Implementation of Maternal Blood Cell Free DNA Testing in Early Screening for Aneuploidies

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Abstract
In the past 9 years, several externally blinded validation and implementation studies have shown that it is now possible, through analysis of cell-free (cf) DNA in maternal blood, to effectively detect a high proportion of fetuses affected by trisomies 21, 18, and 13 at a much lower false positive rate than all other existing screening methods. This article aims to review the technical and clinical considerations for implementing cfDNA testing in routine practice, including methods of analysis, performance of the test, models for clinical implementation, and interpretation of results.

Keywords: Screening; Aneuploidies; First-trimester; Cell-free DNA; Noninvasive

Introduction
Aneuploidies are major causes of perinatal death and childhood handicap. Consequently, the detection of chromosomal abnormalities constitutes the most frequent indication for invasive prenatal diagnosis. However, invasive testing by amniocentesis or chorionic villous sampling is associated with a risk of miscarriage, and therefore these tests should only be carried out in pregnancies considered to be at high risk for aneuploidies.1

In the last 9 years, several externally blinded validation and implementation studies have shown that it is now possible, through analysis of cell-free (cf) DNA in maternal blood, to effectively detect a high proportion of fetuses affected by trisomies 21, 18, and 13 at a much lower false positive rate than all other existing screening methods.2 There is also some evidence that cfDNA testing can detect other autosomal trisomies, sex chromosome aneuploidies, and triploidy and even sequence the complete fetal genome that has led some laboratories to offer screening for fetal chromosomal aberrations of more than 3–7 megabases (Mb) on any chromosome.2–5

Since sensitivity and specificity of cfDNA testing are not 100%, the test should not be considered a diagnostic test to replace invasive testing but a new screening test that identifies a high-risk group requiring further investigation by invasive testing.

This article aims to review the technical and clinical considerations for implementing cfDNA testing in routine practice.

Current practice in screening for aneuploidies

Methods of screening
In the 1970s, the main method of screening for trisomy 21 was by maternal age and in the 1980s by maternal serum biochemistry and detailed ultrasonographic examination in the second trimester. In the 1990s, the emphasis shifted to the first trimester when it was realized that the great majority of affected fetuses could be identified by a combination of maternal age, fetal nuchal translucency (NT) thickness, and maternal serum β-human chorionic gonadotropin and pregnancy-associated plasma protein A. Screening by this combined test can identify about 90% of fetuses with trisomy 21 for a FPR of 5%.6 In many countries all over the world, like the United Kingdom, there is a national program of screening for trisomy 21 based on the combined test and the offer of invasive testing at a certain risk cutoff. However, in most countries there are no national guidelines on screening and individual practitioners offer a variety of first and/or second trimester methods, often driven by market forces and the rules of supply and demand. Consequently, in some countries the rate of invasive testing ranged from 20% to 40% before the introduction of cfDNA testing. From 2012, there has been a rapid widespread introduction of the cfDNA testing in clinical practice, first in the private sector and then in the public sector. However, there are very few countries that have established national policies for offering the cfDNA test and in those that have, different strategies, from universal to contingent screening, have been adopted.

Aneuploidies included in screening
Traditionally, screening for aneuploidies has focused on trisomy 21. However, invasive testing in the screen positive group often leads to the detection of many additional
clinically significant aneuploidies. In the case of some aneuploidies, such as trisomies 18 and 13, triploidy, and monosomy X, their incidence in the screen positive group for trisomy 21 is much higher than in the screen negative group because they have a similar pattern in the expression of biophysical and biochemical markers. Therefore, by using the first trimester combined test for the screening of trisomy 21, detection of other aneuploidies was given at no extra “cost,” meaning with no increase in the FPR. However, this cannot apply to cfDNA testing because for every condition we include in the analysis, we are adding its related FPR; for example, if we test for trisomy 21 alone, the FPR is only 0.04%, but if we include trisomies 18 and 13, the FPR goes up to 0.12%, which, although still extremely low, would continue to increase with every single new condition analyzed.

On the other hand, prenatal detection of fetal anomalies that are potentially associated with genetic conditions necessitates invasive diagnosis and the use of any method of screening, regardless of its accuracy, is not an appropriate option in these cases. Moreover, the lack of sufficient scientific evidence is a burden for including sex chromosome aneuploidies, rare autosomal aneuploidies, or subchromosomal anomalies in routine cfDNA screening. There is also the difficulty in parental counseling when discussing these conditions, either due to the wide spectrum in their clinical manifestation or due to inappropriate understanding of the disease.

For all the reasons above, there is no current recommendation to include any other condition in addition to trisomies 21, 18, and 13 when requesting cfDNA testing for screening of aneuploidies even if it is technically possible.

**Screening for aneuploidies by cell-free DNA testing in maternal blood**

**Performance of the test in screening for trisomies 21, 18, and 13**

A recent meta-analysis in singleton pregnancies reported that in the combined total of 1963 cases of trisomy 21 and 223,932 non-trisomy 21 singleton pregnancies, the weighted pooled detection rate (DR) was 99.7% (95% CI, 99.1%–99.9%) and FPR was 0.04% (95% CI, 0.02%–0.07%); in a total of 563 cases of trisomy 18 and 222,013 unaffected pregnancies, the pooled weighted DR and FPR were 97.9% (95% CI, 94.9%–99.1%) and 0.04% (95% CI, 0.03%–0.07%), respectively; and in a total of 119 cases of trisomy 13 and 212,883 unaffected singleton pregnancies, the pooled weighted DR and FPR were 99.0% (95% CI, 97.2%–100%) and 0.03% (95% CI, 0.02%–0.07%), respectively. Similarly, a recent meta-analysis in twin pregnancies reported that in the combined total of 56 trisomy 21 and 3718 non-trisomy 21 singleton pregnancies, the pooled weighted DR and FPR were 98.2% (95% CI, 96.9%–99.5%) and 0.03% (95% CI, 0.01%–0.06%), respectively; in 119 cases of trisomy 18 and 3143 non-trisomy 18 pregnancies, the pooled weighted DR and FPR were 98.9% (95% CI, 97.2%–99.8%) and 0.03% (95% CI, 0.01%–0.06%), respectively; although the number of twin pregnancies with trisomy 13 (n = 3) was too small for accurate assessment of DR, the average FPR for trisomy 13 of 0.19% (5/2569) seems slightly higher than the values reported for singleton pregnancies. These results show that, by far, cfDNA testing is the best available method for screening of trisomy 21.

**Detection of other aneuploidies**

Studies on a smaller number of confirmed cases have reported the ability of cfDNA analysis in maternal blood to detect sex chromosome aneuploidies, rare autosomal trisomies, triploidy, microdeletion and microduplication syndromes, and even monogenic disorders. However, the exact performance and clinical utility of the test for these conditions require further investigation.

**Methods for analysis**

By parallel sequencing of numerous cfDNA fragments, millions of nucleotide sequences can be amplified and sequenced. This results in a large amount of data that bioinformaticians have to analyze and compare with the reference genome. Two main approaches for analysis have been used in the main clinical studies assessing performance of cfDNA testing: massively parallel shotgun sequencing (MPSS) by which the whole genome is analyzed, and targeted chromosome analysis by next-generation sequencing, custom microarray, or single-nucleotide polymorphisms (SNP) analysis, which is directed and limited only to the chromosomes of interest.

**Massively parallel shotgun sequencing**

Several millions of maternal and fetal cfDNA fragments from maternal plasma are sequenced. Next, the origin of each fragment is established and the number of DNA fragments derived from each of the chromosomes is quantified. In pregnancies with a trisomic fetus, the number of molecules derived from the extra chromosome in proportion to the rest of the sequenced molecules (in general chromosome 3 is used as a reference) is higher than in diploid gestations. It requires a large number of sequences (depth of sequencing or “coverage”) and a great biomathematical effort to examine these numerical changes that, sometimes, are minute.

By this method the molecules of all the chromosomes are examined, so it is potentially able to identify all the aneuploidies. However, since chromosome 21 represents only 1%–2% of the human genome, it is necessary to sequence many millions of molecules from the whole genome to ensure a minimum of chromosome 21 counts that allows differentiation between trisomy 21 and euploid pregnancies. This method has a high performance in the screening of trisomies 21, 18, and 13 and sex chromosome aneuploidies, with a low failure rate (<1%) since not all laboratories systematically determine the fetal fraction.

**Chromosome selective sequencing**

The basic principles are the same as for MPSS but by chromosome-selective sequencing (CSS), the selective assay is directed against specific regions of chromosomes.
Clinical implementation of cell-free DNA testing in maternal blood

In the last 40 years of screening, we have learnt that pregnant women are able to use sophisticated screening information to make scientifically and ethically rational decisions about invasive testing. In the case of trisomy 21, the rate of invasive testing increases exponentially with increasing estimated risk for this aneuploidy and the opposite is also true. Therefore, although the main achievement of the introduction of cfDNA testing as a method of screening is the substantial reduction in the invasive testing rate worldwide, a small proportion of the population at very low risk for aneuploidies still demands invasive testing for an increasing number of conditions made possible by molecular techniques. On the opposite side of the spectrum, some women at a very-high-risk for aneuploidies choose to avoid having an invasive test and for them, cfDNA testing may help reinforce the suspected diagnosis, guide pregnancy care, and prepare the prospective parents.

There are few limitations when offering cfDNA testing because, although most studies were carried out in high-risk pregnancies, increasing number of studies performing the test in routine population have demonstrated that this test is equally effective in low-risk pregnancies. Moreover, the test can be reliably performed at any time during pregnancy starting from 10 weeks’ gestation; therefore, the best approach to implement screening for aneuploidies by cfDNA testing is to take the maternal blood for cfDNA analysis within the first trimester. By doing so, it would be possible to retain the advantages of first-trimester screening: first, early reassurance of the majority of parents that the fetus is unlikely to be aneuploid and the option for first-trimester termination of pregnancy for the few where the fetus is found to be affected, and second, early diagnosis of major fetal defects and assessment of risk for pregnancy complications.

Primary method of screening

There are two possible options: first, to take the blood at 10 weeks, in which case the results of the test would be available at the time of the scheduled first-trimester ultrasound examination, which is ideally performed at 12 weeks; second, to take the blood at 12 weeks after the first-trimester examination. The major advantage of taking the blood sample at 10 weeks is that the results of the test should be available at the time of the first-trimester scan, which will then be solely performed to diagnose major fetal defects and evaluate the risk of pregnancy complications. In addition, it would allow the realization of a rescue first-trimester combined test in those cases in which the cfDNA test has not provided results. However, this model, has
the disadvantage of performing many unnecessary tests for pregnancies that miscarry spontaneously before the 12th–13th week or that are diagnosed of having increased fetal NT or major defects requiring of invasive testing at the time of the ultrasound.28 By taking the blood sample after the first-trimester assessment, these problems would be overcome but with the disadvantage of losing the possibility of performing rescue first-trimester combined test in those cases without cfDNA result, especially if the ultrasound was performed in week 13th.

**Contingent screening based on the results from another method of screening**

An alternative to universal screening by cfDNA testing is to offer cfDNA testing contingent on the results of first-line screening by another method, preferably the first-trimester combined test. cfDNA testing could be offered to the high-risk group as an alternative to invasive testing aiming to reduce invasive testing rate, or to the intermediate-risk group aiming to increase DR of aneuploidies.29 The exact risk cutoffs that define the high- and intermediate-risk groups will depend on the cost of cfDNA testing and, therefore, the proportion of the population that can be offered this test.30

**Interpretation of results from cell-free DNA testing**

If cfDNA testing reports a high-risk for trisomies 21, 18, or 13, it does not mean that the fetus definitely has one of these aneuploidies and it is important to confirm or refute the result by invasive testing. In contrast, if cfDNA testing reports a low-risk, the parents can be reassured that it is highly unlikely that the fetus has one of these aneuploidies. However, these results should always be interpreted together with a detailed ultrasound examination that has excluded increased fetal NT and major malformations. In those cases where fetal NT is above 3.5 mm or there are any major fetal defects, irrespective of the cfDNA results, parents should be offered invasive testing with array analysis not only to exclude the three major trisomies but also other chromosomal and subchromosomal conditions.

Those cases where cfDNA testing does not provide a result must be managed individually. As explained before, the main reason why the test fails to provide a result is a low fetal fraction and the main determinants for this to occur are maternal obesity and a low placental mass. In trisomies 18 and 13, but not in trisomy 21, the fetal fraction is lower and the rate of no-reults is therefore higher than in unaffected pregnancies.31 Consequently, those pregnancies in which a result from cfDNA test is not obtained can be considered at high-risk for trisomies 18 and 13, but not for trisomy 21. The management of these cases will depend essentially on the reason why the test was performed in the first place. If there is a previous screening that has already shown a low-risk result without fetal defects, it is preferable to repeat the cfDNA test explaining to the parents that there is a >60% chance that a result will be obtained in the second attempt. However, some pregnant women will prefer not to perform the test again to avoid the anxiety generated by the inconclusive result of the first one; in these cases and in those in which the test fails for the second time, it is advisable to perform a detailed ultrasound looking specifically for fetal anomalies associated with trisomies 18 and 13 and if these are present, an invasive test should be recommended.31 In cases in which previous screening has already shown a high risk for these conditions but the detailed ultrasound has not detected any findings suggestive of fetal pathology, most patients will choose to repeat the cfDNA test, although some will prefer to perform an invasive test directly.

**Conclusion**

cfDNA analysis of maternal blood is the best available method for screening of trisomy 21, providing reliable results from the first trimester of pregnancy. Since sensitivity and specificity are not 100%, cfDNA testing is not a diagnostic test but a high-performance screening test that identifies a high-risk group requiring further investigation by invasive testing. Therefore, results from cfDNA testing should never be interpreted alone, but with an ultrasound assessment of fetal anatomy.

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**Conflicts of Interest**

None.

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