Sequencing of Circulating Cell-free DNA during Pregnancy

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Sequence analysis of cell-free DNA (cfDNA) fragments that circulate in the blood of pregnant women, along with the translation of this method into screening for fetal chromosome abnormalities, is a success story of modern genomic medicine. In less than a decade, prenatal cfDNA testing has gone from small, proof-of-principle studies to a global transformation of prenatal care. As of late 2017, a total of 4 million to 6 million pregnant women had had DNA from their plasma analyzed to screen for fetal aneuploidy. The exponential growth of the test has been a function of the role of the biotechnology industry in its development and marketing. Here we review what has been learned from the wide-scale implementation of this testing, how it has changed prenatal clinical care, and what ethical concerns have arisen, and we speculate about what lies ahead.

Path to Clinical Implementation

Twenty years ago, cfDNA was identified as a plasma analyte when Y chromosomal “fetal” DNA was extracted and amplified from the blood of pregnant women carrying male fetuses. During pregnancy, DNA fragments are released from the placenta into the maternal circulation as cytotrophoblast and syncytiotrophoblast cells undergo physiologic cycles of fusion and apoptosis (see video). In contrast to the isolation of intact fetal cells from maternal blood, analysis of circulating DNA fragments is performed on a plasma sample that contains both maternal and placental cfDNA. The ratio of placental to total (consisting of maternal and placental) cfDNA is known as the fetal fraction, which increases as pregnancy advances. Testing of cfDNA is generally performed from the 10th week of gestation onward, since this is when the fetal fraction in the maternal circulation reaches the minimum amount needed for an informative test result. The fetal fraction is only one of several variables that affect the sensitivity of the test. Other variables include the number of cfDNA molecules sequenced, the proportion of guanine and cytosine bases in a specific chromosome, and the presence of maternal and fetal copy-number variants.

Two basic sequencing approaches are used to analyze circulating cfDNA: random (whole-genome) and targeted (Fig. 1). In the whole-genome sequencing method, maternal and fetal cfDNA molecules are randomly sampled, sequenced, and mapped to specific chromosomes. The numbers of DNA molecules belonging to different human chromosomes are then counted. For pregnancies involving a fetus with trisomy 21, the proportion of cfDNA molecules derived from chromosome 21 is expected to be higher than that in a reference data set based on samples from pregnant women carrying euploid fetuses. In one targeted method, single-nucleotide polymorphisms (SNPs) on the chromosomes of interest are amplified and...
sequenced. Ratios between heterozygous SNP alleles are compared with those of other targeted chromosomes. Skewing of the ratios is expected when there is aneuploidy of a targeted chromosome. Other non–sequencing-based targeted methods are not addressed in this review.

The sequencing of cfDNA was initially studied in pregnant women who were at high risk for having a fetus with trisomy 13, 18, or 21. In a recent meta-analysis commissioned by the U.K. National Screening Committee,9 sensitivities and specificities for detection of the common aneuploidies in high-risk women were 97% and 99.7%, respectively, for trisomy 21, 93% and 99.7% for trisomy 18, and 95% and 99.9% for trisomy 13. The positive predictive values were 91% for trisomy 21, 84% for trisomy 18, and 87% for trisomy 13. In the low-risk population, the sensitivities and specificities are similar to those found in the high-risk population, but the positive predictive values are lower because of the lower prevalence of fetal aneuploidies. In the meta-analysis described above, the positive predictive values for trisomies 21, 18, and 13 were 82%, 37%, and 49%, respectively. The current standard “multiple marker” prenatal screening for aneuploidy consists of serum biochemical assays and sonographic measurement of fetal nuchal translucency. In three large-scale studies, the test performance of cfDNA sequencing was compared with that of multiple-marker screening in the general obstetrical population.10–12 In all three studies, the false positive rates associated with cfDNA screening were less than one tenth as high as that with multiple-marker screening, and positive predictive values were significantly higher. The clinical significance of the lower false positive rates is that fewer women are made anxious by a falsely abnormal screening test result, and fewer invasive diagnostic procedures that carry a risk of miscarriage, such as amniocentesis and chorionic villus sampling, are needed to determine the fetal karyotype. Some studies have already shown a 40 to 76% reduction in the number of these procedures since 2012.13,14

### Prenatal Screening Applications

Given the available evidence, professional guidelines universally recommend cfDNA testing for trisomy 21, 18, and 13 as an option for pregnant women at high risk for fetal aneuploidy.15–17 Some guidelines also support cfDNA testing for all women, because it is the most sensitive test for these common autosomal aneuploidies.16,17 In fact, the positive predictive values of cfDNA testing among low-risk women are higher than the positive predictive values of multiple-marker screening among high-risk women.16,17 However, cfDNA analysis is more expensive than multiple-marker screening. In the United States, depending on the maternal-risk status, cfDNA aneuploidy screening is accessible through some insurers, some public payers (including state Medicaid programs), and the California state screening program (as follow-up testing), as well as on a self-pay basis. To incorporate cfDNA testing into publicly funded prenatal screening programs, a variety of approaches have been taken by health care systems in other countries.18 These approaches include offering the screening as a first-tier test for all pregnant women (Belgium),19 presenting it as a second-tier test to women with risks higher than 1 in 150, as estimated by first-tier standard prenatal screening (United Kingdom),20 and prospectively studying cfDNA screening for all pregnant women rather than only those deemed to be at high risk (Netherlands).21

The resources and efforts that are required for pretest counseling associated with maternal plasma cfDNA testing are greater than those required for counseling associated with the standard screen. In consenting to a blood test that poses no fetal risk, some women may not be fully aware of the limitations of the test or they may give inadequate consideration to the effect of potentially receiving a positive result.22 Guidelines and tools have been developed to facilitate the counseling process (Table 1).23 It is also important to recognize that the common trisomies account for only one third of the chromosomal aberrations that can be identified with a diagnostic karyotype or microarray study.24 Further investigation is warranted for pregnancies with sonographic features suggestive of aneuploidies despite a negative cfDNA test report.

More recently, the spectrum of chromosomal aberrations that are reportable by cfDNA testing has greatly expanded (Table 2). Most cfDNA tests routinely assess for fetal sex and sex chromosomal aneuploidies. Some laboratories report findings for subchromosomal aneuploidies (also known as copy-number variants), including microdeletion...
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Random/uni0020Sequencing

Targeted Sequencing

Chromosomes

Maternal plasma containing maternal and fetal cfDNA fragments

Amplification by PCR to enrich targeted SNPs on chromosome 21 and other chromosomes of interest

Sequencing of the targeted alleles

Alignment and mapping

Counting of DNA molecules from each chromosome

Percentage of chromosome 21 fragments among sequenced cfDNA is determined and compared with a reference sample; percentage will be elevated in trisomy 21 pregnancies

Allelic ratios of amplified fragments derived from chromosome 21 are compared with those of fragments derived from other target chromosomes. A skewed ratio of the alleles on chromosome 21, as compared with the ratio on other chromosomes, yields a positive result.

Randomly selected cfDNA sample

Genomewide sequencing

Alignment and mapping

Chromosome 1

Chromosome 21

Chromosome X

Chromosome Y

SNP

Calculation of ratios between SNP alleles on chromosome 21

Allele 1 : Allele 2

N : N

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and microduplication syndromes. Prenatal screening for these conditions is hampered by limited prospective clinical studies and incomplete outcome data. For this reason, professional guidelines do not currently recommend it for routine screening. The combined or individual incidence of these rarer syndromes, as well as the potential to provide early neonatal treatments, are the main considerations cited by advocates for expanded testing. For both biologic and analytic reasons, the positive predictive values for detecting these conditions are lower than those of the common autosomal trisomies, resulting in more false positive cases needing invasive confirmatory testing to determine the true fetal karyotype. The need for further evaluation of cfDNA approaches in the screening for these additional conditions remains considerable.

**Figure 1 (facing page). The Two Main Methods of Maternal Plasma DNA Sequencing for Prenatal Screening of Fetal Chromosomal Aneuploidies.**

Testing for trisomy 21 is shown as an illustrative example. In random sequencing, cell-free DNA (cfDNA) fragments originating from any chromosome are sequenced. A random representative selection of maternal (purple fragments) and fetal (orange fragments) cfDNA molecules is sequenced. The DNA molecules belonging to different human chromosomes are counted to determine the proportion of cfDNA molecules derived from chromosome 21. The proportion of chromosome 21 DNA sequences is elevated if the plasma sample was collected from a pregnant woman carrying a fetus with trisomy 21. In targeted sequencing, only cfDNA from specific chromosomes of interest is sequenced. Loci of single-nucleotide polymorphisms (SNPs, highlighted in blue) on targeted chromosomes are amplified and sequenced. Fetal cfDNA from chromosome 21 is indicated by orange fragments with blue highlighting. Maternal cfDNA from chromosome 21 is indicated by purple fragments with blue highlighting. Ratios between heterozygous SNP alleles on the cfDNA from chromosome 21 are compared with ratios similarly computed for other targeted chromosomes. If the fetus is aneuploid, allelic ratios on the aneuploid chromosome are skewed in comparison with other, noneanuuploid chromosomes.

**Table 1. Key Points to Consider When Providing Pretest Counseling.**

- State that testing is optional.
- Clarify that this is a screening test and not a diagnostic test.
- Describe limitations of the test (i.e., what it does not test for).
- Review the clinical features and variability of the conditions being screened.
- Briefly review test methods and reporting formats.
- Define positive and negative predictive values and their clinical significance.
- Recommend that all positive screening tests be confirmed with a diagnostic test to determine fetal or neonatal karyotype.
- Mention the possibility of incidental findings regarding maternal health.
- Refer the patient to specialists in medical genetics for unusual test results.

* Modified from Sachs et al.

**Table 2. Conditions for Which Cell-free DNA Testing Is Clinically Available.**

- Common autosomal aneuploidies
  - Trisomy 21
  - Trisomy 18
  - Trisomy 13

- Sex chromosome aneuploidies
  - 45,X
  - 47,XXX
  - 47,XXY
  - 47,XY

- Rare autosomal aneuploidies
  - Whole-chromosome aneuploidy of any autosome (trisomy 7, 15, 16, and 22 are the most commonly detected)

- Microdeletion and microduplication syndromes
  - 1p36 deletion
  - Wolf–Hirschhorn syndrome (terminal 4p deletion)
  - Cri du chat syndrome (terminal 5p deletion)
  - Langer–Giedion syndrome (8q24 deletion)
  - Jacobsen’s syndrome (terminal 11q deletion)
  - Prader–Willi and Angelman syndromes (15q11.2-q13 deletion)
  - DiGeorge syndrome (22q11.2 deletion)

- Copy-number variants larger than 7 Mb
- Triploidy

* The sex of the fetus is also reported if the patient requests it, but not in all countries.
fall short of the threshold generate a “no call” result. Certain aneuploidies, such as digynic triploidy, have been reported to be associated with low fetal fractions, increasing the risk of false negative results if one does not follow up on the no-call results.

A low fetal fraction occurs more frequently in early gestation and with assisted reproduction. Maternal body weight is negatively correlated with fetal fraction because of the increased inflammation and apoptosis that occur in adipose tissue of obese pregnant women, resulting in an increased release of maternal cfDNA into the circulation. The association of low fetal fractions with maternal thromboembolic disorders, heparin use, and vitamin B12 deficiency is thought to be due to increased maternal cfDNA release as a result of maternal blood clotting and intramedullary hemolysis or possibly to a direct effect on the trophoblasts. Even with an adequate fetal fraction, false negative results may occur because of true fetal mosaicism or an aneuploid fetus with a euploid placenta (Table 3).

### Table 3. Reported Biologic Causes of False Positive and False Negative Cell-free DNA Results

<table>
<thead>
<tr>
<th>Causes of false positive results</th>
<th>Causes of false negative results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Confined placental mosaicism (placenta aneuploid, fetus euploid)</td>
<td>Low fetal fraction</td>
</tr>
<tr>
<td>True fetal mosaicism</td>
<td>Maternal obesity</td>
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<tr>
<td>Death of a twin in utero</td>
<td>Multiple gestation causing low fetal fraction per fetus</td>
</tr>
<tr>
<td>Maternal incidental findings</td>
<td>Maternal medical condition or treatment affecting quality of circulating DNA</td>
</tr>
<tr>
<td>Copy-number variant</td>
<td>Certain fetal chromosomal aneuploidies (e.g., triploidy)</td>
</tr>
<tr>
<td>Chromosome abnormality</td>
<td>Confined placental mosaicism (placenta euploid, fetus aneuploid, or mosaic)</td>
</tr>
<tr>
<td>45,X or 47,XXX</td>
<td>Fetal fraction because of the increased inflammation and apoptosis that occur in adipose tissue of obese pregnant women, resulting in an increased release of maternal cfDNA into the circulation. The association of low fetal fractions with maternal thromboembolic disorders, heparin use, and vitamin B12 deficiency is thought to be due to increased maternal cfDNA release as a result of maternal blood clotting and intramedullary hemolysis or possibly to a direct effect on the trophoblasts. Even with an adequate fetal fraction, false negative results may occur because of true fetal mosaicism or an aneuploid fetus with a euploid placenta (Table 3).</td>
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### Biology of False Positive Results

Clinical practice standards recommend confirmation of positive cfDNA screening results with a diagnostic karyotype or microarray study. The type of diagnostic procedure may be influenced by the type of aneuploidy detected. In a study involving 52,673 women who underwent both chorionic villus sampling and amniocentesis, the likelihood of finding confined placental mosaicism was higher for trisomy 13 and monosomy X than for trisomy 21 or 18. For trisomy 13 and monosomy X, therefore, amniocentesis may more accurately reflect the true fetal karyotype. If the cfDNA result shows trisomy 18 or 21, chorionic villus sampling may be the most appropriate confirmatory test, albeit with a 2% to 4% chance of an inconclusive result.

When the diagnostic fetal karyotype is discordant with the cfDNA screening result (Table 3), a potential explanation may be that there is true imbalance of the test:reference chromosome ratios. If, however, the whole-genome sequencing results are masked, an incomplete or inaccurate interpretation appears in the test report. For example, a rare autosomal trisomy will have excess DNA from a nontarget trisomic chromosome such as 7, 16, or 22, which, when measured relative to the typical chromosomes of interest (13, 18, and 21), skews the ratios and manifests as either a test failure or nonphysiologic results, such as multiple monosomies. Rare autosomal trisomies can be due to confined placental mosaicism or true fetal mosaicism (Table 3). They are increasingly recognized and challenge long-held assumptions regarding fetoplacental
biology because they show that fetuses with rare autosomal trisomies can survive well into the third trimester. Rare autosomal trisomies are also associated with poor obstetrical outcomes, including in utero growth restriction and stillbirth.

The death of a twin in utero (“vanishing twin syndrome”) is another biologic reason for false positive results. Estimated to occur in approximately 0.42% of pregnancies, this phenomenon most commonly manifests as sex discordance, in which Y-chromosomal cfDNA is detected and the fetus appears female on sonographic examination. Another reason for sex-discordant results is that the mother has previously received a bone marrow or organ donation from a male donor.

Because sequencing is performed on a sample that contains both maternal and placental DNA, it is not surprising that many false positive results are of maternal origin. In one study, two thirds of the cases for which follow-up information was available were due to maternal incidental findings. Although professional guidelines recommend discussing the possibility of detection of maternal DNA abnormalities in pretest counseling, in practice, this is not done. Maternal mosaic sex chromosome aneuploidies (45,X and 47,XXX), both constitutional and somatic, are common reasons for the lower positive predictive values observed in cfDNA testing of the sex chromosomes.

Clinically asymptomatic pregnant women have also been shown to have autosomal aneuploidies, such as mosaic trisomy 8 or 18. Benign copy-number variants exceeding 500 kb may be present in as much as 10% of the general population. If bioinformatics algorithms do not account for these variants, an asymptomatic maternal microduplication may generate a false positive result.

When a pregnant woman harbors a malignant tumor, apoptotic cell-free tumor DNA can be shed into the circulation. If this occurs, then whole-genome sequencing techniques may detect genomewide imbalance that can be misinterpreted as fetal aneuploidy. There are increasing reports of a variety of clinically silent malignant neoplasms detected through prenatal screening. The clinical utility of disclosing cfDNA results that are suggestive of cancer is unknown. Follow-up studies are needed to determine the appropriate clinical management. In addition, some uterine leiomyomas will also manifest with genomewide imbalance. Uterine leiomyomas are very common, particularly in black women, but it is not currently known whether there is biologic significance to the subset of these benign tumors that are detected through prenatal cfDNA sequencing.

Analysis of cfDNA is also used to noninvasively test for fetal single-gene disorders in couples who are at high risk because of their personal or family history (Fig. 2). This testing is based on direct detection of paternally inherited or de novo mutant DNA sequences in maternal plasma. The presence of Y-chromosomal DNA sequences in maternal plasma aids further management if the fetus is at risk for a sex-linked disorder. If there is a family history of congenital adrenal hyperplasia, cfDNA results that are suggestive of a female fetus may influence the decision to start prenatal glucocorticoid treatment. With sensitivities as high as 99%, noninvasive rhesus D genotyping in rhesus D-negative women facilitates the management of fetal blood-group incompatibility without increasing the risk of sensitization. Testing of cfDNA has also been used to diagnose skeletal dysplasias in fetuses with suspicious sonographic findings.

To investigate a maternally inherited condition or an autosomal recessive disorder, cfDNA methods are used to assess whether there is proportionally more or less of the maternal mutant allele or haplotype in comparison with its non-mutant counterpart. The allele or haplotype that is present in greater excess is the one inherited by the fetus. Assessment of relative mutation dosages in maternal plasma by means of digital polymerase chain reaction has been shown for beta-thalassemia, sickle cell anemia, and hemophilia. For the analysis of structural mutations (e.g., DNA inversions) and mutations that are complicated by the existence of homologues, an evaluation of relative haplotype dosages is recommended. Sequence data from cfDNA analyses are used to reconstruct the haplotype structure surrounding the potentially mutated locus. The combined quantity of cfDNA originating from the mutant haplotype is compared with that of the opposite haplotype. The haplotype that is more abundantly represented by cfDNA is the one inherited by the fetus. Proof of principle for this approach has been demonstrated for the pre-
natal assessment of congenital adrenal hyperplasia, Hunter’s syndrome, and hemophilia A.\textsuperscript{42}

**ETICAL AND LEGAL CONCERNS**

Testing of cfDNA for aneuploidy has been developed and marketed as a laboratory test that is monitored by the Clinical Laboratory Improvement Amendments program in the United States. At present, the Food and Drug Administration (FDA) does not provide oversight or approval. There are no standards regarding what must be disclosed to the medical community or potential patients regarding the performance characteris-
opportunities for trainees to learn how to perform downstream consequence is that there are fewer has occurred as a result of cfDNA testing, a
commercial laboratories. Efforts are under way to provide unbiased, curated, and easily accessible materials have been developed.46 Another outcome is a reduction in referrals to genetic counseling practices for families affected by single-gene disorders because some practitioners mistakenly think that cfDNA screening tests for all genetic conditions.47

With regard to clinical applications, there is the ethical concern that the testing is being used to determine fetal sex. Lastly, although there has been apprehension among disability advocacy groups that the ease of cfDNA testing would decrease the number of live-born infants with trisomy 21, evidence from recent studies suggests that this is not the case; a recent comparative study shows no evidence that the rates of elective termination have changed.48

**WHAT LIES AHEAD**

Sequencing of cfDNA for detection of the common fetal autosomal aneuploidies is likely to be increasingly adopted by publicly funded programs as a first-tier test for both high-risk and low-risk women because of its superior performance in screening for the common aneuploidies. A major issue posed by the rapid advance of this technology is the question of what should be screened for prenatally. To date, clinical utility, as demonstrated by the reduction of unnecessary invasive procedures, has been reproducibly demonstrated only for the common autosomal aneuploidies. Noninvasive whole-genome sequencing for rare autosomal trisomies could perhaps shed light on the origins of miscarriages that occur after 10 weeks of gestation.

Noninvasive prenatal screening for single-gene disorders is an active area with increasing test volumes. Methodologic strategies have also been achieved for the noninvasive prenatal detection of fetal de novo mutations and decoding of the entire fetal genome.49 Maternal plasma placental RNA sequencing also has potential clinical utility as a screen for preeclampsia and preterm birth.50

**CONCLUSIONS**

Maternal plasma cfDNA sequencing has translated from a research endeavor to clinical care since its feasibility was first demonstrated.7,8 Driven by the desire of pregnant women to have safer prenatal screening and stimulated by commercial incentives, the clinical adoption of cfDNA sequencing for chromosomal aneuploidy screening has already had a global effect. Discordant test results have led to new biologic insights re-
An audio interview with Dr. Bianchi is available at NEJM.org

regarding the fetus, placenta, and pregnant woman. Indeed, the results have led to a new appreciation of what genetic abnormalities may be present in a seemingly healthy pregnant woman. Maternal plasma cfDNA sequencing represents a major advance in genomic medicine that has resulted in more precise screening, reduced invasive procedures, and created multiple ethical challenges. 

Disclosure forms provided by the authors are available with the full text of this article at NEJM.org.

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