive prenatal test compares maternal and fetal cfDNA ratios for chromosomes of interest (i.e., 21, 18, 13, X, and Y) to assess chromosomal aneuploidy. Compared to traditional screening using ultrasound and serum markers, the noninvasive prenatal test has superior test characteristics, including a higher detection rate and positive predictive value, and a lower false-positive rate. The noninvasive prenatal test is already used for primary screening in high-risk women and is rapidly expanding to all women. Given its increasing use, understanding the noninvasive prenatal test's limitations is critical. Discordant results (i.e. noninvasive prenatal test is positive for aneuploidy with a normal fetal karyotype) can occur because of biological processes such as aneuploidy confined to the placenta, a vanished twin, maternal aneuploidy or maternal cancer. Use of the noninvasive prenatal test for screening beyond the most common aneuploidies is not recommended. The noninvasive prenatal test is a major advance in prenatal aneuploidy screening but it is not diagnostic and does not replace invasive testing (i.e. chorionic villous sampling or amniocentesis) for confirmation of fetal chromosomal disorders.

**Keywords** Cell-free deoxyribonucleic acid (cfDNA) · Down syndrome · Fetus · Genetics · Noninvasive prenatal testing · Prenatal · Screening

## Introduction

Prenatal genetic screening has changed dramatically in the last 5 years, driven primarily by the introduction of cell-free DNA (cfDNA) into clinical practice in 2012. Using next-generation sequencing technology, cfDNA in the maternal circulation can be sequenced and an assessment given for risk of common aneuploidies (trisomy 21 [Down syndrome], trisomy 18, trisomy 13, and sex chromosome aneuploidies). Because of the excellent sensitivity of cfDNA screening, the rates of invasive diagnostic tests (i.e. chorionic villous sampling [CVS] and amniocentesis) have decreased significantly since 2012. In high-risk women cfDNA testing detects 98–99% of Down syndrome pregnancies, and less than 1% of women have a positive cfDNA screening result. Women with a positive cfDNA result are still advised to undergo a diagnostic procedure for confirmation of the screening result because false-positive cfDNA results can occur. Additionally, cfDNA testing is not yet validated for chromosomal or genetic abnormalities beyond trisomies 21, 13 and 18, and X and Y abnormalities. Thus diagnostic testing with CVS or amniocentesis is still recommended for women with fetal anomalies detected on prenatal ultrasound and for women who desire genetic screening beyond the common aneuploidies.

In this article, we review the current guidelines for prenatal aneuploidy screening, discuss cfDNA screening technology and test characteristics, examine the reasons for false-negative and false-positive cfDNA test results, and address current clinical dilemmas surrounding cfDNA testing.

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## Current guidelines for aneuploidy screening

The chances of having a live-born baby with Down syndrome increase significantly with increasing maternal age, with a 1 in 85 chance at age 40 [1]. The American Congress of Obstetricians and Gynecologists (ACOG) and the Society of Maternal-Fetal Medicine (SMFM) recommend that all women be offered screening and diagnostic testing for aneuploidy, ideally in the first trimester [1]. Options for aneuploidy screening include maternal serum screening as well as cell-free DNA. Since the introduction of cfDNA in 2012, ACOG and SMFM guidelines have suggested that aneuploidy screening with cfDNA is appropriate for high-risk women with singleton pregnancies [2]. High-risk women are defined as women  $\geq 35$  years old, those with fetal ultrasound findings suggestive of an increased aneuploidy risk, women with prior trisomy-affected offspring, parents with increased risk of trisomy 13 or trisomy 21 offspring from a parental balanced Robertsonian translocation, and women with an increased aneuploidy risk based on first- or second-trimester screening results [1]. In 2015, SMFM updated its screening recommendations to add that if a low-risk woman requests cfDNA and has appropriate pretest counseling, cfDNA could be offered; however, routine screening methods remained the preferred option [3]. Additionally, the 2016 ACOG practice bulletin “Screening for Fetal Aneuploidy” discusses cfDNA as a method for aneuploidy screening without mention of maternal age [1]. Thus, while cfDNA is not yet recommended as a first-line screening option in low-risk women by ACOG or SMFM, cfDNA-based screening tests are being increasingly utilized in all-risk women and are anticipated to become first-line screening tests for all women in the near future.

## Serum screening for Down syndrome

Traditionally, aneuploidy screening in pregnancy has relied on serum screening-based methods that analyze biochemical markers in maternal blood. With serum screening,  $\sim 5\%$  of all pregnant women screen positive for Down syndrome [4, 5]. The positive predictive value (PPV) of serum screening is poor. First-trimester serum screening using nuchal translucency measurement and serum markers (pregnancy-associated plasma protein A [PAPP-A] and free beta human chorionic gonadotropin [hCG]) has a 2–3% PPV, while second-trimester screening using either a triple or quad screen (alpha-fetoprotein [AFP], estriol, beta-hCG with inhibin for quad screen) has a 2% PPV [4, 5]. Combined first- and second-trimester screening (referred to as contingent screening, sequential screening or integrated screening, each with slight differences in testing protocol), all have slightly improved Down syndrome detection rates but still have only 4% PPV [4, 5] (Table 1). Given the low PPV of serum screening, there

was a clear need for the development of a prenatal aneuploidy test with improved test characteristics. Recognition of cell-free fetal DNA in maternal circulation, in combination with improved genetic sequencing technology, would prove to be the key elements that catalyzed a major advance in prenatal aneuploidy screening.

## Cell-free fetal deoxyribonucleic acid (DNA) technology

It was first recognized in 1963 that cells can pass between the mother and fetus and can be extracted, quantified and studied [6]. Cell-free nucleic acids (fragments of DNA and ribonucleic acid [RNA] without cell membranes) were first noted in adult serum in 1948 [7]. These cell-free nucleic acids are associated with inflammatory diseases (i.e. lupus, glomerular nephritis, pancreatitis), rapid cell turnover (as seen in cancer), and tissue injury (as seen in trauma, stroke and myocardial infarct) and are thought to originate primarily from maternal hematopoietic cells [8–10]. This became relevant to pregnancy when it was reported that there is fetal DNA in maternal plasma and serum, as evidenced by the presence of fetus-derived Y sequences in maternal blood [11]. Cell-free fetal DNA is thought to originate from the placenta (specifically, the rapidly dividing syncytiotrophoblasts that undergo apoptosis) because the genetic signatures in cell-free fetal DNA are specific for placental genes [12]. Although cell-free fetal DNA is derived from placental cells, these DNA fragments generally reflect the genetics of the fetus, as well, because the fetus and the placenta arise from the same embryo [12].

The fetal proportion of total cell-free DNA in the maternal blood (referred to as the “fetal fraction”) is variable, but on average there is  $\sim 10\%$  from the fetus and the remainder is maternal [13]. Fetal cell-free DNA can be detected as early as 5–7 weeks of gestation and is rapidly cleared from the maternal circulation within hours [11, 14]. The half-life of fetal cfDNA is about 1 h in healthy women, and almost all fetal cfDNA is eliminated within 48 h of delivery [15, 16]. The fetal fraction is known to be altered by gestational age, maternal body mass index (BMI) and aneuploidy, while it is unaffected by several other clinical and demographic variables (Table 2) [17, 18].

With some uses of cell-free DNA, fetal free nucleic acids are distinguished from maternal free nucleic acids (e.g., discrepant Rh status, presence of sex-determining region Y); in others, analysis is done jointly and nucleic acids from each source are not delineated (e.g., aneuploidy) [1, 19]. All circulating cell-free DNA is fragmented, with fragment size length ranging 50–200 base pairs [20].

Cell-free DNA testing became possible by the development of and advances in next-generation sequencing, also referred to as massively parallel genomic sequencing.

**Table 1** Performance of prenatal aneuploidy screening tests in women >35 years old

	2nd-trimester quad	1st-trimester combined	Integrated screen	NIPT (cfDNA)
Detection rate	80%	90%	95%	≥98%
Screen positive rate	5%	15%	2%	0.2%
Positive predictive value	2%	2–3%	4%	80–99%
Failure rate	<<1%	<1%	<1%	0.3–3%
Complexity	1 blood draw	Ultrasound+ 1 blood draw	Ultrasound+ 2 blood draws	1 blood draw

cfDNA cell-free deoxyribonucleic acid, NIPT noninvasive prenatal test

Samples are multiplexed using identifier codes, then millions of DNA fragments are sequenced simultaneously, with the first ~36 bases of each fragment sequenced [21, 22]. Initially, four commercial labs offered cell-free fetal DNA testing, with detection rates for Down syndrome of 99%, trisomy 18 of 97–99% and trisomy 13 of 80–99% [23]. False-positive rates were reported to be <0.1–1.3% and failure rates (i.e. uninformative or “no call” reports resulting from low fetal fraction, failed quality control, failed sequencing, etc.) were reported to be 0.9–3.0% [13]. Each commercial lab uses a slightly different approach for its cfDNA test. Some labs use counting technology for the specific chromosomes of interest (21, 13, 18, X and Y) and assign the number of reads belonging to each of the chromosomes to different buckets and assess for alterations from the expected number of reads in any given bucket, with lab-specific bioinformatic pipelines. Another lab uses counting of targeted sequences on the chromosomes of interest (i.e. 21, 13, 18, X and Y), while yet another uses single nucleotide polymorphisms (SNPs) to compare maternal SNPs (from maternal leukocyte DNA) to the SNPs in mixed maternal–fetal cfDNA and assess for shifts in the pattern of informative SNPs. The SNP-based cfDNA method cannot be used if the pregnancy is the result of an egg donor, if the mother has received a bone marrow or organ transplant, or if the mother is a gestational carrier [23].

Noninvasive prenatal testing utilizing cell-free fetal DNA for Down syndrome (trisomy 21) screening is a significant advance over serum screening in women >35 years old because it has the highest detection rate (99% vs. 80–95%),

lowest screen-positive rate (0.2% vs. 2–15%), and highest positive predictive value (80–99% vs. 2–4%) [1]. Additionally, the noninvasive prenatal test requires only one blood draw, which can be performed any time after 10 weeks of gestation. One minor drawback is that the overall rate of test failure is slightly higher for the noninvasive prenatal test (0.3–3% vs. <1% for serum screening; Table 1) [23].

When combined, the detection rate for the most common autosomal aneuploidies (i.e. trisomies 21, 18 and 13) using the noninvasive prenatal test is 97% with a false-positive rate of 1.25% [23, 24]. For these aneuploidies, the noninvasive prenatal test is the most sensitive screening test available. Sensitivity is highest for trisomy 21, with a detection rate of 98.6% and false-positive rate of 1.01%. The detection rates for trisomy 18 and trisomy 13 are slightly lower, with a trisomy 18 detection rate of 94.9% and false-positive rate of 0.14%, and a trisomy 13 detection rate of 91.3% and false-positive rate of 0.14% [23, 24], accounting for “no call” results and for successful subsequent repeat tests. Detection rates are lower for the sex chromosome aneuploidies than for the autosomal aneuploidies, with a detection rate of 90.3% for 45,X and a detection rate of 93% for 47,XXY, 47,XYY and 47,XXX [24]. Also, failure rates for sex chromosome aneuploidies are higher, ranging 4–7% [24–27].

**Comparison of noninvasive prenatal testing in high- and low-risk populations**

While some low-risk women are already utilizing the noninvasive prenatal test, it has not been explicitly endorsed for primary Down syndrome screening in low-risk women for many reasons, including its higher cost, issues with the availability of appropriate pre- and post-test genetic counseling, and concerns about pregnancy termination after a positive test result without confirmatory diagnostic testing [3]. Currently, more low-risk women receive the noninvasive prenatal test as an advanced screen following abnormal serum screening or detection of a fetal abnormality. This strategy is useful for reducing the number of normal fetuses lost to invasive procedures; the screen-positive rate of the noninvasive prenatal test is much lower than that of serum screening and the positive predictive value of a

**Table 2** Factors influencing the fetal fraction of cell-free deoxyribonucleic acid (DNA) in maternal blood (adapted from [1])

Affects fetal fraction	Does NOT affect fetal fraction
Gestational age	Maternal age
BMI	Race
Aneuploidy	Parity
	Mode of conception
	Smoking
	Placental volume

BMI body mass index

noninvasive prenatal test is much higher (Table 1) [23]. However the use of the noninvasive prenatal test as an advanced screen does not improve the overall detection rate of Down syndrome because of the lower detection rate of serum screening (80–90% vs.  $\geq 98\%$  for the noninvasive prenatal test). This means that when serum screening is used as the primary screen, up to 20% of fetuses with Down syndrome fail to be screen-positive and, consequently, these pregnancies do not have advanced screening with noninvasive prenatal test [23].

There has also been hesitation to switch to the noninvasive prenatal test for all women because maternal serum screening provides additional information on other fetal disorders that would be lost if only the noninvasive prenatal test were performed. Of women who screen positive on maternal serum screening and have fetal chromosomal abnormalities detected with subsequent invasive testing, 17–23% have fetal chromosomal disorders that would be missed by current noninvasive prenatal test [28, 29]. The chromosomal abnormalities missed by the noninvasive prenatal test include smaller deletions and duplications, other aneuploidies, mosaicism and triploidy/tetraploidy. Overall there is an approximately 2% risk of the fetus having a karyotypic abnormality not detected by the noninvasive prenatal test but identified on maternal serum screening [28]. Some of these fetuses would be anticipated to have identifiable ultrasound anomalies but these fetal abnormalities might not be seen until the 18-week fetal survey.

When the noninvasive prenatal test is used in low-risk women, it is important to remember that the PPV of the test will be lower because the PPV depends on the a priori risk of the condition in a given population. Thus for women <35 years old, the PPV may be  $\sim 50\%$  overall; however this is significantly higher than the serum screening PPV of 2–4%. In studies that have examined the noninvasive prenatal test in all-risk women, the PPV ranges 45–91% (vs. a PPV of 2.4–4.2% for serum screening) [30, 31]. Additionally, the overall false-positive rate for trisomies 13 and 18 is higher in clinical practice than indicated by many of the commercial companies [32]. In contrast to the autosomal aneuploidies, the PPV for Turner syndrome (45,X) using the noninvasive prenatal test is expected to be similar in younger and older women because Turner syndrome is not associated with maternal age [33].

## Reasons for false-positive and false-negative noninvasive prenatal test results

### False positives

The noninvasive prenatal test's false-positive rate for Down syndrome is 0.1% (meaning that the cfDNA test is positive for an abnormality but the fetus is later determined to be unaffected) [2]. Most false positives result from the presence of increased chromosome 21-specific DNA, whose origin does not

reflect the chromosome composition of the fetus in the ongoing pregnancy. Possible origins of this increased DNA include the placenta (from confined placental mosaicism), a vanishing twin, maternal mosaicism and other maternal medical conditions.

### Confined placental mosaicism

Confined placental mosaicism is defined as a pregnancy with a normal fetal karyotype and a placenta that has both normal and aneuploid cell lines. Approximately 1–2% of all pregnancies at 10–12 weeks have confined placental mosaicism [34]. The primary source of fetal cfDNA in the maternal circulation is placental cells (specifically originating from the rapidly dividing syncytiotrophoblasts), and thus confined placental mosaicism can lead the cfDNA test to be positive when the placenta is aneuploid but the fetus is euploid. Confined placental mosaicism is associated with adverse pregnancy outcomes including intrauterine growth restriction and the possibility of uniparental disomy in the fetus as a result of trisomy rescue [35–37]. Many cases of confined placental mosaicism leading to false-positive noninvasive prenatal test results have been reported but the overall prevalence has not been systematically determined.

### Vanishing twins

Approximately 12–15% of all pregnancies begin as a twin gestation and, of these twins, up to 20–30% spontaneously reduce to a singleton. It has been estimated that 1–3% of singletons originate from a pregnancy with a vanished twin [38, 39]. Vanishing twins have been documented as the cause of discordant noninvasive prenatal test results, including discordant trisomy 13 results and detection of two paternal haplotypes (using the single-nucleotide-polymorphism-based noninvasive prenatal test methodology) [35, 40]. The positive noninvasive prenatal test is a result of the placenta of the vanished twin continuing to shed DNA into the maternal circulation as fragments of cfDNA following the demise. If the demise of the vanished twin occurs very early in gestation, the twin pregnancy might have never been clinically detected and, consequently, will not be recognized as a source of a false-positive noninvasive prenatal test result.

### Maternal chromosomal abnormalities

Because most noninvasive prenatal test technologies assess maternal and fetal cfDNA together, the test assumes that the maternal karyotype is normal. However results can be positive because of unrecognized sex chromosome aneuploidy in the mother. Most commonly, this is either maternal mosaic 45,X/46,XX or maternal 47,XXX [41–43]. Even women who were born with normal 46,XX karyotype have a proportion of their

cells lose an X chromosome over time, leading to some 45,X cells [43, 44]. Overall 8.6% of positive noninvasive prenatal test results for sex chromosome aneuploidy are from maternal sex chromosome mosaicism [43]. The presence of maternal mosaicism can be confirmed by performing a karyotype on a maternal peripheral blood sample [44]. In addition to maternal aneuploidy, maternal copy number variants can lead to false-positive noninvasive prenatal test results, especially when there are larger maternal duplications on the chromosomes of interest. For example, maternal duplications on chromosome 18 have led to false-positive results for trisomy 18 [45].

#### *Maternal cancer*

Women with known or undiagnosed cancer can have circulating cfDNA in their peripheral blood with one or several chromosomal alterations. This can lead their noninvasive prenatal test to return positive for multiple chromosome abnormalities or can demonstrate widespread genomic imbalance. This type of result is observed in approximately 0.03% of all noninvasive prenatal test screens [46]. In these cases follow-up diagnostic testing on the fetus reveals a normal fetal karyotype but the pregnant woman is then found to have an undiagnosed malignancy. Of all women with multiple chromosomal abnormalities on noninvasive prenatal test results and euploid fetus on diagnostic testing, approximately 18% are found to have an undiagnosed malignancy [46–48]. Documented types of maternal malignancies leading to abnormal noninvasive prenatal test results include breast, leukemia, lymphoma, colorectal, anal and neuroendocrine. While standard management recommendations are lacking for follow-up after a woman receives a noninvasive prenatal test result with multiple chromosomal abnormalities and has a confirmed euploid fetus, an oncology referral seems prudent.

#### *Maternal transplant recipients*

If a pregnant woman has received a bone marrow or organ transplant from a male donor, noninvasive prenatal test results can indicate a male fetus (due to detection of Y chromosome material from the transplant) even when the fetus is actually a female [49]. Even a recent blood transfusion (within the last 4 weeks) from a male donor can lead to indication of a male fetus on noninvasive prenatal test when the fetus is actually a female [50]. Given this, a detailed medical history should be obtained prior to performing cfDNA-based prenatal aneuploidy screening on any patient.

#### *Other issues*

False-positive results can also be caused by statistical chance or technical issues, such as incorrect sample labeling and other lab-based errors in running the test.

### **False negatives**

The noninvasive prenatal test's false-negative rate for Down syndrome is thought to be quite low (meaning that the cfDNA test indicates no chromosomal abnormality but the fetus is affected). While it is difficult to ascertain the true rate of these false-negative results, false-negatives cases have been reported for all of the common aneuploidies [51–53]. False-negative results are more common when the fetal fraction of cfDNA is low and when placental mosaicism is present.

Most laboratories that perform cfDNA screening quantify the fetal fraction, and some specify the fetal fraction on the reported results; a cutoff of  $\leq 4\%$  fetal fraction is generally used. Borderline fetal fractions of 4–5% increase the risk of failing to detect an aneuploidy because there is only a small difference between the observed and expected amounts of DNA fragments from the aneuploid chromosome. Overall there are four main reasons for low fetal fraction: early gestational age, maternal obesity, fetal aneuploidy, and issues with sample quality.

#### *Early gestational age*

The fetal fraction is much lower prior to 10 weeks' gestation. At 10 weeks' gestation, the fetal fraction is  $\sim 13\%$  of the total cfDNA and increases throughout the rest of gestation [22, 54, 55]. Thus if a woman's estimated gestational age is overestimated and noninvasive prenatal test is sent prior to 10 weeks' gestation, the noninvasive prenatal test is likely to have a lower fetal fraction.

#### *Maternal obesity*

The higher the maternal body mass index (BMI) is, the lower the fetal fraction. The lower fetal fraction is thought to result from dilutional effects from the larger maternal plasma volume of obese women, as well as increased maternal cfDNA secondary to increased inflammation. Both lead to lower fetal fraction and an increased rate of both false-negative and unreportable results [13, 22]. In contrast to the noninvasive prenatal test, when serum analyte screening is performed, maternal weight is adjusted for mathematically. Overall, obese women (weighing  $>180$  lb) have a 3–4-fold increased risk of having a failed or inaccurate cfDNA result [13]. Although women with an increased BMI can still have a noninvasive prenatal test, they should be counseled about the possibilities of test failure and false-negative results when informed consent is obtained.

#### *Fetal aneuploidy*

Fetuses with trisomy 13 and trisomy 18 who survive pregnancy and are live born have been shown to have mosaicism in their placentas, meaning that there are both euploid and aneuploid cell lines present in the placenta [56]. In these aneuploid

pregnancies, up to 40% of the placenta can have a normal euploid karyotype. Additionally, the fetal fraction of cfDNA is decreased in some aneuploid pregnancies compared to euploid pregnancies [18]. Both of these factors can lead to a false-negative or unreportable noninvasive prenatal test in aneuploid pregnancies. Regarding specific aneuploidies, the fetal fraction is lower in pregnancies with trisomies 13 and 18, as well as monosomy X, and is higher in pregnancies with Down syndrome (trisomy 21) [57]. Specifically, at 10–20 weeks of gestation pregnancies with trisomy 18 have an average 9% fetal fraction, compared with an average 13% in euploid pregnancies [57]. In trisomy 21 pregnancies, the average fetal fraction is 15%, and significant placental mosaicism (as observed in trisomies 13 and 18 pregnancies) has not been reported [56, 58]. Pregnancies with trisomy 13 are also more difficult to detect by some noninvasive prenatal test methodologies because chromosome 13 is technically more challenging to sequence; this is because of the low gene density of chromosome 13 and resultant low G+C content compared to other autosomes [59]. Triploid pregnancies are associated with very low fetal fractions [58, 60], leading to frequent noninvasive prenatal test failures in these pregnancies.

#### *Sample quality issues*

If a few white blood cells degrade from the maternal blood, the fraction of maternal cfDNA in a noninvasive prenatal test sample is increased and dilutes the fraction of fetal cfDNA. To prevent this complication, maternal specimens for noninvasive prenatal tests must be appropriately collected and stored to prevent DNA degradation.

### **Clinical dilemmas**

#### **What should be done with no-call results?**

Overall, approximately 1–5% of all noninvasive prenatal test specimens have no reportable result. Up to 50% of these are because the fetal fraction is below an acceptable level (i.e. <4%) [23]. Other failures are from insufficient numbers of cfDNA fragments sequenced or aligned to reference chromosomes. As discussed, women with a high BMI have lower fetal fraction, leading to more no-call results in this group. The percentage of no-call results increases with the degree of obesity, with 20% of women greater than 250 lb and 50% of women greater than 350 lb having a no-call result [13, 22]. When the first sample leads to a no-call result, a repeat sample leads to a reportable result in ~60% of cases. It is important to note that there is an increased risk of aneuploidy with a no-call result (as high as 20% of all samples with no result are karyotypically abnormal) [18, 61, 62]. Women with failed noninvasive

prenatal test have several options: they can have a repeat test, they can proceed with serum screening, or they can have an invasive procedure. Given the increased risk of aneuploidy in pregnancies with failed noninvasive prenatal test, it is reasonable to encourage diagnostic testing as follow-up.

#### **What should be done with twin pregnancies?**

Some companies offer use of noninvasive prenatal test in twins, although there are fewer data for cfDNA test performance in twin pregnancies than singleton pregnancies. Available data suggest that the fetal fraction is lower and failure rate is higher in twin vs. singleton pregnancies [63, 64]. Although the mechanism is not clear, the noninvasive prenatal test failure rate is especially increased in pregnancies that occurred as a result of in vitro fertilization [63, 64]. Specific data on the detection rate of aneuploidy in twin pregnancies are still lacking because of the low numbers of aneuploid twins in the studies assessing noninvasive prenatal test performance in twins.

#### **Should noninvasive prenatal testing be expanded beyond the common aneuploidies?**

Most companies offering the noninvasive prenatal test now include the option of testing for microdeletion and microduplication syndromes. These syndromes are of clinical importance because, taken together, the five most common occur in approximately 1 in every 1,000 neonates, the most frequent of which is 22q11.2 deletion syndrome (i.e., DiGeorge syndrome) [65]. The use of cfDNA-based technology to screen for microdeletions and microduplications is an emerging technology without significant clinical validation and is not currently supported by either ACOG or SMFM [1, 66–69]. Specifically, the high reported rate of both false-positive and false-negative noninvasive prenatal test results for subchromosomal abnormalities precludes the clinical utility of cfDNA-based screening for these disorders at this time. Additionally, one testing platform now offers cfDNA-based chromosome-wide screening up to a 7 megabase pair (Mb) level (MaterniT GENOME; Sequenom, San Diego, CA), which is at the resolution most equivalent to a noninvasive karyotype. There has been no independent clinical validation of this chromosome-wide noninvasive prenatal test and sensitivity and specificity remain unknown [70]; given these limitations, this chromosome-wide noninvasive prenatal test is also not currently endorsed by ACOG or SMFM.

#### **What is the current role for noninvasive prenatal testing?**

Cell-free DNA-based aneuploidy tests are appropriate as first-line screening for trisomy 21 in women of advanced maternal age (>35 years old) and other women who qualify as high-risk

[1]. The noninvasive prenatal test has been validated in singletons, but there are much fewer data on its performance in twins. The noninvasive prenatal test is not appropriate for screening microdeletion or microduplication syndromes, and it is not a replacement for diagnostic testing (i.e. CVS or amniocentesis). Diagnostic testing should be recommended following the detection of fetal anomalies on prenatal ultrasound. While the noninvasive prenatal test has not been specifically endorsed as a first-line screening test for all-risk women, SMFM advises that low-risk patients can elect cfDNA-based tests for aneuploidy screening. Because there are increasing published data on the performance of the noninvasive prenatal test in all-risk women, it could soon become the first-line screening test for all women.

### What is the role for the noninvasive prenatal test in women <35 years old?

The use of the noninvasive prenatal test for Down syndrome screening is appealing as a first-line test for all women for many reasons. Specifically, it has higher sensitivity, a very low rate of false positives and a higher PPV than standard screening with measurement of nuchal translucency and biochemical analytes [71]. The noninvasive prenatal test involves only one blood test and can be performed anytime starting at 10 weeks of gestation. Caution is still advised because the test's PPV depends on the incidence of the condition, and thus PPV is lower in all-risk women than in the high-risk population. Additionally, there is concern about eliminating Down syndrome screening based on serum analytes because additional conditions beyond aneuploidy can be detected with serum screening (e.g., elevated AFP from open neural tube defects, very low estriol associated with Smith Lemli–Opitz syndrome) [1]. If all chromosome abnormalities are considered, serum screening has a higher overall detection rate for chromosomal abnormalities than the noninvasive prenatal test in all-risk women [72]. This information would be lost if only the noninvasive prenatal test were performed. Moreover, cost efficacy of the noninvasive prenatal test versus traditional serum screening has not been demonstrated; however the cost differential has been reduced dramatically by rapidly decreasing sequencing costs.

Even if the noninvasive prenatal test would become the first-line aneuploidy screening test in all-risk women, it would not replace the need for fetal ultrasound and invasive testing when fetal anomalies are detected. Despite increased sensitivity and PPV, the noninvasive prenatal test is still a screening test, not a diagnostic test. The noninvasive prenatal test has demonstrated sensitivity for major aneuploidies (i.e. trisomies 13, 18 and 21, as well as sex chromosome aneuploidies), but these abnormalities only account for ~80% of the abnormal karyotypes [28]. The value of ultrasound screening for fetal anomalies and detection of other fetal syndromes has not

changed and should not be confused with Down syndrome screening. The detection of fetal anomalies on prenatal ultrasound should prompt a genetic counseling referral and offer of diagnostic testing (i.e. CVS or amniocentesis). The noninvasive prenatal test is not recommended as the first-line test in this setting [1, 2].

### Follow-up after noninvasive prenatal testing

Women with a screen-positive noninvasive prenatal test result should be offered invasive testing. Because CVS is also a sample of placental cells, some advocate waiting for amniocentesis (after 15 weeks of gestation) to obtain a true sample of the fetal genotype. However if the result is time-sensitive, offering a CVS with subsequent analysis of cultured cells is a reasonable option [73]. In general women with a screen-negative noninvasive prenatal test result are not offered follow-up testing. However, women who are diagnosed with a fetal anomaly at 18 weeks (after negative noninvasive prenatal test earlier in the pregnancy) should be offered diagnostic testing [1, 2].

### Management of Down syndrome pregnancies

Initially when the noninvasive prenatal test was released, there was concern that the number of terminations for pregnancies with Down syndrome would increase because of improved trisomy 21 detection by this test. However recent data suggest that termination rates for pregnancies with trisomy 21 are decreasing [74]. At our institution women who have the noninvasive prenatal test and test positive for trisomy 21 have the lowest termination rate when compared with women who undergo invasive tests and have a positive trisomy 21 result. Specifically, the percentage of women who terminate after a positive trisomy 21 result is 94% with CVS, 71% with amniocentesis and 63% with the noninvasive prenatal test [75]. Thus, women who choose the noninvasive prenatal test might be the least likely to terminate even with a positive result.

For women with known Down syndrome pregnancies, many questions arise regarding the meaning of an increased nuchal translucency on first-trimester ultrasound. In trisomy 21 pregnancies with an increased nuchal translucency, most increased nuchal translucencies resolve by the second trimester and, beyond the association with trisomy 21, the increased nuchal translucency is not predictive of whether the trisomy 21 pregnancy has a concurrent cardiac anomaly or might end in miscarriage [76, 77]. Therefore, the increased nuchal translucency should just be considered a marker of trisomy 21 in these pregnancies but not predictive of other outcomes.

The question frequently arises as to how likely pregnancy loss is following the diagnosis of trisomy 21. The rate of loss in trisomy 21 pregnancies is about 10% in the timeframe between CVS and amniocentesis and about 20% in the

timeframe from amniocentesis to term [78]. Additionally, the rate of stillbirth is increased in trisomy 21 pregnancies [78]. Overall ~25% of trisomy 21 pregnancies have growth restriction, which is independent of fetal anomalies and maternal age [79]. At our institution, in trisomy 21 pregnancies with ongoing fetal surveillance, 35% of women with trisomy 21 pregnancies were delivered for new onset non-reassuring testing; the non-reassuring testing was independent of fetal anomalies, growth restriction and maternal age [80].

At the time of delivery, if only the noninvasive prenatal test (but not diagnostic testing) has been done in the antepartum period and was positive for trisomy 21, the appropriate newborn genetic testing is a fetal karyotype, not microarray, because karyotyping allows for detection of unbalanced translocations that result in Down syndrome. While microarray detects the presence of increased copies of chromosome 21, it does not provide information on the arrangement of chromosomes. If the karyotype reveals that trisomy 21 is the result of an unbalanced translocation (3–4% of Down syndrome pregnancies [81, 82]), follow-up parental testing should be performed to evaluate for balanced maternal or paternal translocations. Such testing allows for appropriate counseling on recurrence risk.

### Implications of cell-free DNA for prenatal imaging

Since the advent of cfDNA for aneuploidy screening, there has been increasing debate surrounding the utility of nuchal translucency assessment in the first trimester. SMFM recently released guidelines for the use of ultrasound imaging in women who have undergone cfDNA screening [69]. The guidelines suggest that in women with a negative cfDNA result, ultrasound at 11–14 weeks specifically for the purpose of nuchal translucency assessment is not indicated; however first-trimester ultrasound remains important for determining viability and number of fetuses, confirming gestational age and identifying major fetal anomalies. Additionally, in women with negative cfDNA, the presence of an isolated soft marker (e.g., choroid plexus cyst, echogenic intracardiac focus) should be considered a normal variant and diagnostic testing should not be recommended for this indication [69]. In contrast, women with a fetal structural abnormality should be offered diagnostic testing with chromosomal microarray (performed on CVS or amniocentesis sample) even in the setting of a negative cfDNA screening result [69].

### Resources for cell-free DNA screening

Many resources surrounding cell-free DNA screening are available and summarized on the ACOG website: <http://www.acog.org/Resources-And-Publications/Committee-Opinions/Committee-on-Genetics/Cell-free-DNA-Screening-for-Fetal-Aneuploidy/More-Information>.

### Conclusion

Given the complexities of cfDNA screening, including the possibility both false-negative and false-positive results (as well as incidental findings), appropriate genetic counseling should be provided prior to testing and following abnormal or failed testing results. It is essential that patients be counseled about differences between screening and diagnostic tests. In particular, women should be advised that the noninvasive prenatal test has been validated as screening for trisomies 13, 18 and 21, as well as sex chromosome aneuploidies; however data are limited on screening for other conditions including other chromosomal abnormalities and microdeletion and microduplication syndromes. If women desire more detailed information about all potential chromosomal abnormalities, they should be encouraged to pursue diagnostic testing (i.e. either amniocentesis or CVS) and chromosomal microarray. Diagnostic testing should be encouraged as the next step in evaluation when there are fetal anomalies. Ultimately genetic screening is optional and is at the discretion of each individual patient.

### Compliance with ethical standards

**Conflicts of interest** None.

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