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María M. Gil[1,2,3] · Kypros H. Nicolaides[1]

[1] Fetal Medicine Research Institute, King's College Hospital, London, UK

[2] Obstetrics and Gynecology Department, Hospital Universitario de Torrejón, Torrejón de Ardoz, Madrid, Spain

[3] School of Health Sciences, Universidad Francisco de Vitoria (UFV), Pozuelo de Alarcón, Madrid, Spain

Implementation of maternal blood cell-free DNA testing in early screening for aneuploidies

Introduction

Aneuploidies are major causes of perinatal death and childhood handicap. Consequently, the detection of chromosomal disorders 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 of aneuploidies [1].

Several externally blinded validation and implementation studies carried out over the last 9 years 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 (FPR) than all other existing screening methods [2]. There is also some evidence that cfDNA testing can detect other autosomal trisomies, sex chromosome aneuploidies, triploidy, and even sequence the complete fetal genome, which has led some laboratories to offer screening for fetal chromosomal aberrations of more than 3–7 megabases (Mb) on any chromosome [2–5].

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

This article is aimed at reviewing technical and clinical considerations of 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 it was 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, maternal serum β-human chorionic gonadotropin (β-hCG), and pregnancy-associated plasma protein A (PAPP-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 UK, 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 cut-off. 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 20–40% before the introduction of cfDNA testing. Since 2012, there has been a rapid and widespread introduction of cfDNA testing into clinical practice, first in the private and then in the public sector, but similarly, very few countries have established national policies for offering cfDNA and those that have done so have adopted different strategies, from universal to contingent screening.

Aneuploidies included in screening

Traditionally, screening for aneuploidies has been 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 Turner syndrome, 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 [6–9]. 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% [2] which, although still extremely

low, would continue to increase with every single new condition analyzed.

On the other hand, prenatal detection of fetal anomalies 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, not only the lack of sufficient scientific evidence is a burden for including sex chromosome aneuploidies, rare autosomal aneuploidies or subchromosomal anomalies in routine cfDNA screening but also the difficulty in parental counselling when discussing these conditions, either because of the wide spectrum of their clinical manifestation or because of inappropriate understanding of the disease.

For all the reasons above, there are no current recommendations 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 [10, 11].

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 the 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 65.8–100%) and 0.04% (95% CI 0.02–0.07%) [2]. Similarly, a recent meta-analysis in twin pregnancies reported that in a combined total of 56 trisomy 21 and 3,718 non-trisomy 21 twin pregnancies, the pooled weighted DR and FPR were 98.2% (95% CI 83.2–99.8%) and 0.05% (95% CI 0.01–0.26%), respectively. In a total of 18 cases of trisomy 18 and 3,143 non-trisomy 18 pregnancies, the pooled weighted DR and FPR were 88.9% (95% CI 64.8–97.2%) and 0.03% (95% CI 0.00–0.33%) respectively. Although the number of twin pregnancies with trisomy 13 ($n = 3$) was too small for accurate assessment of the DR, the average FPR for trisomy 13 of 0.19% (5 out of 2,569) seems slightly higher than the values reported for singleton pregnancies [12]. These results show that cfDNA testing is by far the best available method for screening of these conditions.

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 [5, 13–15]. 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 bioinformatics 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, either by next-generation sequencing, custom microarray or single-nucleotide polymorphisms (SNP) analysis, which is directed and limited only to the chromosomes of interest.

MPSS

Several millions of cfDNA fragments from maternal plasma are sequenced, of both maternal and fetal origin. 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 [16, 17]. 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; thus, it is potentially able to identify all the aneuploidies. However, as 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 pregnancies with trisomy 21 and euploids. 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%), as 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 21, 18, 13, X, and Y before sequencing. CSS evaluates SNPs in other chromosomes to estimate the fetal fraction [18]. The advantages of this technique are, in the first place, the theoretical reduction in cost, as the number of regions that need to be sequenced is substantially lower than when sequencing the whole genome and, second, the simultaneous calculation of the fetal fraction in the same assay. The disadvantage is that the failure rate in providing results may be somewhat higher (2%) than with the MPSS, although a recent meta-analysis did not show significant differences [2].

Microarray

Recently a new technique has been developed that is substituting CSS, in which, instead of using next-generation sequencing as a counting method, a custom microarray is utilized [19]. This method has shown results comparable with those obtained by CSS, but more cost-effectively and with a shorter time to obtain results [19, 20].

SNPs

Single-nucleotide polymorphisms are variations of DNA that help distinguish among different individuals. An SNP represents a difference in a single nucleotide (a base) within a certain DNA sequence, which for everything else is identical among individuals. The SNP-based method of analyzing cfDNA in maternal blood is based on the principle that the fetus has different SNPs than the mother. The maternal plasma is analyzed, which contains a mixture of maternal and fetal DNA, and the DNA of the buffy coat, which is only of maternal origin. Using a conventional polymerase chain reaction (PCR) variant, the multiplex PCR, more than 13,000 polymorphic loci are quantified simultaneously on chromosomes 21, 18, 13, X, and Y [15]. As the mother and fetus have different specific SNP patterns, these small differences can be used throughout the genome to estimate whether the fetal distribution in comparison to the mother's is consistent with monosomy, disomy or trisomy. As a method itself, this technology would be expected to be the most accurate, even at lower fetal fractions. However, this has not been shown in published studies, with reported performance for the detection of trisomies 21, 18, and 13 similar to that of MPSS or CSS, but with a nonsignificantly higher failure rate (3–5%) [2].

Others

Even before the spread of next-generation sequencing, many groups were already working on the development of a cfDNA test for the screening of aneuploidies based on PCR, such as real-time PCR or digital PCR [21]. There are already laboratories that offer the test using this method and, although the validation studies show results comparable with those obtained by MPSS, no large-scale prospective validation study has yet been published in the general population. More recently, a proof-of-principle study on a method based on highly specific chromosomal fluorescent labeling has been published [22].

Limitations of the test

There are two main limitations of cfDNA testing in the implementation of this method of screening for aneuploidies. First, although the cost of the test is similar to that of invasive testing and karyotyping, it is considerably higher than that of the currently available screening methods. Second, there is a rate of failure of the test to provide results of about 1% [2]. An important cause of not getting a result from cfDNA testing is a low fetal fraction, which is often a consequence of maternal obesity but also secondary to a small placental mass [23, 24].

Clinical implementation of cell-free DNA testing in maternal blood

Over 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 [25]. 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 [25]. 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 to reinforce the suspected

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Abstract

Several externally blinded validation and implementation studies in the last 9 years 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 (FPR) than all other existing screening methods. This article is aimed at reviewing 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

Cell-free DNA · Non-invasive prenatal testing · Trisomies · Prenatal screening · Aneuploidies

Implementierung von Tests zur Analyse zellfreier DNA im mütterlichen Blut in das Frühscreening auf Aneuploidien

Zusammenfassung

Verschiedene extern verblindete Validierungs- und Implementierungsstudien aus den letzten neun Jahren haben gezeigt, dass es inzwischen mithilfe der Analyse von zellfreier (cf) DNA im mütterlichen Blut möglich ist, einen hohen Anteil der Feten mit Trisomie 21, 18 und 13 mit einer viel niedrigeren Falsch-Positiv-Rate (FPR) zu entdecken als bei allen anderen angewandten Analysemethoden. Ziel dieses Artikels ist es, die technischen und klinischen Überlegungen für die Implementierung von cf-DNA-Tests in die Routinepraxis zu prüfen, einschließlich der Analysemethoden, Testdurchführung, Modelle für die klinische Umsetzung sowie der Interpretation der Ergebnisse.

Schlüsselwörter

Zellfreie DNA · Nichtinvasive Pränataldiagnostik · Trisomien · Pränatales Screening · Aneuploidien

diagnosis and be helpful for pregnancy care and preparation for the parents.

There are few limitations when offering cfDNA testing because, although most studies were carried out in high-risk pregnancies, an increasing number of studies performing the test in the routine population have demonstrated that this test is equally effective in low-risk pregnancies [2]. Moreover, the test can be reliably performed at any time during pregnancy, starting at 10 weeks; thus, the best approach to implementing screening for aneuploidies by cfDNA testing in the routine population is to take the maternal blood during 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 the risk for pregnancy complications [26].

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 to evaluate the risk for 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 [27]. 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 as having increased fetal NT or major defects requiring 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 a rescue first-trimester combined test in those cases without a cfDNA result, especially if the ultrasound was performed in week 13.

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 the invasive testing rate, or to the intermediate-risk group, aiming to increase the DR of aneuploidies [29]. The exact risk cut-offs that define the high- and intermediate-risk groups 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 cfDNA testing

If cfDNA testing reports a high-risk for trisomy 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 where there are major fetal defects, irrespective of the cfDNA results, parents should be offered invasive testing with array analysis, to exclude not only 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-results 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 in these cases will depend essentially on the reason why the test was performed in the first place. If there was a previous screening that had already shown a low-risk result without fetal defects, it is preferable to repeat the cfDNA test, explaining to the parents that there is >60% chance that a result will be obtained in the second attempt. However, some pregnant women prefer not to undergo 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 had already shown a high risk for these conditions but the detailed ultrasound had not detected any findings suggestive of fetal pathology, most patients choose to repeat the cfDNA test, although some prefer to undergo an invasive test directly.

Corresponding address

Prof. Kypros H. Nicolaides
Fetal Medicine Research Institute, King's College Hospital
16–20 Windsor Walk, Denmark Hill,
SE58BB London, UK
Kypros@fetalmedicine.com

Compliance with ethical guidelines

Conflict of interest M.M. Gil and K.H. Nicolaides declare that they have no competing interests.

For this article no studies with human participants or animals were performed by any of the authors. All studies performed were in accordance with the ethical standards indicated in each case.

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