

Advances in Non-invasive Prenatal Diagnosis

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Abstract. *The invasive procedures amniocentesis and chorionic villus sampling are routinely applied in pregnancies at risk for fetal genetic disorders and the results obtained are the gold standard for prenatal diagnosis. These procedures have an approximately 0.5-1% risk for fetal loss and are mainly used in cases at risk for fetal chromosomal abnormalities and single-gene disorders. Identification of cell-free fetal nucleic acids (DNA and RNA) in maternal plasma and the recognition that they represent a useful source of fetal genetic material for prenatal diagnosis has led to intensive efforts to develop non-invasive prenatal testing. This review summarizes recent developments in the field of non-invasive prenatal diagnosis through the use of cell-free fetal nucleic acids in maternal circulation during pregnancy and provides an overview of the possibilities for future clinical applications.*

Cell-free Nucleic Acids in Maternal Plasma

The year 1997 is a landmark for non-invasive prenatal diagnosis (PD) as it was hailed the identification of the presence of cell-free fetal DNA (cffDNA) in the maternal circulation that opened new horizons for non-invasive PD (1). cffDNA originates from the placenta as a result of trophoblastic apoptosis. A less likely source, due to its scarcity, is fetal cells that undergo apoptosis in the maternal circulation. It can be found in low concentrations in the maternal plasma and cffDNA molecules are outnumbered 20:1 by maternal cell-free DNA molecules. cffDNA constitutes approximately 3-6% of the total cell-free DNA

present in the maternal circulation (2). Recent data, however, show that its actual proportion is approximately 19% (3). It can be detected in maternal plasma from the 6th week of gestation, with increasing concentration throughout gestation. A large number of studies showed that *postpartum*, cffDNA is rapidly cleared from the maternal circulation with a half-life of approximately 16.3 min (4). Moreover, it has a relatively smaller size compared to maternal cell-free DNA, offering an alternative enrichment option (5). Its size varies between individuals and because half of the fetal genome is of maternal origin, its use in the diagnosis of fetal aneuploidies and single gene disorders is problematic.

Three years after the discovery of cffDNA Poon *et al.* isolated cffRNA from maternal plasma using a reverse-transcriptase polymerase chain reaction (PCR) (6). In contrast to the instability of RNA molecules, cffRNA is relatively stable because of its storage within apoptotic microvesicles in the plasma, which protect it from RNase degradation. It appears in the maternal circulation as early as the 4th week of gestation and rapidly disappears from maternal plasma after delivery, having a median half-life of 14 minutes. The main advantage of cffRNA over cffDNA is that it is possible to select for placenta-specific mRNA sequences not expressed by any maternal tissues (7).

Approaches to cffDNA and -RNA Analysis

Both maternal plasma and serum contain cffDNA, plasma however is the material of choice for PD since it contains less maternal background. Plasma DNA isolation is performed manually with commercially available kits, although automation of the process has also been reported (8).

The scarcity of cffDNA in maternal plasma and its co-existence with maternal DNA represent the two major limitations for the use of cffDNA for diagnosis. Various methods have been used to overcome these limitations, including those based on the fact that fetal fragments tend to be of shorter length than maternal fragments (5). Efforts to increase the relative proportion of fetal DNA compared to

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the larger maternal fraction have also included the use of formaldehyde to prevent lysis of maternal cells during the isolation of the maternal plasma (9). Formaldehyde enrichment however, has not yet been reproducible by other laboratories.

Different methodologies have been used for the detection of cffDNA, *e.g.* conventional PCR, restriction analysis, quantitative fluorescence (qF)-PCR, real-time quantitative PCR (RT-qPCR), massively parallel sequencing (MPS), mass spectrometry and digital PCR (3, 10-16). Since RT-qPCR is very sensitive, enabling the detection of very low copy numbers of DNA, it represents the optimal method for reliable non-invasive PD based on the detection of fetal-specific sequences in maternal plasma. The technique is less time consuming and has an extra level of protection against contamination. A wide range of cycle threshold (Ct) values in each RT-qPCR and poor repeatability of some replicates has been reported, partly due to the variability of target copy numbers in maternal plasma. It is, therefore, recommended to perform several replicates from each sample in order to increase the probability of cffDNA detection and avoid false-negative results (17).

Universal Fetal Markers

An ideal non-invasive PD method would be accurate, applicable in clinical practice, cheap, with repeatable results and independent of gender, ethnicity and polymorphisms. However, such a target has not been yet achieved. The two main steps for the exploitation of cffDNA and -RNA as a source of genetic information are much the same between the different techniques that have been developed. Firstly, cffDNA and cffRNA have to be isolated and purified from maternal plasma. The second step is to verify the presence of fetal material in the maternal circulation. Various methods have been used with most of them focusing on the detection of paternally inherited sequences *e.g.*, sequences located on the Y chromosome. As cffDNA is routinely determined by quantifying Y chromosome-specific sequences sex-determining region Y (*SRY*) and testis-specific protein Y-linked 1 (*DYS14*), alternative, gender-independent approaches have been tested to overcome this limitation. Therefore, recent research has investigated the usefulness of paternally inherited single-nucleotide polymorphisms (SNPs), and variable regions of repeated DNA such as short tandem repeats and epigenetic markers. One potentially appropriate epigenetic marker is the maspin gene, which is specifically hypomethylated in the fetus (18). Hypomethylated maspin sequences can be detected in maternal plasma and could thus represent a universal fetal DNA marker in the maternal circulation. An alternative is offered by the promoter sequence of Ras association domain-containing protein-1 (*RASSF1A*) which, contrary to maternal cells, is

hypermethylated in the placenta (19). Hypomethylated *RASSF1A* sequences can be removed from maternal plasma using methylation-sensitive restriction enzyme digestion, revealing only the fetal hypomethylated target.

Clinical Applications

To date, due to technical challenges, non-invasive PD is only practiced in some centers for fetal sex determination in women at high risk of sex-related disorders and for fetal RhD status determination in pregnancies at risk of hemolytic disease of the newborn (20). Other clinical applications that are being developed include detection of aneuploidies, single-gene diseases and pregnancy-related complications.

Fetal sex determination. Fetal sex determination was the first clinical application of non-invasive PD. The test relies on the identification of Y chromosome sequences in maternal plasma and has approximately 100% accuracy. This approach enables reliable detection from the 7th week of gestation onwards, which means that the fetal sex can be determined non-invasively prior to performing chorionic villus sampling (CVS) or ultrasound assessment.

The main drawback of this method is that female fetuses are detected indirectly, which raises the possibility of false-negative results. It is, therefore, important to confirm the presence of cffDNA when a negative result for Y chromosome sequences is obtained. Recently an efficient alternative to currently applied protocols was reported using a multiplex PCR based protocol for the simultaneous application of *SRY*, *DYS14*, *RASSF1A* and beta-actin (*ACTB*) (21). Following enzymatic digestion using a methylation-specific restriction enzyme, this assay directly demonstrates the presence of cffDNA, providing reassurance and preventing reporting of false negative results (Figure 1). In this protocol, *ACTB* is used as an internal control system for the detection of incomplete enzyme digestion, which could potentially lead to false-positive results.

Fetal sex determination is indicated for families at high risk for X-chromosome-linked disorders, family history of conditions associated with ambiguous development of external genitalia, endocrine disorders such as congenital adrenal hyperplasia and abnormal ultrasound genitalia findings. Non-invasive fetal sex determination limits invasive testing and its side-effects and prevents the development of sex-related diseases (7). A recent cost-effective analysis showed that the costs for the test can be off-set by the cost savings in the reduction of invasive tests (22).

Rhesus blood grouping. Prenatal determination of the fetal RhD status can be achieved by PCR amplification of *RHD* sequences in amniotic fluid or CVS. These invasive procedures, however, carry not only the risk of miscarriage

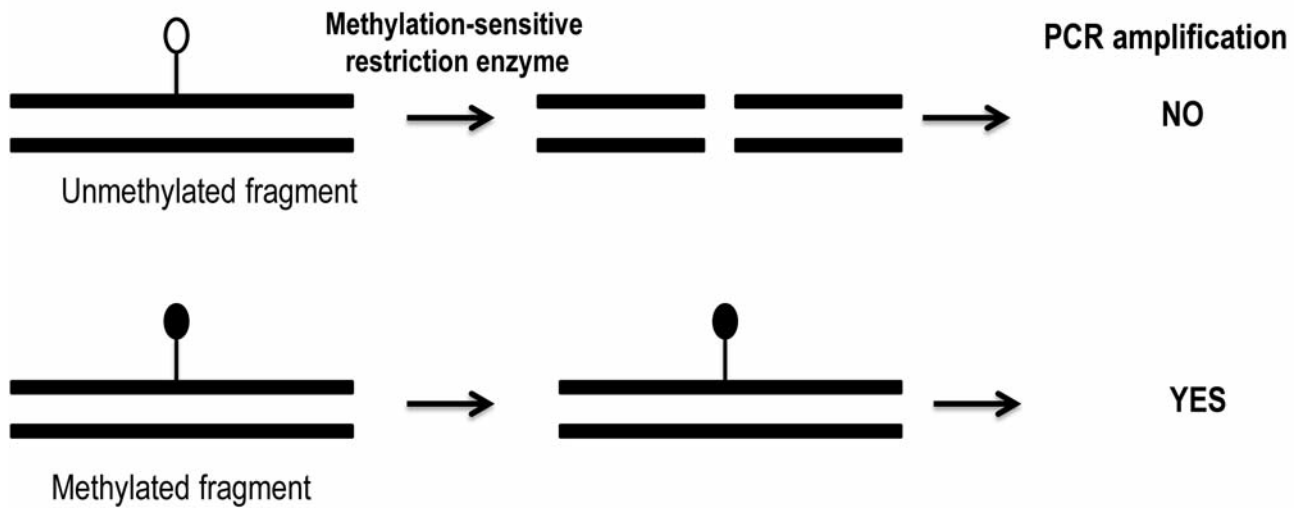


Figure 1. General schema of cell-free fetal (cff) DNA methylation analysis using methylation-sensitive restriction enzymes that selectively digest unmethylated DNA, so that only methylated fragments remain intact for detection.

but most importantly, testing RhD-negative pregnant women may lead to immunization due to feto-maternal hemorrhage.

The benefits of mass testing antenatally for fetal RhD status by analysis of cffDNA in maternal plasma of RhD-negative mothers could reduce the use of anti-D and the number of anti-D donors exposed to blood products for hyperimmunization. Women carrying a RhD-negative fetus (approximately 40%) would be spared unnecessary exposure to anti-D, with its associated discomfort and risk from viral (hepatitis C) or prion (variant Creutzfeld-Jacob disease) contamination.

There are multiple reports of high degrees of accuracy for non-invasive PD of the fetal RhD status, but to date, clinical application has been confined to those women known to be at high risk for hemolytic disease [reviewed in (23)]. RT-qPCR technology is considered the optimal method for the reliable detection of *RHD* sequences using cffDNA. Efforts have also been made for non-invasive PD of fetal RhD status by mass spectrometry (24). Validation of the assay showed the presence of 2.5% RhD-positive genomic DNA in a background of RhD-negative genomic DNA. Five out of 178 samples examined were incorrectly diagnosed as RhD-negative. The main advantage of this approach over RT-qPCR is that it has the potential for multiplex analysis of several different loci in a single assay but further studies are necessary in order to determine the clinical utility of the technique.

The most widespread approach used in prenatal *RHD* diagnosis is an assay that detects at least two different exons of the *RHD* gene. Many laboratories prefer to include amplification of *exon 7* because it contains the most sequence difference to the *RHCE* gene, thus facilitating specificity for *RHD* and allowing for the detection of fetal

RHD even in the 7th week of gestation, without giving false-positive results [reviewed in (23)].

Single-gene diseases. Diagnosing single-gene diseases with the aid of non-invasive methods is a challenging and technically difficult approach. The detection of paternal mutations related to autosomal dominant diseases is possible owing to the absence of the mutation in the maternal genome. Diagnosis however, of autosomal recessive diseases or maternally transmitted autosomal dominant diseases is more demanding as at the moment is not possible to distinguish the differences between maternal and fetal genes, nor between paternal and maternal genes that carry the same mutation. Much effort has been made in this field lately, in order to detect and prevent diseases such as achondroplasia, myotonic dystrophy, Huntington's disease, cystic fibrosis and β -thalassemia. Most studies used β -thalassemia as a model since it has high-frequency and well-characterized mutations in each ethnic group. Various approaches have been investigated (predominantly applying PCR and mass spectrometry), namely: i) detection of the paternally inherited mutation, ii) detection of paternally inherited SNPs, and iii) detection of the maternally inherited mutant allele. The latter approach is based on a new and innovative assay labeled relative mutation dosage that compares the relative amounts of the mutant and normal alleles of a genetic locus (25).

Fetal aneuploidies. Non-invasive PD of fetal aneuploidies is considered technically challenging. One aspect of the challenge relates to the presence of the background of large maternal DNA which interferes with the analysis of the fetal

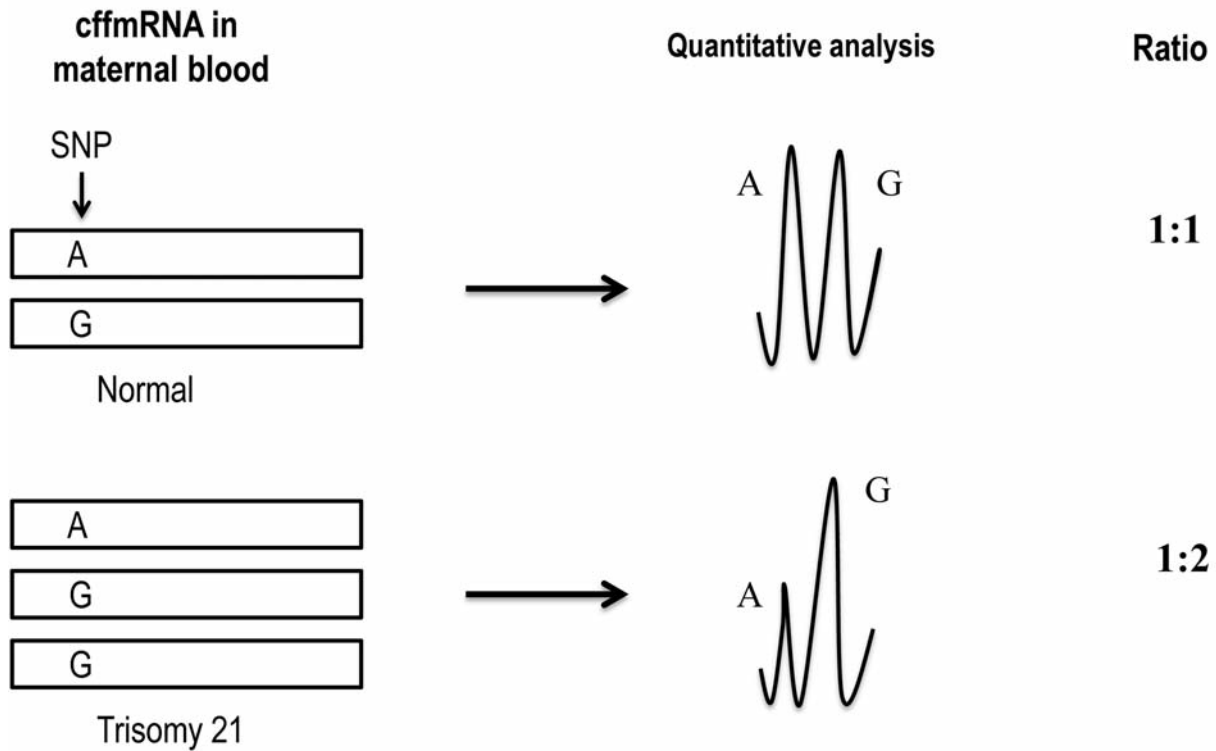


Figure 2. Calculation of fetal chromosome dosage using the allelic ratio of a specific heterozygous single nucleotide polymorphism on cell-free fetal mRNA.

DNA in maternal plasma, while the other is related to the extracellular nature of cffDNA that causes difficulty in determining the chromosome dosage of the fetus.

In order to overcome the maternal background effect and to determine the relative chromosome dosage, researchers targeted placentally expressed mRNA molecules in maternal plasma. The mRNA of placenta-specific 4 (*PLAC4*) and serpin peptidase inhibitor clade B member-2 (*SERPINB2*) have been used for the detection of trisomy 21 and 18 respectively [reviewed in (26)]. An RNA-SNP allelic ratio test was developed for the non-invasive PD of trisomy 21 by determining the ratio between polymorphic alleles of (*PLAC4*) mRNA, a transcript on chromosome 21, in maternal plasma. The target gene, *PLAC4*, has high expression levels in the developing placenta, with correspondingly high levels of circulating RNA. Deviation of the *PLAC4* mRNA SNP allelic ratio was observed in plasma of trisomy 21 pregnancies compared with the expected 1:1 ratio in heterozygous euploid fetuses (Figure 2). Sensitivity of 90% and specificity of 96% for the non-invasive detection of trisomy 21 were achieved by using the *PLAC4* RNA-SNP test alone; this is comparable to many of the currently used screening tests. This approach enables for the direct detection of fetal chromosomal aneuploidies, but is only applicable to fetuses with certain

genotypes. For example, RNA-SNP tests are only informative for fetuses heterozygous for the specific gene; thus, a panel of coding SNPs is required to increase the population coverage of those tests.

Other differences, such as size fragmentation and epigenetic modifications were also investigated. Researchers have sought differences in the methylation patterns of maternal and fetal DNA. For example *SERPINB5* (encoding maspin) located on chromosome 18 is hypo-methylated in the placenta and hyper-methylated in maternal blood cells. These differences have been exploited for the development of an epigenetic allelic ratio approach similar to that of RNA-SNP.

A newly-discovered and promising strategy combining methylated DNA immunoprecipitation with RT-qPCR allows for the non-invasive PD of fetal trisomy 21, and probably other aneuploidies, through the identification of fetal-specific methylation regions. The analysis of the differentially methylated regions determines the chromosome dosage in a gender and polymorphism independent manner (27). Another methodology is the epigenetic-genetic dosage approach which allows for the determination of chromosome dosage through the comparison of a fetal epigenetic marker located on an aneuploid chromosome with a fetal genetic marker located on an euploid reference chromosome, independent of

gender and polymorphisms. The epigenetic-genetic dosage approach has been used for the detection of trisomy 21 and of trisomy 18 (26).

Single-molecule counting techniques known to have superior analytical precision compared with conventional PCR methods have been used for non-invasive PD of fetal aneuploidies. Digital PCR and MPS can precisely quantify the small increments in the total number of DNA molecules derived from the aneuploid chromosome by counting one molecule at a time. Digital PCR was used in an approach called relative chromosome dosage where the number of plasma DNA molecules from chromosome 21 was compared with that of a reference chromosome, *e.g.* a chromosome expected to have a normal dosage in the fetus [reviewed in (26)]. The chromosome 21 to reference chromosome ratio is expected to be elevated in trisomy 21 fetuses and the increase depends on the fetal DNA concentration. This analytical platform however, needs further improvements to reliably determine small changes.

Recently, non-invasive PD of fetal aneuploidies has been achieved with the use of MPS which can identify and quantify millions of DNA fragments in a high throughput manner [reviewed in (26)]. This technique sequences the first 36 bases of millions of DNA fragments to identify their specific chromosomal origin and determine the proportion of molecules derived from a potentially aneuploid chromosome, *e.g.* chromosome 21. If the fetus has a third chromosome 21, the percentage of chromosome 21 fragments is slightly higher than expected. However the results from measurements on trisomy 13 and 18 were less precise. Subsequently the diagnostic accuracy of MPS for NIPD of trisomy 21 was evaluated in large-scale clinical studies (28, 29). The cffDNA sequencing test was performed for pregnancies that were identified as high risk using current screening protocols and proceeded to invasive testing with full karyotyping results available. A detection rate of at least 98% was achieved at a false-positive rate of 2% or lower. Therefore, it was concluded that the maternal plasma DNA sequencing test could be implemented as a second-tier screening to be performed for pregnancies that have been identified as high-risk based on the current screening protocols.

Recently, Sequenom developed its MaterniT21 test. In a study funded by Sequenom MaterniT21 test was applied for fetal karyotyping in 212 Down's syndrome and 1484 matched, normal pregnancies. The test detected 98.6% of the Down's syndrome pregnancies, with a false-positive rate of 0.2% (30). Currently the test, as recommended by the manufacturer, can be used as an adjunct to current screening for high-risk patients including women of advanced maternal age and those who have a family history of Down's syndrome, an ultrasound finding suggestive of a fetal chromosome abnormality, or unusual results on other biochemical screening tests.

Future Perspectives

Throughout the last decade, advances made in the technical possibilities for non-invasive PD based on cffDNA in maternal plasma are considerable. Non-invasive PD of fetal sex determination and Rhesus haplotyping are currently in routine use in many European countries. One step forward was also taken in the direction of NIPD of fetal aneuploidies and single-gene disorders (7, 20). In small studies and model system strategies, techniques for potential non-invasive aneuploidy tests have been demonstrated. For wide implementation, a test has to be robust, preferably gender- and polymorphism-independent, be possible to implement from the first trimester onwards, and have reasonable costs.

Nevertheless, certain ethical issues arise from the possibility of massive application of non-invasive PD. One major concern is whether pregnant women make informed and voluntary choices. Another important consideration is the degree of access that certain social groups will have to such a probably expensive test. Moreover, we will possibly experience a rise in pregnancy terminations due to the increased diagnosis of genetic diseases. Finally, the relative ease with which the test is performed could expand testing for non-medical reasons, such as fetal sex or paternity test, and for life-threatening medical conditions that may appear in the future, such as cancer and heart diseases. Hopefully, cffDNA may alter the way PDs are performed.

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