Non-invasive aneuploidy detection using free fetal DNA and RNA in maternal plasma: recent progress and future possibilities

Attie T.J.I. Go1,3,*, John M.G. van Vugt1, and Cees B.M. Oudejans2

1Department of Obstetrics/Gynaecology, VU University Medical Center, De Boelelaan 1117, 1081 HV Amsterdam, The Netherlands
2Department of Clinical Chemistry, VU University Medical Center, De Boelelaan 1117, 1081 HV Amsterdam, The Netherlands
3Present address: Department of Obstetrics/Gynecology, Erasmus Medical Center, Rotterdam, The Netherlands

*Correspondence address. E-mail: attiego@xs4all.nl
Submitted on July 16, 2010; resubmitted on September 2, 2010; accepted on September 22, 2010

TABLE OF CONTENTS

• Introduction
• Focus of this review
• Methods
  • Fetal DNA in maternal plasma
  • Fetal RNA in maternal plasma
  • New approaches with great potential
• Conclusion

BACKGROUND: Cell-free fetal DNA (cff DNA) and RNA can be detected in maternal plasma and used for non-invasive prenatal diagnostics. Recent technical advances have led to a drastic change in the clinical applicability and potential uses of free fetal DNA and RNA. This review summarizes the latest clinical developments in non-invasive prenatal diagnosis in the context of the latest technical developments.

METHODS: We searched PubMed with the search terms ‘prenatal’, ‘non-invasive’, ‘fetal DNA’, ‘mRNA’ and cross-referenced them with ‘diagnostics’, ‘microRNA’, ‘aneuploidy’, ‘trisomy’ and ‘placenta’. We also searched the reference list of the articles identified by this search strategy.

RESULTS: Genome-wide methods have been, or can be, successfully applied on total DNA (DNA-seq), methylated DNA immunoprecipitation (with tiling array), microRNA (Megaplex) and total RNA (RNA-seq). Chromosome- or gene-specific assays have been successively applied on placenta RNA (allele ratio) or DNA multiplex ligation-dependent probe amplification (MLPA). These methods are reviewed for their merits and pitfalls with consideration of the placental biology. For the purpose of clarity, the technical and clinical characteristics are limited to non-invasive prenatal detection of chromosomal aneuploidies, with emphasis on trisomy 21.

CONCLUSIONS: The technical advances for non-invasive aneuploidy tests based on cff DNA and placental mRNA in maternal plasma have been enormous. Multimarker assays including genome-wide approaches with the option of qualitative information on variation (polymorphism or mutation) besides quantitative information are the preferred methods of choice. The time for population-based, double blind, large-scale clinical cohort trials has come.

Key words: non-invasive / free fetal DNA / RNA / placenta / trisomy
Introduction

For the diagnosis of aneuploidy and other chromosomal abnormalities of the fetus, invasive procedures are necessary to obtain fetal cells. These procedures, amniocentesis or chorionic villus sampling, carry a risk of miscarriage due to the procedure. Non-invasive procedures that retain the same specificity and sensitivity are urgently needed. Trisomy 21, related to advanced maternal age, is the most common reason for women to choose prenatal diagnosis. Screening tests like the first trimester combination test are nowadays offered to all pregnant women. Risk calculation is based on maternal age, nuchal translucency (NT) measurement by sonography and two serum markers: pregnancy-associated plasma protein-A (PAPP-A) and free beta hCG (free β-hCG). Both are proteins produced by the outer layer (syncytiotrophoblast) of the placenta, i.e. the layer in direct contact with the maternal circulation. Although the test properties are rather good with a detection rate of ≈85–90% with a false-positive rate of 5–9% (Spencer et al., 1999; Wald et al., 2003; Wapner et al., 2003; Go et al., 2005), the test lacks diagnostic power.

However, non-invasive tests that yield a definitive diagnosis rather than a probability score have now become within technical and clinical reach, by direct targeting for and analysis of fetal DNA and RNA in maternal plasma rather than through an analysis of placental proteins that provide indirect information. This possibility was considered a realistic and testable option a decade ago following the exciting landmark discovery that the blood of pregnant women contains fetal DNA and mRNA from early pregnancy onwards, that appear in the maternal circulation of all pregnancies as a normal consequence of placental physiology and can be isolated and subjected to molecular analysis (Lo et al., 1997; Poon et al., 2000). As this genetic material is identical to the genetic blueprint of the fetus and representative of the transcriptional activity of the placenta, it provides a reservoir of possible biomarkers for the development of non-invasive prenatal tests with diagnostic power (Tsui et al., 2004). These landmark discoveries were followed by technically challenging but accurate methods. These methods allow adequate correction of biological variation (based on allele ratio’s) (Lo et al., 2007a) and genome wide, all-in-one approaches by technology-driven breakthroughs [massively parallel sequencing (MPS)] (Bentley et al., 2008; Voelkerding et al., 2009).

Finally, the biological information gained by targeting for fetal DNA and RNA has enormous implications and great potential for the future; i.e. for biology in general and for prenatal diagnostics in particular. The nucleic acid approach, contrary to proteomic and metabolomic approaches, permits the analysis of non-coding RNA (such as the large gene family of microRNAs) and regulatory DNA (such as differentially methylated DNA), which exceeds that of conventional markers by a factor 4–10 (Ponting et al., 2009).

Focus of this review

Today, the development of a robust non-invasive diagnostic test for fetal aneuploidy is within technical and clinical reach. To assure clinical implementation, essential or desirable qualities of such a non-invasive diagnostic test are the following:

(1) specificity and sensitivity levels each approaching 100%,
(2) eligibility for and applicability in all pregnancies,
(3) the possibility to perform from the first trimester onwards,
(4) eligibility for population-based implementation with large-scale screening,
(5) reasonable costs,
(6) practically executable in a clinical setting, while
(7) meeting the technically demanding criteria allowing analysis in a routine laboratory setting.

This review summarizes the latest developments in the use of free fetal DNA and RNA in maternal plasma for non-invasive prenatal detection of fetal chromosomal aneuploidies, with emphasis on trisomy 21. This focused review aims to provide the reader with a roadmap to overview possible clinical application of non-invasive aneuploidy detection; those that are within reach and those that lie ahead using free fetal DNA and RNA (Table I). The various approaches are discussed in the context of placental biology as specific genetic, epigenetic and biological features unique or specific for the placenta affect the approach to be used and the modifications required.

Methods

Excellent reviews have appeared recently on the nature, origin and characteristics of cell-free fetal DNA (cfd DNA) and RNA along with their use for various prenatal applications (Lo, 2009; Wright and Burton, 2009; Hall et al., 2010). We searched PubMed with the search terms ‘prenatal’, ‘non-invasive’, ‘fetal DNA’, ‘mRNA’ and cross-referenced them with ‘diagnostics’, ‘microRNA’, ‘aneuploidy’, ‘trisomy’ and ‘placenta’. We also searched the reference list of the articles identified by this search strategy and selected those that we judged to be relevant. Review articles are cited to provide readers with more details than this review provides.

Fetal DNA in maternal plasma

Procedural enrichment of fetal DNA

A biological, and therefore technical, limitation of circulating fetal DNA, discovered to be present in maternal plasma (Lo et al., 1997), is that the proportion of cfd DNA is only 3–6% of the total amount of cell-free DNA. Later studies showed that the actual amount is somewhat higher (19%) (Lun et al., 2008), but still a minor fraction of the total amount is in maternal plasma. The majority of cell-free DNA is maternal. This implies that either quantitative and/or qualitative corrections are needed, or selective enrichment techniques applied to be able to measure fetal DNA. An alternative approach is to perform no selection at all and to execute a quantitative genome-wide all-in-one sequence analysis of all, both maternal and fetal, DNA sequences present (see later section on genome-wide sequencing).

The notion that maternal cell-free DNA originates from normal or procedural leakage from peripheral blood cells, led to the idea to reduce the procedural contribution by formaldehyde pretreatment (Dhallan et al., 2004). Although initially claimed to be successful, no enrichment by this pretreatment was reported by other investigators (Dhallan et al., 2004; Chinnapagari et al., 2005; Chung et al., 2005). However, formaldehyde treatment of the fetal DNA does permit another option: cross-linking of the proteins complexed with fetal DNA allowing immunoselection (see later section on nucleosomes).

As fetal DNA is on average smaller in size compared with maternal DNA, this may allow size fractionation as a way to enrich fetal DNA.
(Chan et al., 2004; Li et al., 2004, 2005). Size fractionations by conventional gel electrophoresis followed by isolation of the smaller-sized DNA fraction prior to analysis, is time-consuming and prone to contamination, which prevents its widespread use. However, fetal DNA is enriched for small fragments of specific nature, i.e. nucleosomal fragments, as shown by MPS approaches (Fan et al., 2008), and there is theoretically another option; the targeting of nucleosomes, as will be discussed in a later section.

<table>
<thead>
<tr>
<th></th>
<th>Clinical and technical characteristics of chromosome- or gene-specific versus genome-wide prenatal diagnostics using free fetal DNA or RNA.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1. Chromosome or gene specific</strong></td>
<td></td>
</tr>
<tr>
<td><strong>A. RNA</strong></td>
<td>Type of RNA</td>
</tr>
<tr>
<td>Subtype</td>
<td>mRNA</td>
</tr>
<tr>
<td>Gene</td>
<td>PLAC4, C2orf105</td>
</tr>
<tr>
<td>Method</td>
<td>Q-RT–PCR</td>
</tr>
<tr>
<td>Gender independent</td>
<td>Yes</td>
</tr>
<tr>
<td>Polymorphism independent</td>
<td>Yes</td>
</tr>
<tr>
<td>Tested on plasma samples</td>
<td>Yes</td>
</tr>
<tr>
<td>Advantage</td>
<td>Simple</td>
</tr>
<tr>
<td>Disadvantage</td>
<td>Variation</td>
</tr>
<tr>
<td><strong>B. DNA</strong></td>
<td>Type of DNA</td>
</tr>
<tr>
<td>Subtype</td>
<td>Total</td>
</tr>
<tr>
<td>Gene</td>
<td>SERPINB5</td>
</tr>
<tr>
<td>Method</td>
<td>MLPA</td>
</tr>
<tr>
<td>Gender independent</td>
<td>Yes</td>
</tr>
<tr>
<td>Polymorphism independent</td>
<td>Yes</td>
</tr>
<tr>
<td>Tested on plasma samples</td>
<td>Yes</td>
</tr>
<tr>
<td>Advantage</td>
<td>Sensitive</td>
</tr>
<tr>
<td>Disadvantage</td>
<td>Maternal DNA</td>
</tr>
<tr>
<td><strong>2. Genome wide</strong></td>
<td></td>
</tr>
<tr>
<td><strong>A. RNA</strong></td>
<td>Type of RNA</td>
</tr>
<tr>
<td>Subtype</td>
<td>Polymorphic</td>
</tr>
<tr>
<td>Method</td>
<td>cDNA-seq</td>
</tr>
<tr>
<td>Gender independent</td>
<td>Yes</td>
</tr>
<tr>
<td>Polymorphism independent</td>
<td>Yes</td>
</tr>
<tr>
<td>Tested on plasma samples</td>
<td>No</td>
</tr>
<tr>
<td>Advantage</td>
<td>Complete</td>
</tr>
<tr>
<td>Disadvantage</td>
<td>Method</td>
</tr>
<tr>
<td><strong>B. DNA</strong></td>
<td>Type of DNA</td>
</tr>
<tr>
<td>Subtype</td>
<td>Hypomethylated</td>
</tr>
<tr>
<td>Method</td>
<td>DNA-seq</td>
</tr>
<tr>
<td>Gender independent</td>
<td>Yes</td>
</tr>
<tr>
<td>polymorphism independent</td>
<td>Yes</td>
</tr>
<tr>
<td>Tested on plasma samples</td>
<td>Yes</td>
</tr>
<tr>
<td>Advantage</td>
<td>Spec/sens</td>
</tr>
<tr>
<td>Disadvantage</td>
<td>GC-bias</td>
</tr>
</tbody>
</table>

MLPA, multiplex ligation-dependent probe amplification.
Targeting of fetus specific genetic and epigenetic sequences

The demand for enrichment is unnecessary when the fetal nucleic acids of interest are only expressed by the fetus. This allows selective targeting and thereby selective analysis. The Y chromosome is an obvious choice and is used as a target for validation of new methods (Chiu et al., 2008; Fan et al., 2008; Lun et al., 2008; Papageorgiou et al., 2009). Other examples are prenatal diagnosis of Rhesus antagonism and paternally inherited mutations, which have been reviewed elsewhere (Lo, 2009; Wright and Burton, 2009). However, straightforward discrimination between cff DNA and maternal DNA in maternal plasma for quantification of chromosomes, other than the Y chromosome, is not yet possible since the fetus inherits half its DNA from the mother.

One way to solve this problem is to target epigenetic rather than genetic features. The epigenetic properties carried by certain DNA sequences (mostly CpG islands) appeared to be an interesting strategy. Epigenetics involve processes of heritable changes in gene expression that occur without changes in the DNA sequences (Wolffe and Matzke, 1999). Methylation of CG DNA sequences is an example of an epigenetic change. This approach is based on differential DNA methylation profiles between placenta and maternal cells, and has been demonstrated as a feasible strategy (Poon et al., 2002).

Chim et al. subsequently described a chromosome 18-specific marker with these discriminating properties (Chim et al., 2005). They discovered that the maspin gene (SERPINB5), located on chromosome 18, is hypomethylated in the placenta and hypermethylated in maternal blood cells. By using bisulphite DNA sequencing, the methylation status of the maspin gene promoter in placental tissues and paired maternal blood cells from pregnant women was analyzed. Bisulphite converts unmethylated cytosine in uracil while leaving methylated cytosine unchanged (Chim et al., 2005). Subsequently, the feasibility of trisomy 18 detection was successfully tested in a model system using this bisulphite modification followed by methylation-specific PCR and primer extension to assess the allelic ratios. This generated a sensitivity of 100% and a false-positive rate of 9.7% (Tong et al., 2006).

Subsequent systemic searches for placental DNA methylation markers on chromosome 21 with differential methylation between placenta and maternal blood cells resulted in the detection of multiple genes (Chim et al., 2008a, b). Although the great advantage that the method is gender- and polymorphism-independent, while the low concentration of DNA can be overcome by PCR and genome-wide screening is possible, one major drawback remains. Bisulphite conversion, needed for the majority of methylated markers, destroys a large proportion of the input DNA (Grunau et al., 2001). Unless better methods become available that reduce or prevent this destruction, this approach will remain less favorable for clinical introduction.

However, the reverse situation may provide the solution: where the fetal marker is hypermethylated in placenta and hypomethylated in the maternal tissue (Chan et al., 2006) bisulphite treatment would not damage the DNA of interest. The existence of chromosome 21 markers with this differential methylation pattern (CpG islands of C21orf63 and C21orf29) has been described, but not yet tested in maternal plasma (Old et al., 2007; Chim et al., 2008a, b).

The search for potential markers for aneuploidy detection has been explored with a microarray-based approach. With this approach it is possible to focus on virtually every CCGG site (MspI/HpaII) on chromosomes 21, 13 and 18 for differential CpG methylation patterns between chorionic villus samples and maternal blood cells. The resulting data were consecutively tested for a highly polymorphic single nucleotide polymorphism (SNP) within 150 bp of the identified MspI/HpaII site and in this way numerous potential biomarkers could be identified (Chu et al., 2009). In a model system, the microarray approach was also tested for assessment of genomic methylation to predict the degree of methylation measured with bisulphite-conversion PCR. The microarray signal associated with the trisomic chromosome 21 and 18 was tested in placental tissue and maternal blood and differs significantly from that of other chromosomes (Brown et al., 2010).

Selective targeting by differentially methylated markers in placenta and maternal blood cells has recently been combined with microfluidics digital PCR for non-invasive detection of fetal trisomy 21. Chromosome dosage analysis was performed by comparing the dosage of an epigenetic chromosome 21 marker (HLCS, a hypermethylated fetal-DNA marker) with that of reference chromosomes, RASSF1A on chromosome 3 and ZFY on the Y chromosome. The ratio of HLCS to RASSF1A shows great overlap between euploid and trisomy 21 samples. The comparison between HLCS and ZFY was discriminative, but limited to women carrying male fetuses (Tong et al., 2010).

An interesting new technique, not requiring bisulphite pretreatment, has been tested to detect methylation differences between placental tissue and whole blood by methylated DNA immunoprecipitation (MeDIP) coupled with high-resolution tiling oligonucleotide array analysis. By this approach genome-wide screening is theoretically possible. With this technique the DNA amount is not impaired. Methylation patterns of chromosomes 21, 18, 13, X and Y were analyzed and led to the additional identification of previously unreported fetal epigenetic hypermethylated markers (Papageorgiou et al., 2009). This selective targeting technique combines the advantages of being gender- and polymorphism-independent does not involve loss of input DNA and is discriminative for chromosomes 21, 18, 13, X and Y. Although the method has the right properties to target convenient biomarkers for aneuploidy detection, its clinical value remains to be proven.

It should be noted that the MeDip procedure in its present form uses whole blood DNA rather than cff DNA from plasma. This is most likely related to the amount of input DNA (2.5 μg) needed for MeDIP. In addition, in contrast to fetal and adult tissues, the placenta is subject to hypomethylation. This implies that the number of placenta-specific hypermethylated markers will be less favorable than predicted from or compared with other tissues and applications.

Fetal RNA in maternal plasma

The problem of discrimination between fetal and maternal genetic material can be addressed and solved in a straightforward manner by targeting RNA. As a consequence of normal biology, the level of RNA molecules expressed and circulating is much higher than that of fetal DNA, although variable for different individual genes. On the other hand, RNA isolation is technically more demanding given the additional variation in expression level caused by intrinsic and
extrinsic degradation and by high inter- and intra-individual biological variation. For instance, many genes, consistently increasing in number, show genotype-dependent variation in expression levels leading to normal high, intermediate and low expression level differences among individuals.

**Targeting of placental specific mRNA transcripts**

It is known that each tissue in the body contains a characteristic profile of mRNA. If the presence of fetal-specific mRNA in maternal plasma can be demonstrated, it might provide another source of potential fetal specific biomarkers. The placenta is the most obvious organ that represents the fetus and is in close contact with the maternal circulation. Placental mRNA when released in a tissue-specific or tissue-restricted manner in the maternal circulation, thereby being absent in the plasma of non-pregnant individuals, qualifies as fetal-specific and therefore as potential biomarker for aneuploidy detection during pregnancy. The proof of principle of fetal RNA in maternal plasma was first demonstrated in pregnant women carrying a male child using Y chromosome-specific zinc finger protein mRNA (Poon et al., 2000). Besides the demonstration of fetal RNA, this finding demonstrated another feature of the cff RNA approach, it allows the detection of transcription factor RNA, i.e. molecules hardly accessible by conventional antibody-based assays.

The presence and detectability of fetal (placental) RNA in all pregnancies were subsequently demonstrated a few years later, through the analysis of placental-specific mRNA, human placental lactogen (hPL) and the β subunit of human placental chorionic gonadotrophin (β-hCG) mRNA (Ng et al., 2003).

Placental mRNA in maternal plasma has been demonstrated to be stable. Filtration studies indicate that the particle-associated nature of the RNA- and DNA-containing microparticles circulating in the maternal blood is the secret of the stability of plasma RNA that protects them from degradation by RNase. Studies for systematic identification of placental mRNA markers, detectable in maternal plasma, followed. Gene expression profiles between placental tissues and peripheral blood from pregnant women were compared in the first and third trimesters by oligonucleotide microarray followed by real-time quantitative RT–PCR (Tsui et al., 2004). In this study six genes with the pattern of interest could be identified: hPL, β-hCG, CRH, TFP12, KISS1 and PLAC1. In another study, a set of 80 genes, selected for their confirmed or predicted placental specificity, was tested using RT–PCR for their presence in early placenta-tissue, absence in non-pregnant plasma and presence in pregnant plasma (Go et al., 2004). In this study eight genes with the pattern of interest (placental expression, present in maternal plasma and absent in non-pregnant plasma) could be identified: GOM1, ZDHHC1, PAPPA, PSG9, PLAC1, β-hCG and LOC90625. Not only was rapid screening of a large set of potential new markers demonstrated, it allowed the detection of markers not accessible by conventional antibody-based assays (Go et al., 2004; Tsui et al., 2004).

**Targeting of chromosome-specific placental RNA**

The search also focused on potential markers for trisomy 21 detection. Oudejans et al. were the first to describe a chromosome 21-specific mRNA present in maternal plasma, LOC90625 (nowadays called C21orf105) (Oudejans et al., 2003). As a consequence of the direct dosage-related difference in expression of chromosome 21-encoded genes between normal and Down syndrome pregnancies, message quantification of C21orf105 in maternal plasma could theoretically discriminate between trisomy 21 and normal pregnancies. However, due to the large biological variation between and within individuals, along with the low expression profile of C21orf105, C21orf105 quantification initially appeared unsuccessful despite correcting for variation using a housekeeping gene (Go et al., 2007a, b).

However, based on the same direct principle by targeting for chromosome 21-encoded transcripts, Lo et al. developed a simple and intelligent strategy to correct for this variation: RNA allelic ratio assessment using SNP (Lo et al., 2007a, b). The gene target in these first studies, PLAC4, has high expression levels in the developing placenta with corresponding high levels of circulating RNA.

What is the principle of the allelic ratio approach? If a fetus is heterozygous for a certain SNP on the chromosome of interest it possesses two different alleles that are distinguishable. If the fetus is euploid, having two copies of the chromosome of interest, the ratio of these two SNP alleles would be 1:1. When the placenta releases its mRNA into maternal circulation, the ratio of placental mRNA in maternal plasma that is transcribed from each of these two alleles would also be 1:1. However, if the fetus has a trisomy 21 and is heterozygous for a SNP on a chromosome 21-transcribed mRNA, then the RNA-SNP allelic ratio would become 1:2 or 2:1 (Fig. 1). In Lo’s study it was hypothesized that the difference in allelic ratio present in the fetus could be measured in the plasma of the pregnant women by a placenta- and chromosome 21-specific mRNA, PLAC4, using extension primers and mass spectrometry detecting the extension products. PLAC4 was chosen for its abundant expression. This strategy was tested on placenta tissue and plasma samples from euploid and trisomy 21 pregnancies. The sensitivity was 90% and the specificity 96.5%. These scores were higher than the scores of the combined multiple marker serum test despite the use of a single marker (Lo et al., 2007a, b). This RNA-SNP allelic ratio strategy has therefore significant potential given its high accuracy. The placental mRNA tested is completely fetal-specific. It was successfully tested in trisomy 21 pregnancies and in a model system for trisomy 18 (Tsui et al., 2009), and is theoretically applicable for trisomy 13 as well. However, although gender-independent, the method is polymorphism-dependent, which is an important disadvantage. The heterozygosity rate differs per SNP and between different populations. PLAC4 SNP (rs8130833) has a heterozygosity rate of 0.45 in the population (Chinese and Caucasian) studied. This means that only in about half of the pregnant females tested, the allelic ratio test will be informative. The population coverage can theoretically be broadened by combining several SNPs of genes transcribed from chromosome 21. The search for other convenient genes had led to the finding of placental mRNA targets with potential different SNPs (Go et al., 2007a, b).

Still making use of one SNP, encoded by the PLAC4 gene, a strategy combining informativity (as provided by the allele ratios) with high sensitivity (as provided by digital PCR) was applied to late first trimester, euploid and trisomy 21, plasma samples. With mass spectrometry (MS) and digital PCR the RNA-SNP allelic ratio strategy analyzed in heterozygous samples has been applied in diagnostic tests (Tsui et al., 2010).
The advantage of digital PCR is that it is especially useful in samples with low mRNA concentrations. On the remaining homozygous samples, quantification by quantitative real-time PCR or quantification by digital PCR was applied as a screening instrument. The diagnostic sensitivity and specificity of 100 and 89.7% were achieved for both methods, the MS and the digital PCR. This group contains 58 euploid and 4 trisomy 21 samples. In the ‘homozygous’ group the area under the receiver operating characteristic curves were 0.859 for the real-time PCR quantification and 0.833 for the digital quantification (Tsui et al., 2010). As a screening test with a deduced false-positive rate around 17%, this approach is not yet suitable for clinical implementation.

The methods tested above require highly specialized equipment, which prohibits the widespread implementation of the RNA-SNP strategy. An alternative technique has been described. The quencher extension technique (QEXT) was adapted and applied for the allelic ratio strategy (Go et al., 2008). The QEXT reaction uses the same principle of allelic ratio determination, but is a single-step, real-time method to quantify SNPs and, most importantly, is directly adaptable to current real-time PCR equipment. The method was tested in a model system with early second trimester placenta tissue (Go et al., 2008). An SNP (rs 2187247) located in exon 2 of C21orf 105 was used as marker. Allelic ratio differences between bi-allelic and tri- allelic cases in the trisomy 21 model system could successfully be demonstrated. It remains to be seen whether this technique is feasible and discriminative in maternal plasma, but if so, QEXT might facilitate implementation of a diagnostic test based on SNP-RNA allelic ratio differences, which is low cost and less labor-intensive than other techniques.

**New approaches with great potential**

**Digital PCR**

Several recent developments and breakthroughs are technology-driven. For example, digital PCR, a highly sensitive technique that...
uses limiting dilution to isolate single template DNA molecules to be amplified. Digital PCR was tested in a model system for molecular detection of fetal trisomy 21 (Lo et al., 2007a, b). It is noteworthy that in this way fetal-derived DNA is not specifically distinguished from maternal DNA. Chromosome dosage was determined by analysis of a non-polymorphic chromosome 21 locus relative to a non-polymorphic locus on a reference chromosome, in this study chromosome 1. Artificial mixtures containing placenta DNA in a background of maternal blood cell DNA were analyzed. Based on these results, the estimation was made that correct classification in 97% of the cases could be made with samples containing 25% fetal DNA (Lo et al., 2007a, b). However, an input of 15 ml maternal blood was needed to reach a sufficient amount of DNA after extraction. For implementation in a routine clinical test this would be undesirable. Thus while the method was demonstrated to be feasible, the technique is demanding and an enrichment step would be necessary.

MPS

A breakthrough with enormous potential is MPS. Millions of short sequence tags can be sequenced by high-throughput shotgun sequencing technology, enabling deep sequencing (Fan et al., 2008; Mardis, 2008). Fan et al. tested this technique on cell-free DNA from plasma of pregnant women with a gestational age of 10–35 weeks (Fan et al., 2008). Over- and under-representation of chromosomes were measured. It was possible to discriminate all fetuses with trisomy 21 (n = 6), trisomy 18 (n = 1) and trisomy 13 (n = 1) from the six normal cases. A drawback of this study was that the samples were collected 15–30 min after invasive procedures. Puncture, necessary to obtain fetal material, disrupts the barrier between maternal and placental circulation and artificially influences the amount of fetal DNA in the maternal circulation (Samura et al., 2003).

Chiu et al. tested the same technique, but followed a different strategy for data analysis (Chiu et al., 2008). They tested this strategy for trisomy 21 and for the Y and X chromosomes difference between male and female fetuses. They tested an algorithm to calculate the percentage unique sequences for the chromosome of interest in the test sample compared with the reference population of that same chromosome. They were able to discriminate trisomy 21 from disomy 21 samples. In this study 11 of the 14 trisomy 21 samples were collected after an invasive prenatal procedure (range 2–22 days). The other samples were collected before the invasive procedure. Nevertheless, both studies demonstrated the feasibility of deep sequencing for non-invasive prenatal diagnosis.

Measurements of the genomic representations for chromosomes 13 and 18 were less precise (Chiu et al., 2010). The latter appears a consequence of the variable mean GC percentages of the individual autosomes and dependent in the MPS method used. However, when this hurdle is taken either by adaptations in prehandling or during sequencing, for example by single-molecule detection approaches, it will be just a matter of time, till MPS approaches allow simultaneous detection of all trisomies in a single assay.

The important advantage of the MPS technique is that it is gender- and polymorphism-independent, applicable in all pregnancies and likely to allow analysis of all frequent forms of aneuploidies in the same test. Currently the technique is technically demanding, the costs per tested sample are high and the throughput per instrument is low (16 samples per week). This prevents its use as a regular test for all pregnant women. However, given the considerable attention this approach is receiving for prenatal diagnosis and other purposes, and the huge efforts undertaken by the companies involved, the throughput both in time, sample number and costs will all improve drastically in the coming 5 years. One point of concern is that a possible change in the composition of the cff DNA might interfere with the reliability of this quantitative test. The concentrations of circulating cell-free DNA and cff DNA in maternal plasma are variable in normal pregnancies (Zhong et al., 2000) while in women who developed pre-eclampsia increased fetal DNA concentrations were found prior to the onset of pre-eclampsia in the first half of the second trimester (Leung et al., 2001). Although a higher amount of fetal DNA is likely to facilitate early testing, potentially a change in the composition of the cff DNA, due to a pathological process in the placenta, might interfere with the reliability of these quantitative tests (Cotter et al., 2004). On the other hand, it could be highly informative when this change is pathognomonic for the disease of interest.

It should be noted that the MPS approach also permits detection of sequence variations (polymorphism, mutation and copy number variations). Provided that the sequence coverage is large, the probability score for a correct call is >0.99, >0.98 and >0.95 for coverages of 30, 25 and 20, respectively (Heap et al., 2010). This implies that the allelic ratio approach is theoretically possible for fetal DNA by the MPS method provided that sequence coverage is large.

In summary, MPS is certainly one of the most promising approaches. Yet, population-based studies involving prospective studies in low-risk populations (i.e. normal populations) have to indicate if the technique is robust.

Placental microRNA

MicroRNAs (miRNAs) are short, 19–25 nucleotides, single-stranded, non-coding RNAs (Lee and Ambros, 2001; Lagos-Quintana et al., 2001). Among other regulatory processes, they regulate gene expression by binding to the 3′ untranslated region of the target mRNAs. Multiple microRNAs can regulate a single gene, while a single microRNA can regulate multiple genes. Since their first description (Lee and Ambros, 2001; Lagos-Quintana et al., 2001), miRNAs have been studied because of their regulatory role in gene expression and currently the number of miRNAs known in humans is approaching 1000 (www.mirbase.org). miRNAs, if placenta-specific, qualify as a biomarker. The search for placenta-specific miRNAs, present in maternal blood, has started. A systematic search of 157 well-established placental miRNAs in maternal plasma was performed by real-time quantitative RT–PCR and physical properties of the miRNAs were investigated (Chim et al., 2008a, b). Comparison was made between placental tissue obtained after a term delivery and maternal blood cells; samples collected in the first, second and third trimester and 24 h post-delivery. From the 157 placental miRNAs tested, 17 occurred at concentrations more then 10-fold higher in placentas then in maternal blood. miR-141, miR-149, miR-299-5p and miR-135b were the four most abundant of these placental miRNAs present in maternal plasma.

First trimester and term placenta tissues were sequenced to identify placenta-specific miRNAs. Luo et al. identified placenta-specific
miRNAs, such as miR-517A, abundant in plasma of pregnant women and rapidly cleared after delivery.

miR-517A belongs to the chromosome 19 microRNA cluster, which is the largest human miRNA gene cluster described so far. This primate-specific miRNA cluster spans ≏100 kb at human chromosome 19q13.41 and comprises ≏46 tandemly repeated miRNA genes exclusively expressed in the placenta. Recently, this cluster was demonstrated to be subject to genomic imprinting with only the paternally inherited allele being expressed in the placenta (Marie et al., 2010). Genomic imprinting indicates by definition involvement in gene dosage compensation. The absence of microRNAs on the Y chromosome, the presence of X-linked primate-specific microRNA clusters, and the male-biased expression of miRNAs on the X chromosome indicate a specific role for microRNAs in gene dosage compensation for the sex chromosomes as well (Mishima et al., 2008; Li et al., 2010). For both situations, autosomes and sex chromosomes, this indicates an as-yet unexplored role, either diagnostic or functional, of microRNAs for diseases with gene dosage anomalies (such as trisomy) or caused by gain- or loss-of-function of susceptibility genes (such as pre-eclampsia).

Further tests suggest that miRNAs are released by exosomes and are exported from the syncytiotrophoblast into maternal circulation (Luo et al., 2009).

The presence of placenta-derived miRNA in maternal plasma has been shown to decrease within the 24 h after delivery (Chim et al., 2008a, b). It is imperative for a useful placenta-specific marker that the marker cannot be detected after pregnancy or in the blood of non-pregnant women. If a marker can be measured after pregnancy, either the marker is not placenta-specific or is not ‘cleared’ after pregnancy; both might interfere with results in the same or the next pregnancy. miR-141 and miR-149 showed a significant reduction, but not a reduction to zero like placental miRNA in maternal plasma. The presence of miRNAs was studied in serum as well; comparison was made between pregnant and non-pregnant women. MicroRNAs associated with human placenta were significantly elevated in serum of pregnant women and their levels correlated with pregnancy stage (Glad et al., 2008). Although levels of miR-141 and miR-149 were not as different between non-pregnant and pregnant women when measured in serum rather than plasma as found in the study of Chim et al., it can be stated that based on these studies miRNAs can be detected in both maternal plasma and serum.

This new class of circulating nucleic acids might provide a source of biomarkers for non-invasive aneuploidy detection and pregnancy associated (pathological) processes. An additional advantage is that miRNAs appear extremely stable while methods are available allowing real-time quantification of all currently known microRNAs (Chim et al., 2008a, b).

**Placental macroRNA**

In parallel with the revolution on miRNA, the existence of large non-coding RNA transcripts, such as macroRNA (maRNA) and large intergenic non-coding RNA (lincRNA) has been demonstrated (Latos and Barlow, 2009). The lincRNA termed HOTAIR (Rinn et al., 2007) was discovered, and is encoded antisense to the HOXCl cluster at the exact juncture of a 40 kb domain of heterochromatin and a 60 kb domain of euchromatin. However, HOTAIR does not serve to regulate this boundary. HOTAIR affects the global epigenetic state of the HOXD cluster located on a separate chromosome. This means that lincRNAs of this class serve as chromatin modifiers and function as epigenetic ‘air-traffic controllers’. It is likely that the developing human placenta expresses several (as yet unidentified) maRNAs or lincRNAs that are specific for the placenta and control its underlying biological processes. Despite their large size, given the fact that placental RNA as circulating in the maternal plasma is fragmented, it can be expected that these RNA family will be as informative as conventional RNA for prenatal diagnosis, most likely for pregnancy associated diseases with placental origin.

**Targeting of nucleosomes**

The actual peak fragment size of cff DNA is on average 169 bp (Fan et al., 2008). This size corresponds to the length of DNA wrapped in a chromatosome, which is a nucleosome bound to a H1 histone and in accordance with the finding that majority of cell-free DNA in the plasma is derived from apoptotic cells (Rochman et al., 2009; Furusawa and Cherukuri, 2010). This generates an interesting option. High mobility group N (HMGN) proteins are the only nuclear proteins known to specifically recognize the generic structure of the nucleosome core particle. HMGN proteins are involved in epigenetic regulation by modulating chromatin structure and levels of post-translational modifications of nucleosomal histones. Expression of HMGN proteins is developmentally regulated. One of these, HMGN5, has a unique molecular structure and expression pattern. Its expression and function appear restricted to and important for the placenta as well as the reproductive system (Rochman et al., 2009; Shirakawa et al., 2009; Furusawa and Cherukuri, 2010). This means that HMGN5 immunoprecipitation of circulating DNA might enrich for trophoblast-specific nucleosomes, that when analyzed by MPS (HMGN5-ChiP-seq) provide a better (i.e. selective) coverage and thereby information of fetally derived sequences.

**Microarray analysis**

The possibilities of microarray analysis for prenatal diagnostic analysis of fetal DNA were recently tested using a genome-wide 250 K SNP array platform (Faas et al., 2010). Although analyzed on fetal DNA obtained after termination of pregnancy or birth, or intruterine fetal DNA, the study showed that in 16% of fetuses with ultrasound anomalies and a normal or balanced karyotype, causal submicroscopic aberrations were detected. This would have gone undetected with most-targeted approaches thereby underscoring the added value of a genome-wide approach. The question then arises whether microarray or next-generation sequencing will remain the method of choice for genome-wide approaches. Microarrays have the following intrinsic limitations: (i) Requires a priori knowledge of the genome; (ii) Cross-hybridization between similar sequences; (iii) Detection of low-abundance targets is difficult and (iv) Micrograms of input DNA are needed. Next-generation sequencing approaches offer remedies to these problems. (i) Knowledge of the genome is helpful but not required; (ii) No experimental bias and cross-hybridization issues; (iii) NGS approaches are equally adept at detecting changes in rare and highly expressed sequences in the same sample; (iv) Nanograms rather than micrograms are required. And finally all next-generation platforms have the same output (sequences) (Hurd and Nelson, 2009). Although these features strongly favor the use of MPS, one
can envision a powerful symbiosis. Arrays might remain useful when a low-cost, quick look is required for clinical isolates. Combined options where the microarray is used as pre-selection of DNA analyzed by MPS, such as for exon-sequencing, have become reality. For use in prenatal diagnosis using cff DNA or RNA, given the reasons above (low input, unbiased and complete information, etc.), MPS methods will certainly become the method of first choice.

**RNA sequencing**

The same arguments are true for prenatal diagnosis of fetal RNA rather than DNA. RNA following the enzyme-mediated conversion of single-stranded RNA into copy DNA allows the same approach: genome-wide analysis of all sequences expressed (transcriptome) in a particular sample (RNA-seq or cDNA-seq). It allows the detection of single-nucleotide variations in expressed sequences, allelic expression imbalances with insight in the transcriptional landscape of a particular sample, tissue or cell (Chepelev et al., 2009; Tang et al., 2010; Tuch et al., 2010). The prediction is justified that RNA-seq approaches applied on placental RNA isolated from maternal plasma will be performed and published in the immediate future and be expected to have the same impact as DNA-seq.

**The all-in-one approach**

Given the preference for genome-wide approaches, and the need for complete information on genetic variations, both genetically and epigenetically and both for DNA and RNA of any nature (non-coding), one might consider an all-in-one approach. This approach, at this stage extremely costly, but likely to be reduced in cost and practicality in the near future, would consist of the following protocol. From EDTA blood of a pregnant female taken during the first trimester, preferably at two sequential time points, the plasma anduffy coat are separated by centrifugation. The buffy coat DNA is subjected to MeDIP or related specific immunoprecipitations. The plasma is subjected to fractionated DNA and RNA isolation with selective affinity-based enrichments of total DNA, small (<200 bp) and large RNA (>200 bp). All fractions (n = 4) are subjected to DNA or cDNA library construction, and analyzed by deep sequencing. This will provide a complete overview of the genome, epigenome and transcriptome with both quantitative and qualitative information of the free DNA and RNA present in maternal blood. Comparison of all data between the two time points will permit correction for biological variation in addition to selection for fetus-specific information.

**Implementation in clinical practice**

What if a reliable, cost-effective, diagnostic non-invasive test for trisomy 21 (and other aneuploidies) was available? The main advantage is that the iatrogenic fetal loss due to invasive procedures like amniocentesis and chorionic villous biopsy would be avoided. Invasive procedures for reason of advanced maternal age would be replaced. It would be logical that screening tests on Down syndrome would be replaced as well, not only the first trimester combined test, but also the triple and integrated test. It can reasonably be expected that this diagnostic test to be will be independent of gestational age. This fact would be a great advantage for daily practice. A second advantage is the fact that one test can replace two others (NT measurement and the serum test) and a risk calculation is redundant. A lot of time would be saved. However, what can definitely not be replaced is the pre-test counseling. It is likely that explanation of a diagnostic test is easier then explanation of a screening test. The impact of the result should be clearly discussed and thought over before the patient chooses to have the test. The risk of an easy and low-threshold diagnostic test is that thinking starts after the test result. The result in this case can mean a serious prenatal diagnosis for the unborn child. Counseling (pre- and post-test) and informed choice is, and must, remain an important part of it. In the report of the UK expert working group on ‘cff nucleic acids for non-invasive prenatal diagnosis’, a move toward a rigorous informed consent model is advocated (Wright, 2009).

Can we abandon the NT measurement? An increased or thickened NT is not only associated with trisomy 21, but also with other chromosomal abnormalities, congenital malformations or syndromes of the fetus. If the non-invasive diagnostic test can diagnose aneuploidies (trisomies 18, 13, 45X), it has to be studied and discussed what the remaining reasons would be for carrying out screening by NT measurement. In the future, besides a full karyotype, formation of a screening panel for DNA mutations, microdeletions or duplications indicating syndromes or diseases associated with a thickened NT is conceivable. It might be well possible that the NT measurement will be offered in parallel to a non-invasive diagnostic test for aneuploidies. But it must be discussed what the role of this additional screening test would be.

What would be the ideal timing to perform such a test? Of course as such a test is not yet available, this has not been rigorously investigated. Until about 10 weeks of gestation, depending on maternal age, miscarriage is a regularly occurring phenomenon. It is also likely that the concentration of circulating nucleic acids in maternal plasma is lower in cases of pregnancy demise, and that might interfere with the feasibility of performing the test. A potential optimum time for conducting such a test might therefore be between 10 and 15 weeks of gestational age.

**Conclusion**

Throughout the last decade advances made in the technical possibilities for non-invasive aneuploidy tests based on cff DNA and placental mRNA in maternal plasma are considerable.

In small studies and model systems strategies and 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. The RNA-SNP allelic ratio strategy seems to be the most feasible test at present with the desirable quality that the result is based on fetal-specific genetic material, but has the disadvantage of polymorphism-dependence. Using several markers might help to enlarge population coverage. MPS is promising, but at the moment too labor-intensive for routine clinical implementation and is very costly. One great advantage of MPS is the fact that the test is gender- and polymorphism-independent, and several aneuploidies can be tested at the same time.

Combining techniques and strategies might have a place in the future. However all strategies and techniques have to be further tested to demonstrate or validate their diagnostic potential and their
cost-effectiveness for aneuploidy detection. The time for population-based, double blind and clinical trials has come. Only then might the promise of these technical advances become reality.

**Authors’ roles**

A.T.J.G., J.M.G. van V, and C.B.M.O. were all involved in reviewing the literature and the preparation of the manuscript.

**Funding**

The authors did not receive funding for this work.

**References**


