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REVIEW

INNOVATIONS IN GYNECOLOGY AND OBSTETRICS

Update in non-invasive prenatal testing

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ABSTRACT

Non-invasive prenatal testing (NIPT) has revolutionized the approach to prenatal diagnosis and, to date, it is the most superior screening method for the common autosomal aneuploidies, mostly trisomy 21. This screening is having a significant population-wide impact on the uptake of conventional screening and diagnostic testing. In recent years, emerging genomic technologies, largely based around next generation sequencing, have expanded the analyses to the sub-chromosomal aneuploidies. However, further clinical validation studies are needed to better characterize this technology. These tests bring advantage through providing a higher diagnostic yield, without risks of miscarriage than previously available diagnostic test, but also raise the question of harms related to an increase in uncertain and unknown results. In view of the revolution brought about by the NIPT, numerous scientific societies have published recommendations regarding the appropriate application of cell-free DNA screening in pregnancy. In this review, we discuss the progress that has been made to date in NIPT.

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The goal of prenatal screening for fetal aneuploidies is to identify pregnancies at increased risk for chromosomal disorders using tests with a high detection rates and low false-positive results. In the last decades, we assisted to remarkable advances in prenatal screening for aneuploidy, particularly in the identification of Down Syndrome.[1] Definitive prenatal diagnosis for chromosomopathies requires invasive sampling followed by karyotype analysis. However, invasive tests are costly and pose a risk of procedure-related complications including miscarriage,[2] and therefore they can not be used as a routine in general population. The most common chromosome condition affecting live births is trisomy 21 (Down Syndrome), with an incidence of around 1 in 700 live births. The risk of a live term infant increases with maternal age from ~1 in 1500 at age 20 years to 1 in 85 at age 40 years.[3] Starting from 1980, many strategies for the diagnosis of trisomy 21 in low risk population followed one another[4] (Table I). In many developed countries invasive prenatal tests were offered to pregnant women with advanced age (>35 years), but this strategy was not efficient because fewer than one third of Down syndrome pregnancies were diagnosed prenatally and of those undergoing invasive prenatal diagnosis only about 2% had fetal karyotype abnormalities.[5] The introduction of second-trimester serum analyses improved

Table I.—*Evolution and performance of different screening test for trisomy 21.*

Year	Methods of screening	Detection rate (%)
1970s	MA	30
1980s	MA+ maternal serum marker	55-60
	MA + serum AFP, hCG (double test)	60-65
	MA + serum AFP, free β-hCG (double test)	60-65
	MA + serum AFP, hCG, uE3 (triple test)	
1990s	MA + NT	75-80
2000s	MA + NT + free β-hCG and PAPP-A (Combined test)	85-90
2001	Combined test + NB or tricuspid flow or DV flow	93-96
2011	cfDNA measured in maternal plasma	99

MA: maternal age; AFP: alpha-fetoprotein; hCG: human chorionic gonadotropin; uE3: unconjugated estriol; β-hCG: β-human chorionic gonadotrophin; NT: nuchal translucency; PAPP-A: pregnancy-associated plasma protein-A; NB: nasal bone; DV: ductus venosus; cfDNA: cell-free DNA.

the screening performance for aneuploidy from a 30% of the advanced maternal age to 60-84% for a false-positive rate of 5%.[6, 7] The proportion of Down syndrome pregnancies diagnosed were more than doubled and a chromosomal abnormality was found in as many as 4% of those designated as "screen-positive."[8] Despite the enhancement, this screening strategy was considered still not satisfactory for the high rate of false negative and the late diagnosis of chromosomal abnormalities that could imply pregnancy termination at an advanced gestational age, that could be stressful for the couple. Only with the introduction of the combined test in early 2000s there was a substantial development in screening performance. This test, performed during the first trimester (11-13 weeks +6), consisted in ultrasound measurement of nuchal translucency thickness (NT) together with maternal serum concentration of placental proteins free beta human chorionic gonadotropin (hCG) and pregnancy-associated plasma protein-A (PAPP-A).[6, 9] Other aneuploidies, including trisomy 18 (Edwards Syndrome), trisomy 13 (Patau Syndrome) and monosomy X (Turner Syndrome), were also being detected with the widespread use of these screening tests. In 2011, the paradigm of prenatal screening shifted with the introduction of cell-free DNA (cfDNA) test (Table I). The presence of cfDNA in maternal blood has enabled the development of highly sensitive screening tests for fetal aneuploidy, diagnostic tests for fetal RHD type and monogenic disorders.[10]

In this review, we discuss the progress that has been made to date in the analysis of cfDNA in maternal blood for non-invasive prenatal testing (NIPT).

Fetal cfDNA for prenatal diagnosis of aneuploidies

The presence of fetal DNA in maternal plasma was first reported in 1997 by Lo *et al.* using conventional polymerase chain reaction techniques to identify Y chromosome-specific DNA sequences.[11] This study was the landmark for the development of cfDNA screening test.

The discovery of circulating cfDNA in maternal blood has enabled the development of genomics-based non-invasive prenatal testing NIPT to analyze the fetal genome.

This new technology allowed for significant improvements in the detection of pregnancies with trisomy 21 as well as the less common chromosomal disorders. In October 2011, the first commercial laboratory began offering NIPT using cfDNA for screening of common aneuploidy conditions.

Physiology and techniques

Plasma cfDNA fragments originate from apoptosis of various cells, mostly from hematopoietic cells.[12] During pregnancy, there is a combination of both maternal and feto-placental derived cfDNA circulating in women plasma. The origin of fetal cfDNA (fetal fraction) is the placental trophoblasts.[13] Fetal fraction increases with gestational age and amount to ~10% of the cfDNA in maternal blood.[14] A fetal fraction greater than 4% is required for reliable analysis, and this value is reached at about 10 weeks of gestation. The most common reason for an interpretable result is a relatively low quantity of placental cfDNA in maternal blood, or low fetal fraction. The cfDNA is no longer available in the maternal circle a few hours after childbirth and is probably eliminated

through renal excretion. DNA fragments derived from placenta are shorter in length (143 base pairs) than maternally derived DNA fragments (166 base pairs). This difference is very important to improve the accuracy of prenatal aneuploidy screening.[15]

In 2008, in two studies fetuses with chromosomal aneuploidy were identified using massively parallel sequencing of cfDNA from maternal plasma. There are different methods developed for aneuploidy detection: massive parallel sequencing (MPS), chromosome-selective (or targeted) sequencing (CSS) and single nucleotide polymorphism (SNP) based sequencing.

Whole genome MPS process is based on the random or shotgun sequencing of DNA molecules in maternal plasma.[16, 17] Apart from abnormalities involving the entire chromosome, MPS-based analysis of maternal plasma DNA has also been shown to be useful for detecting Down syndrome caused by Robertsonian translocation as well as microdeletions and microduplications.[18] The whole genome sequencing method allows detection of differences on any chromosome and, depending on the depth of sequencing, detection of less common aneuploidies and subchromosomal abnormalities.[19] MPS methods statistically calculate the standard deviation of the expected count from each chromosome and allocate a "Z-score" for each chromosome. For example, if the number of DNA fragments from chromosome 21 in the test sample is z-score >3 this is considered a high-risk result for trisomy 21.[20]

In the CSS approach, the plasma DNA fragments, that are unique to the targeted chromosome, undergo an enrichment process involves a PCR-based reaction. One disadvantage of this approach is that off target chromosomal aneuploidies will not be detected. The statistical method used for CSS combined the woman's prior risk of aneuploidy (based on maternal and gestational age), the target chromosome counts, and the fetal fraction to calculate a final risk using an odds ratio approach. A value of 1 in 100 or greater is considered at high risk.[21, 22]

The method, at least two orders of magnitude greater than other reports of multiplexed PCRs SNP-based approaches, employ a massively multiplexed PCR amplification targeting 19,488 SNPs in a single reaction and sophisticated informatics analysis to identify fetal chromosomal copy number.[23] Among NIPT, SNP-method demonstrates the highest accuracy with sex chromosome aneuploidy detection. It is also the only method capable of detecting triploidy.[24] Also, SNP-based NIPT will not detect off-target abnormalities.

Biological limits

Some variables could influence NIPT results. The most common reason for the defined "no call" results is a relatively low fetal fraction. The fetal fraction is low in all those conditions in which there is an increase in the maternal cellular turnover without increasing the placental cellular component.[24] For example, maternal obesity is a condition associated with a two-fold increase of maternal cfDNA levels in plasma with no significant difference in fetal cfDNA. This fact is due to the increased apoptosis and necrosis present in adipose tissue.[25] Autoimmune disease could also cause an increase in cell turnover. Indeed, non-pregnant patients affected by systemic lupus erythematosus show elevated levels of circulating cfDNA.[26] Other statistically significant associations of maternal factors with fetal fraction have been reported, including maternal aneuploidy (47, XXX), maternal mosaicism (45X/46XX), maternal copy number variations, prior organ transplant, maternal medications, smoking and pre-existing hypertension.[23] In the same case reports maternal malignancy was related to discordant NIPT results. A case series obtained from a total cohort of 125,426 women provided details on 10 women with discordant NIPT results due to an undiagnosed maternal cancer. The cancer types included lymphoma, leukemia, colorectal and anal cancers. In another cohort of the 39 cases with NIPT result indicating multiple aneuploidies, seven were due to asymptomatic maternal malignancies. Also, benign tumors such as uterine fibroids can cause abnormalities in NIPT.[26] Conditions that could determine "biological false-positives" are confined placental mosaicism, demise of a previously undetected co-twin, maternal copy number variants, or maternal aneuploidy.[23, 27] Patients should be counseled of these possibilities before proceeding with screening.

Clinical validity

cfDNA screening is the most superior screening method for trisomy 21 and has the highest detection rate with the lowest false-positive rate, when compared to other conventional screening tests. An updated meta-analysis, which included studies between January 2011 to January 2015, reports detection rates for common aneuploidies as follows: trisomy 21 (99.7%), trisomy 18 (98.2%), and trisomy 13 (99%), with a combined false-positive rate (FPR) of 0.13%. They also report pooled weighted detection rate of 95.8% for monosomy X with a false-positive rate of 0.14%. Other sex chromosome aneuploidies had a pooled weighted detection rate of 100% and FPR of 0.003%[28] (Table II).

Subchromosomal disorders

Subchromosomal disorders result from submicroscopic genomic imbalances that are too small to be detected by standard karyotyping. Subchromosomal abnormalities (microdeletions and duplications) may result in physical and/or intellectual impairments that can be highly variable and depend on the specific chromosome region and the amount of genetic duplicated or deleted material.[29] Unlike the risks of aneuploidy that is associated with nondisjunction, the incidence of subchromosomal copy number variations (CNVs) is independent of maternal age. Clinically relevant microdeletions and duplications occur in 1-1.7% of all structurally normal pregnancies.[30] Because some infants with subchromosomal abnormalities may benefit from early therapeutic intervention, prenatal detection is important for optimal management. Array comparative genomic hybridization (array-CGH) is a molecular technology used for the analysis of subchromosomal human karyotype aberrations. Array-CGH allows genome analysis with a resolution of <1 Mb, while conventional cytogenetic examination allows for a 5-10 Mb resolution.[31]

A meta-analysis conducted by Hillman *et al.* affirmed that array-CGH detected 3.6% additional genomic imbalances when conventional karyotyping was normal. This value increased to 5.2% when the referral indication was a structural malformation on ultrasound.[29] However, with the introduction of NIPT for aneuploidy screening, many women who previously would have had invasive testing are choosing to avoid these procedures because of the small risk of pregnancy loss.

Submicroscopic genomic alterations are harder to detect non-invasively because of their small size. Therefore, the introduction of a highly accurate non-invasive prenatal screening test that would identify women who are at high risk for microdeletions or duplications would be useful. Proof-of-concept studies have reported the possibility of expanding cfDNA screening to include well-defined microdeletion syndromes (Table III).[32-34] Recently, a single-nucleotide polymorphism (SNP)-based NIPT was validated for detection of five important, clinically significant microdeletion syndromes: 22q11.2, Prader-Willi, Angelman, cri-du-chat and 1p36 deletion. For all these five disorders, the detection rate for the large causal deletions was >97%, with a specificity of >99%.[32] Two retrospective cohort studies have been published;[35, 36] however, due to the small number of affected fetuses, heterogeneous populations, and incomplete outcome data, the true sensitivity and NPVs in clinical practice are unknown. No prospective clinical trial has been completed for the use of this technology for the detection of microdeletion syndromes. More studies on the argument are needed.

TABLE II.—*Performance of cfDNA for aneuploidies.*

Aneuploidy	Detection rate (95% CI)[a]	False positive rate (95% CI)[a]
Trisomy 21	99.7% (99.1-99.9%)	0.04% (0.02-0.08%)
Trisomy 18	98.2% (95.5-99.2%)	0.05% (0.03-0.07%)
Trisomy 13	99.0% (65-100%)	0.04% (0.02-0.07%)
Monosomy X	95.8% (70.3-99.5%)	0.14% (0.05-0.38%)
Other sex chromosomal aneuploidy	100% (83.6-100%)	100% (83.6-100%)

[a]Pooled weighted detection rate and false-positive rate from Gil *et al.*.[25]

TABLE III.—*Microdeletion validated for NIPT according Wapner* et al.[32]

Disorder	Position of deletion	Incidence	Detection rate	Specificity	Phenotype
DiGeorge Syndrome	22q11.2	1:1000	>97%	>99%	Cardiac abnormalities; thymic aplasia; immune conditions; endocrine, genitourinary and gastrointestinal problems; developmental delay
Prader-Willi Syndrome	15q11.2-q13	1:10,000-30,000	>97%	>99%	Hypotonia; feeding difficulties in early infancy; obesity; hypogonadism; short stature; behavioral difficulties
Angelman Syndrome	15q11.2-q13	1:12,000-20,000	>97%	>99%	Severe developmental delay; speech impairment; ataxia; happy demeanor and excessive laughter
Cri-du-chat syndrome	5p	1:15,000-50,000	>97%	>99%	Developmental delay; microcephaly; dysmorphic features; hearing defects; short statures; ADHD; a high pitched, cat-like cry
1p36 deletion	1p36	1:5000	>97%	>99%	Developmental delay; dysmorphic craniofacial features; hypotonia; seizures; congenital heart defects

Fetal cfDNA for prenatal diagnosis of monogenic disorders

New emerging genomic technology has allowed an improvement of NIPT application in prenatal diagnosis. The rapid development of next generation sequencing (NGS) technologies such as whole genome/exome sequencing, opens a new opportunity to provide genetic analysis for diagnosis of recessive, X-linked and dominant inherited conditions. The sequencing-based new approach could be used to detect rare diseases, including monogenetic diseases in a non-invasively manner. This approach has great potential to be wildly used in the worldwide with the decreasing in sequencing costs, and therefore play an incredible role to prevent rare diseases. The research of monogenic disease shows advantage because the analysis is targeted and in presence of positive results could be considered diagnostic, reducing the access to invasive testing.[37] Differently, when testing for aneuploidy, the confirmation of an abnormal result is required throughout invasive testing because, as sovramentioned, cfDNA is a mix of maternal and fetal cfDNA. The fetal component released from the placenta may reflect confined cell lines ("confined placental mosaicism").[11-13] The first success of NIPT for monogenic disorder diagnosis was the exclusion or identification of paternally inherited variants or de novo mutations.[38] For these disorders NIPT is based on the detection or exclusion of the paternal mutation in the cfDNA. This approach has been used in the diagnosis of Huntington's disease;[39, 40] myotonic dystrophy[41] and early onset primary dystonia I.[42] In disorders associated to trinucleotide repeat expansions, detection of closely linked polymorphic regions has been used to overcome parents' similar allele size. Li *et al.* used NIPT to identify point mutations inherited from the father for beta-thalassemia. They reported high sensitivity (100%) and specificity (93.8%) by size-fractionating cfDNA in maternal plasma followed by a PCR approach that did not amplify the normal maternal allele.[43] Detection of a fetus with an autosomal dominant disorder with a maternally inherited mutation is much more technically difficult because the fetal genotype in the cfDNA needs to be identified in the presence of an excess maternal DNA. There are some autosomal dominant disorders where a new mutation is relatively common and the detection of the mutation in cfDNA can provide a diagnosis. Some of these mutations could be suspected by ultrasound findings like in skeletal dysplasia. One such example is achondroplasia where a single mutation in the *FGFR3* gene, c.1138G>A (p.Gly380Arg), accounts for 98% of all cases.[44] Ultrasound findings can sometimes be suggestive of achondroplasia and a non-invasive test that looks specifically for this mutation in cfDNA can be carried out.[45, 46] Initially polymerase chain reaction with restriction enzyme

digest (PCRRED) was used for clinical research in NIPT for a small proportion of mutations. However, as interpretation may vary between operators, it had an inconclusive rate of around 8%, and was not universally adaptable and applicable to all mutations.[47] Another drawback is that only one mutation at a time can be detected and this may be not useful when offering prenatal diagnosis to find an unknow mutation, for example in presence of ultrasound abnormalities that may be caused by a number of different mutations. The use of NGS improve the diagnosis for paternally inherited and de-novo dominant disease through the use of panels designed to detect multiple mutations and detected mutations in cases where PCR-RED had not.[30] This approach was introduced into UK NHS practice in 2014.

When both parents are carriers for an autosomal recessive disorder, mutation absence would confirm an unaffected carrier or normal fetus. To identify an affected fetus is otherwise necessary to quantify the relative numbers of the alleles present in the cf-DNA and establish that there is a statistically significant excess of one type over another, consistent with a presence of one of the two mutations being present in the fetus.[48] This problem is solved by the assessment of the relative quantities of mutant and wild-type alleles, which can be achieved by using relative mutation dosage (RMD) and relative haplotype dosage analysis (RHDO).[49, 50] A sex-linked or sex-limited genetic disorder can frequently be excluded early in pregnancy simply by establishing that the fetus is not of the at-risk gender. The detection of cfDNA derived from the Y-chromosome provides a highly accurate determination of fetal sex from as early as 7 weeks gestational age which is earlier than gender can be reliably determined by ultrasound. Additional diagnostic testing can then be limited to only those at-risk cases. This practical approach has been used for a broad range of X-linked disorders such as hemophilia and Duchene muscular dystrophy.[38, 51-53]

There are several limitations when using NIPT: first, the low concentration of fetal cfDNA circulating with the relatively abundant maternal cfDNA; the need for highly sensitive methodologies with a broad applicability for different families; and finally other very important limitation is the cost. Next generation sequencing is also beginning to improve diagnosis in fetuses with unexpected abnormalities that are identified in around 3% of pregnancies.[54] Whole exome sequencing (WES) sequences the DNA regions containing the protein-coding exons, which contain >85% of all disease-causing mutations. In recent studies emerges that use WES is as a tool to successfully identify pathogenic variants in fetuses euploid with structural sonographic abnormalities.[55] Benefits of exome sequencing include improving parental counselling, pregnancy and postnatal management;[56, 57] prenatally identification of molecular genetic disorders may also facilitate targeted in-utero treatment. Problems associated with exome sequencing correlate with counselling issues, for example identification of secondary or incidental findings unrelated to the fetal phenotype like cancer predisposition genes. WES offers incomplete coverage of many genes and this may compromise the interpretation of sequencing results. The cost of prenatal WES has not been formally evaluated, but it is high.

Current guidelines for the use of NIPT

SMFM (the Society for Maternal Fetal Medicine), ACOG (the American Congress of Obstetricians and Gynecologists) and numerous professional societies have published recommendations regarding the appropriate application of cfDNA screening in pregnancy.

The first guidelines of all major societies were published in 2011 and suggested to limit the use of cfDNA screening only to those pregnancies at increased risk of aneuploidy:

- age 35 years or older at the time of delivery;
- ultrasound findings that suggest an increased risk of aneuploidy;
- history of a previous pregnancy with trisomy;
- positive first or second trimester screening tests for aneuploidy;
- parental balanced Robertsonian translocation with increased risk of trisomy 21 or 13.[58, 59]

In a 2015 committee opinion, ACOG and SMFM acknowledged that patients may choose cfDNA screening regardless of their risk status and should receive pre-test and post-test coun-

seling.[60] Pre-test counseling should include both information about commonly detected chromosome arrangement, personal risk factors, both information on the difference between screening and diagnostic testing with a review of available testing options and information about detection rates, false-positive rates, and positive and negative predictive values.[61]

Post counseling advice should be offered in all cases. For patients with negative results, reassurance should be provided along with a discussion that a negative result does not eliminate the possibility that the pregnancy has a genetic condition or birth defect. Patients with positive results on any screening test should be referred for additional genetic counseling to discuss available diagnostic testing options. All patients with discordant results should be referred to an appropriately trained professional for further counseling and review of additional testing options.[62]

The SMFM also recommended that cfDNA microdeletion screening should not be routinely offered;[21] indeed, screening for conditions with such low prevalence will inevitably result in false positive results.

Finally, both SMFM and ACOG do not recommend the use of cfDNA in multiple gestations as suggested by preliminary studies since larger prospective studies are needed.[63]

NIPT and multifetal gestations

Using any maternal blood-based marker as a screening tool can be challenging in twin pregnancies because in both biochemical and DNA-based screening, the abnormal levels of analytes or DNA fragments from an aneuploid fetus may be masked by the euploid co-twin.[63]

In literature, only five studies have reported the performance of cfDNA screening in twin pregnancies.[64-68] Whereas pooled detection rates in the most recent meta-analysis are reported to be 100%, the number of studies included limits the generalizability of these results.[28]

Since there is no data regarding the performance of cfDNA aneuploidy screening in higher-order multiple gestations, it should not be offered and aneuploidy screening for these pregnancies should be limited to ultrasound markers only.

In conclusion, the use of NIPT for the diagnosis of monogenic disorders is not suitable in multiple pregnancies because a conclusive diagnosis is currently not possible to determine whether one or both twins were affected; its use could be useful in cases where discordant ultrasound findings are present.[37]

Economic and social impact

cfDNA screening has transformed the landscape of prenatal screening for chromosomal disorders. This technology is continuing to expand with many studies currently on-going on this topic to improve screening for genome-wide alterations. It is of the utmost importance for obstetric providers receive appropriate genetics training. Clear and concise patient educational brochure should be available in medical centers where the test is offered.

cfDNA screening has already had a significant population-wide impact on the uptake of conventional screening and diagnostic testing and is rapidly becoming a first-line screening test in some high-income populations.[69] The very low false-positive rates associated with cfDNA screening have led to a reduction in the number of diagnostic invasive procedures performed. One study in the USA reported that the number of amniocenteses performed fell by 23.6-50% and CVS by 14.2-65.7% after the introduction of cfDNA screening.[70] Similarly, in Australia the total number of amniocenteses fell by 51% and CVS by 37%, representing the largest fall in number of invasive procedures in 20 years.[71]

Government-funding screening programs are rapidly incorporating the use of this new technology into their screening algorithms.[72, 73] In countries without universal health coverage, despite the high detection rate, at present the cost of NIPT remains still too high to be used as primary test of screening for fetal aneuploidies. Therefore, cfDNA test could be used in association to first trimester combined screening in a contingent model where first-trimester combined testing is offered to all patients as a triage and assessment by cfDNA as a secondary test in a smaller proportion of pregnancies.[74] The inclusion of a contingent screening policy could make cfDNA cost-effective as demonstrated recently in literature.[75]

Conclusions

The NIPT is making revolution in prenatal diagnosis. CfDNA based screening for the common autosomal aneuploidies is the best screening method for trisomy 21 to date. However, women who choose fetal cfDNA technology should be counseled that the test remains a screening test for aneuploidy at this time. Many commercial providers now offer analyses for sub-chromosomal aneuploidies, but we are still at the beginning. No provider has thoroughly validated their tests to a statistically significant level, due to the rare occurrence of these chromosomal abnormalities. Emerging genomic technologies, largely based around next generation sequencing, are offering promise for safer prenatal genetic diagnosis, however these innovative approaches will improve screening for fetal aneuploidies of single gene disorders at an early gestational stage without the need for invasive testing and improve our ability to detect monogenic disorders as the aetiology of fetal abnormalities. This represents a challenge as well as an oppurtunity for clinicians and scientists. Furthermore, as larger proportions of patients with genetic disease are identified we should be ready to offer appropriate genetic counselling to families and potential parents.

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