Non-invasive prenatal diagnosis of aneuploidies: new technologies and clinical applications

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Abstract
Non-invasive prenatal diagnosis (NIPD) has substantial medical importance as it targets the development of safer and more effective methods to avoid the risk of fetal loss associated with currently used invasive methods. Several approaches have been demonstrated as being proof-of-concept for NIPD of chromosomal aneuploidies. These approaches include cell-based and cell-free detection methods, involving the investigation of fetal cells in the maternal circulation, formaldehyde treatment of maternal plasma, DNA methylation studies using sodium bisulfite or restriction enzymes, protein-based studies, identification of fetal-specific mRNAs and digital polymerase chain reaction (PCR) approaches, and recently next-generation sequencing and methylated DNA immunoprecipitation real-time quantitative PCR-based approaches. Although all these NIPD methods have both advantages and limitations, some are moving closer to clinical implementation. Biotechnology companies dedicated to the development of NIPD tests such as the sequencing- or methylation-based approaches are finalizing large clinical trials. It is expected that these new technologies will facilitate safer, more sensitive and accurate prenatal diagnostic tests in the near future. In this review, we highlight the most recent advances in methods for NIPD of aneuploidies, and we discuss their future implications in clinical practice.

Keywords fetal DNA, fetal-specific methylation, next generation sequencing, NIPD clinical implementation, non-invasive prenatal diagnosis

Prenatal diagnosis of aneuploidies
Prenatal diagnosis was introduced in the early 1970s with the primary aim of diagnosing aneuploidies such as trisomy 21 (Down syndrome), trisomy 18 (Edwards syndrome) and trisomy 13 (Patau syndrome), as well as aneuploidies related to X and Y chromosomes (for example, Klinefelter syndrome, trisomy X and Turner syndrome) [1-3]. The majority of cases with aneuploidy result in termination of the developing fetus [4] and aneuploidy is responsible for at least 35% of miscarriages [5]. Nevertheless, there can be cases of live birth, with the most common being trisomies 21, 18 and 13 [4]. The highest survival rate occurs with trisomy 21, which affects 1 in 800 births, whereas trisomy 18 affects 1 in 6,000 births, and trisomy 13 affects 1 in 10,000 births [6]. Prenatal diagnosis has been of great importance since it provides parents with the choice to abort a fetus with the diagnosed condition or to prepare psychologically, socially, financially and medically for a baby with a health problem or disability, or for the likelihood of a stillbirth.

Prenatal diagnosis is achieved through the collection and testing of fetal material during the first, second or third trimester of pregnancy. The most common methods currently used for sampling fetal material are chorionic villi sampling (CVS) during the first trimester and amniocentesis during the second trimester [7,8]. The diagnostic accuracy of these methods is estimated to be 98 to 99% [9]. However, both sampling procedures are invasive, and independent studies indicate that they are associated with a significant risk of fetal loss, which occurs in 0.5 to 1% of all tested cases [1,10]. The risk of fetal loss could be even higher, especially during CVS, if the doctor who performs the sampling procedure is not highly experienced [11]. For this reason invasive prenatal diagnosis tests are currently performed only in high-risk pregnancies or in pregnancies with increased maternal age and/or family history of having a child with an inherited disease.

The limitations of these invasive methods have led to research efforts towards the development and implementation of non-invasive prenatal diagnostic (NIPD) methods for common aneuploidies and other fetal chromosomal abnormalities. NIPD has been shown to have no associated risk of miscarriage and could therefore be
implemented for any pregnant woman, irrespective of age or risk of having an affected child [9].

NIPD methods that have been described to date are divided into two categories: cell-based and cell-free approaches, with the second being the most commonly used. The discovery of free fetal DNA (fDNA) in the maternal circulation during pregnancy [12] was probably the most important step in opening the way towards the development of NIPD approaches. fDNA has been used successfully for the determination of fetal sex and fetal Rhesus D status in maternal plasma [13,14], and these methods are currently applied as routine tests in a number of diagnostic laboratories worldwide. However, direct analysis of the limited amount of fDNA (3 to 6%) [15] in the presence of an excess of maternal DNA is a great challenge for NIPD assessment of fetal aneuploidies. Recent studies suggest that fetal DNA accounts for 10 to 20% of total DNA in the maternal circulation [16]. In aneuploidies, one of the chromosomes is present with additional or fewer copies. For example, in trisomy 21, three copies of chromosome 21 are present instead of two. Therefore, the ability to distinguish normal cases from trisomy 21 cases would depend on the ability to detect the extra copy of chromosome 21. The relatively high levels of maternal DNA in the maternal circulation compared with the limited amount of fetal DNA further complicate quantification.

Over the past decade a large number of different methods have been applied to enable discrimination of fDNA from circulatory maternal DNA or fDNA enrichment [17,18]. These include DNA-based approaches that directly target fDNA sequences, such as sequencing approaches [19,20]. Additional studies have focused on the development of epigenetic approaches, with the majority of these investigating the methylation status of fetal DNA using either sodium bisulfite DNA treatment [21], methylation-sensitive restriction enzymes [22] or antibodies specific to the 5-methylcytosine residues of CpG dinucleotides in the genome [23]. Alternative approaches have targeted fetal-specific mRNA [24] or have focused on the investigation of fetal-specific proteins [25].

In this review, we discuss methods for NIPD of aneuploidies, highlighting the most recent advances, their advantages and disadvantages, and their future use in clinical practice.

Developments in aneuploidy detection

Cell-based detection methods

NIPD was initially attempted through the isolation and investigation of intact fetal cells from the maternal circulation during pregnancy. A number of different fetal cell types have been found in maternal peripheral blood, including fetal trophoblasts, leukocytes and nucleated red blood cells (NRBCs). Trophoblast enrichment and isolation is hindered by the lack of placental-specific antibodies as well as the presence of multinucleated morphology [26,27]. Leukocytes may persist from previous pregnancies, which complicates their evaluation in a subsequent pregnancy. Furthermore, leukocytes lack unique cell markers that could be used to discriminate fetal from maternal leukocytes [28]. However, NRBCs have a short half-life of 25 to 35 days and are therefore unlikely to persist from previous pregnancies [29]. Moreover, they have unique cell morphology and a complete chromosomal complement. Consequently, the majority of studies based on fetal cell recovery in maternal circulation have focused on the investigation of NRBCs [30,31].

Techniques such as fluorescence in situ hybridization (FISH) and polymerase chain reaction (PCR) have shown that there is an average of one fetal NRBC per milliliter of maternal peripheral blood, with a maximum of six fetal cells per milliliter of maternal peripheral blood [32,33]. Due to this limited number of fetal NRBCs, both FISH- and PCR-based approaches have difficulties in isolating and investigating these cells. Using FISH, the majority of NRBCs are identified as maternal and only a small proportion (if any) are identified as being of fetal origin [30,31]. Single-cell PCR approaches have shown that at least 50% of the NRBCs are of maternal origin [34]. In addition, the majority of these cells undergo apoptosis, which leads to unstable and fragmented DNA that is not suitable for molecular cytogenetic analysis [35,36]. PCR analysis of these cells is also associated with allele dropout (failure to amplify or detect an allele). To overcome these difficulties, a higher number of fetal cells would need to be analyzed (for example, five to six fetal cells) [37].

Fetal cell enrichment strategies

Enrichment to increase the number of fetal cells isolated from the maternal circulation has been of great importance in reducing the complexity of evaluating fetal cells. The first step involves Ficoll-Paque or Percoll-gradient density centrifugation to remove mature red blood cells and granulocytes, leaving peripheral mononuclear cells [34,38]. Further enrichment is usually achieved using methods such as fluorescence-activated cell sorting (FACS) [31,39], magnetic-activated cell sorting (MACS) [40,41] or charge flow separation [42]. The general principle of these methods is the separation and isolation of fetal cells from maternal cells using monoclonal antibodies that target fetal-specific cell surface antigens. The first monoclonal antibody used for enrichment of fetal cells was directed against CD71, the transferrin receptor [30]; however, purity after FACS was low. An improvement was later made by using MACS depletion of anti-CD45 prior to selection of CD71-positive
shown that cell-free DNA is detectable from the first trimester blood during pregnancy [12]. Subsequent studies have described for the first time the presence of cell-free fetal DNA (cff DNA) in maternal peripheral blood. Based on this observation, in 1997 Lo and colleagues described for the first time the presence of cell-free fetal DNA (cff DNA) in maternal peripheral blood during pregnancy [12]. Subsequent studies have shown that cffDNA is detectable from the first trimester of pregnancy in maternal plasma at an abundance of 3% of total plasma DNA. This value increases during pregnancy, reaching 6% towards the end of the pregnancy [15,54,55], although more recent studies have demonstrated a range of 10 to 20% of fetal DNA [16]. The studies that refer to a higher percentage of fetal DNA are more recent and they have been using more advanced technologies (digital PCR) compared to previous studies that have used real time quantitative PCR (qPCR) technologies. The first non-invasive diagnostic efforts aimed to identify Y chromosomal regions and thereby determine fetal sex, which would facilitate the early detection of hereditary X-linked disorders [56]. In addition, the use of DNA markers such as single nucleotide polymorphisms (SNPs) has allowed the detection of fetal genetic abnormalities. One such example is the detection of paternally inherited SNPs located in the β-globin locus for NIPD of β-thalassemia [57,58]. Furthermore, the application of PCR technology in combination with mass spectrometry (MS) has allowed the detection of fetal monogenic mutations present in the maternal circulation [57-63]. One such application was the use of the MassARRAY system, a matrix-assisted laser desorption ionization time-of-flight MS system designed for the detection of primer-extended PCR products [62,64]. The MassARRAY system has been used successfully for the reliable exclusion of β-thalassemia mutations in maternal plasma.

Nevertheless, the development of NIPD for aneuploidies remains the greatest challenge, and the primary target has been trisomy 21, the most common aneuploidy [6]. One of the first difficulties to overcome was the limited amount of cffDNA in the maternal circulation (3 to 6% [15] or 10 to 20% based on recent studies [16]). For this reason, the primary focus has been on the development of methods for the enrichment of cffDNA to enable accurate quantification of copy number changes that are associated with fetal aneuploidies.

**Detection of aneuploidies**

An early prospective multi-center study to evaluate the utility of fetal NRBCs for NIPD of aneuploidies was initiated in 1994. The study, which was completed after 5 years and included 2,744 maternal samples, demonstrated that only 41.1% of male euploid cases could be identified, whereas the success rate was higher in aneuploid cases (74.4 %), indicating that fetal cells are more abundant in aneuploid pregnancies and therefore more easily detected [44]. Independent studies using PCR methods have confirmed these findings, especially in trisomy 21 cases, and have reported sixfold higher numbers of fetal cells in maternal blood in trisomy 21 cases compared with normal cases [45].

Although the use of NRBCs was initially promising for prenatal diagnosis, their extremely small number in the maternal circulation, as well as the fact that the vast majority are of parental origin, has not allowed the successful implementation of fetal cell-based approaches [45-47]. Another approach is the investigation of intracellular fetal-specific proteins; however, there is not much evidence yet to support this method [48].

An alternative source of intact fetal cells is the distal endocervical canal. Fetal cells released into the uterine cavity during early pregnancy are a potential source of material for NIPD [49], and are most probably extravillous trophoblasts [50,51]. In a study of 28 normal pregnancies and five trisomy 21 cases at 12 to 13 weeks of gestation, abnormal fetal cells were identified in all abnormal pregnancies, and one abnormal cell was detected in a normal pregnancy using dual FISH labeling and automated microscopy [52]. Nevertheless, larger-scale validation studies are required to evaluate the sensitivity and specificity of this approach for NIPD of aneuploidies.

It is evident that once a highly sensitive method for isolating intact fetal cells is developed, the advantages for NIPD would be great. Intact fetal cells contain the complete fetal genome, which could be investigated using high throughput methods such as microarray- and sequencing-based technologies for screening and diagnosis of aneuploidies as well as other genomic disorders.

**Cell-free detection methods**

The discovery of free nucleic acids in the circulation of adults in 1948 [53] has been of great importance in the field of NIPD. Based on this observation, in 1997 Lo and colleagues described for the first time the presence of cell-free fetal DNA (cffDNA) in maternal peripheral blood during pregnancy [12]. Subsequent studies have shown that cffDNA is detectable from the first trimester...
An alternative approach for cfDNA enrichment and development of NIPD for trisomy 21 was the treatment of maternal peripheral blood with formaldehyde. The theory behind this was that stabilization of maternal white blood cells using formaldehyde would lead to reduced maternal cell lysis, and therefore less maternal DNA would be released into the maternal plasma [67,68], leading to reduced maternal background and therefore indirect enrichment of cfDNA in maternal plasma. This approach was performed in 60 pregnancies including three trisomy 21 cases, and 56 of 57 normal cases and two of the three trisomy 21 cases were correctly classified. Although the initial findings of this approach were promising, they were not reproducible by independent research groups (Table 1) [69,70].

**DNA methylation-based methods**

A different approach adopted by a large number of groups has been the investigation of epigenetic differences between fetal DNA and maternal DNA. Methylation is one of the most common epigenetic mechanisms under investigation, especially in cancer research [71,72]. The identification of tumor-specific DNA methylation patterns in the plasma of cancer patients [73-75] has raised the possibility of identifying fetal-specific methylation patterns in the maternal circulation. The aim of this approach is the identification and selection of fetal-specific markers that have the potential to be developed into NIPD diagnostic markers. One of the most widely used methods for investigating DNA methylation patterns is treatment with sodium bisulfite (NaHSO₃), and this has been used by the majority of methylation-based studies for NIPD. This technique relies on the conversion of non-methylated cytosine to uracil, which allows the discrimination of methylated from non-methylated DNA sequences. The study of both methylated and non-methylated sequences is achieved by the design of primers that target either the methylated or non-methylated regions followed by methylation-specific PCR [76].

In 2002, Poon and colleagues demonstrated for the first time the presence of DNA methylation differences between maternal and fetal DNA [18]. Three years later, the same team reported the first gene to be shown to have differential methylation patterns between maternal and fetal DNA: SERPINB5 (encoding serine protease inhibitor B5), located in chromosomal region 18q [21]. To achieve this, sodium bisulfite conversion of DNA was applied in combination with methylation-specific PCR using placental (fetal) DNA samples and DNA samples from non-pregnant women. The promoter region of SERPINB5 was shown to be hypomethylated in fetal DNA and hypermethylated in non-pregnant female DNA. Furthermore, the same group detected these hypomethylated DNA sequences in cell-free DNA extracted from maternal plasma during pregnancy, indicating the presence of cfDNA in the maternal circulation.

Alternative approaches have used sodium bisulfite-converted DNA in combination with digital PCR and/or MS analysis for the successful quantification of cfDNA in maternal plasma [77]. Furthermore, studies have shown that the combination of sodium bisulfite DNA treatment with the identification of SNPs within differentially methylated regions (DMRs) can facilitate cfDNA quantification [22,78-80]. Specifically, this technique requires the presence of at least one SNP that is homozygous in the mother and heterozygous in the fetus, and therefore is only possible in cases with such informative SNPs. However, the successful and full conversion of non-methylated cytosine to uracil is rarely achieved. Moreover, DNA degradation occurs after treatment with sodium bisulfite [81] and further complicates the quantification of extremely low amounts of cfDNA (Table 1).

An additional methylation-based approach is the use of methylation-sensitive restriction enzymes, which can either be methylation-specific (recognize and cut specific methylated sites) or non-methylation-specific (recognize and cut specific unmethylated sites). The use of non-methylation-specific restriction enzymes [82] can result in enrichment of methylated genomic regions and the decomposition of non-methylated DNA. Therefore, when using such enzymes to treat maternal plasma, the sequences that are hypermethylated in fetal DNA are enriched. A significant number of studies have been based on the use of such restriction enzymes [22,77,79]. However, this approach is limited by the number of regions that are suitable for testing, since the possibility of having a methylation-sensitive restriction site within the feto-maternal DMRs of interest is rare (Table 1) [18,22,79,83-85]. In these conventional methylation-based approaches, a large number of feto-maternal methylation differences have been detected, primarily on chromosome 21 [22,23,77]. Although these methods are cost-effective and do not require prior knowledge or expensive infrastructure, they can only be applied to CpG islands and promoter regions, which cover only a small fraction of the genome.

**Protein detection methods**

In addition to approaches aimed at detecting fetal-specific nucleic acids in the maternal circulation, efforts have been made to detect fetal-specific proteins in maternal plasma. The purpose of this approach was the discrimination between normal and abnormal pregnancies based on protein quantification. However, the presence of high levels of maternal proteins in maternal plasma makes the quantification of fetal-specific proteins challenging. Therefore, studies have now shifted to
implement methods to reduce the ratio of maternal proteins relative to fetal proteins [86]. Following indirect enrichment of fetal proteins, techniques such as two-dimensional gel electrophoresis enable the separation and detection of rare proteins of fetal origin in maternal plasma [25,87]. However, there is at present no convincing evidence of a commonly identified fetal-specific protein in maternal plasma, which is probably due to the high complexity of the plasma proteome, which derives from every tissue of the body.

A new era of non-invasive prenatal diagnosis for aneuploidies
To address the limitations of previous applications, a number of revolutionary and novel approaches have been developed (Table 1).

| Technology | Sensitivity/specificity (%) | Cost | Complexity | Reproduced by other groups* | Advantages | Technical and clinical challenges |
|------------|-----------------------------|------|------------|-----------------------------|------------|----------------------------------|
| Formaldehyde treatment [67,68] | 98.2% sensitivity 66.6% specificity | Low | Simple | No | Indirect enrichment of fetal DNA | Requires a large number of informative SNPs |
| DNA methylation studies using sodium bisulfite [21,78-80] | Small independent studies | Low | Simple | Not tested | Direct enrichment of fetal DNA | DNA degradation, full conversion is rarely achieved |
| DNA methylation studies using restriction enzymes [22,77,79] | Small independent studies | Low | Simple | Not tested | Indirect enrichment of fetal DNA | Limited to the investigation of regions with restriction sites |
| Protein-based studies [25,87] | Small independent studies | Low | Simple | Not tested | Direct discrimination of fetal proteins | Requires accurate quantification to distinguish normal from abnormal pregnancies |
| Next-generation sequencing [19,20,89,91] | 99.2 to 100% sensitivity 97.9 to 99.7% specificity | High | Complex | Yes | Reliable | Time consuming (more than one week to obtain the result), laborious, requires technical expertise, requires expensive equipment and infrastructure |
| MeDIP real time qPCR-based approach [98,99] | 100% sensitivity 100% specificity | Low | Simple | Not tested | Results obtained within 3 to 4 days | Requires 100% antibody performance, requires extensive quality control of reagents prior to use |
| Identification of fetal-specific mRNAs [104,106,108,109] | 100% sensitivity 89.7% specificity | Low | Simple | Not tested | Direct discrimination of fetal RNA from maternal RNA | Requires a large number of informative SNPs, limited by mRNA stability |
| Digital PCR-based approach [110,111] | Proof-of-principle | High | Complex | Not tested | Accurate quantification of DNA molecules | Requires technical expertise, simplification is achieved by the use of microfluidic devices, which are expensive and not widely available |
| Epigenetic-genetic chromosome-dosage approach [78,79] | 96.9% sensitivity 92.8% specificity | High | Complex | Not tested | Use of digital PCR | Requires a large number of informative SNPs, simplification is achieved by the use of microfluidic devices, which are expensive and not widely available |

*No* refers to the presence of published literature indicating failure to reproduce the results by independent groups; *Not tested* refers to the absence of published literature indicating reproduction of the results by independent groups. MeDIP, methylated DNA immunoprecipitation; PCR, polymerase chain reaction; qPCR, quantitative polymerase chain reaction; SNP, single nucleotide polymorphism.

Next-generation sequencing approaches
The implementation of next-generation sequencing (NGS) technologies in the development of NIPD of aneuploidies has revolutionized the field. In 2008, two independent groups demonstrated that NIPD of trisomy 21 could be achieved using next-generation massively parallel shotgun sequencing (MPSS) of maternal plasma [19,20]. Follow-up studies have demonstrated that the sensitivity of the approach is restricted by the number of sequencing reads [88].

Large-scale validation studies were then performed by research groups and biotechnology companies. One of the first studies performed included 753 pregnancies, of which 86 included fetuses with trisomy 21 [89]. However, 7.1% of the samples were excluded from the analysis.
(Table 2). Nevertheless, the samples tested demonstrated 100% sensitivity and 97.9% specificity of the approach. An independent study performed by a biotechnology company prospectively tested 480 pregnancies, of which 39 were in women carrying a fetus with trisomy 21 [90]. In this study the sensitivity and specificity were calculated to be 100% and 99.7%, respectively. However, the authors’ exclusion criteria meant that 6.4% of the samples were excluded from testing and/or analysis (Table 2). The same biotechnology company has recently performed a larger scale international clinical study to validate their laboratory-developed test (LDT). The study included 4,664 pregnancies of which only 1,696 were analyzed. The sample exclusion was based on a number of criteria (Table 2). Among the selected samples, 212 were obtained from trisomy 21 pregnancies. The sensitivity and specificity of the test were calculated to be 99.2% and 98.6%, respectively [91]. In addition, several pilot studies have shown the ability of MPSS to detect trisomy 18 and trisomy 13 non-invasively, although with more variable results [92-94].

An alternative NGS approach was adopted by a different biotechnology company. The study was performed with 298 pregnancies collected from first, second and third trimester pregnancies, and included 39 trisomy 21 cases and seven trisomy 18 cases [95]. The samples were analyzed using a novel, highly multiplexed assay termed digital analysis of selected regions (DANSR).

Cell-free DNA from maternal blood samples was analyzed using DANSR assays for loci on chromosomes 21 and 18. This study showed correct classification of all abnormal cases tested (Table 2). Furthermore, an independent research group has described a new approach based on single molecule DNA sequencing of cell-free DNA in maternal plasma [96]. In this study, 20 maternal plasma samples were tested, of which 11 were obtained from trisomy 21 pregnancies. The samples were obtained at 9 to 16 weeks of gestation. The results indicated that all cases were correctly classified. Furthermore, trisomy 21 fetuses could be more clearly distinguished from euploid fetuses compared with the other sequencing approaches (Table 2). In addition, the proof of concept for NIPD of fetal microdeletion syndromes via sequencing has recently been described [97].

**Methylated DNA immunoprecipitation real-time qPCR-based approach**

An alternative approach for the development of NIPD of aneuploidies has recently been described by our group. The approach is based on the application of methylated DNA immunoprecipitation (MeDIP), which takes advantage of the methylation differences that are present in maternal and fetal DNA. MeDIP application allows enrichment of fetal-specific hypermethylated regions by using an antibody that targets the 5-methylcytosine residues of CG dinucleotides [71,84]. In 2009, our team applied the MeDIP approach in combination with high-resolution microarrays and identified a large number of feto-maternal DMRs on chromosomes that are associated with the development of the most common fetal aneuploidies (chromosomes 13, 18, 21, X and Y) [98].

NIPD of trisomy 21 was then achieved by testing a small number of selected DMRs using the MeDIP approach in combination with real time qPCR. The study was performed with 80 pregnancies, of which 34 were obtained from women carrying a trisomy 21 fetus. The pregnancies tested were in the range of 10 to 13 weeks of gestation. Correct diagnosis was obtained for all normal and abnormal cases, demonstrating 100% sensitivity and specificity of the approach (Tables 1 and 2) [99]. Subsequently, a large-scale clinical trial including about 700 cases is currently in progress.

**mRNA detection methods**

An alternative approach with the potential to be developed into an NIPD test for Down syndrome was based on the identification of fetal-specific mRNA in the maternal circulation during pregnancy. The primary aim was the identification of mRNAs that are actively transcribed only in the fetus and are released into the maternal circulation. The presence of fetal-specific mRNA in the maternal circulation was first described in 2000 [100]. Subsequent studies have investigated the stability of mRNA in maternal plasma [101-103]. In addition, one of the most important discoveries in this area is that the majority of mRNA molecules in the maternal circulation derive from the placenta [24]. Lo and colleagues successfully identified mRNA molecules that could be used for NIPD [104,105]. A large panel of fetal-specific mRNAs, actively transcribed only in the fetus but not in the mother, was identified. Subsequent studies showed that fetal-specific mRNA of genes located on chromosome 21 can be detected in maternal plasma [106,107]. One such gene is *PLAC4* (encoding placenta-specific 4 protein, and located on chromosome 21), which is expressed in the placenta and is specific for the fetus in maternal plasma [106]. *PLAC4* mRNA was used to develop NIPD for trisomy 21 using a so-called RNA-SNP strategy using MS. In this approach, informative SNPs (heterozygous in the fetus and homozygous in the mother) located in *PLAC4* mRNA needed to be identified and used for NIPD of trisomy 21 [106]. This study was performed in 10 trisomy 21 cases and the diagnostic sensitivity and specificity of the approach were calculated to be 96.5% and 90%, respectively. An improvement of the diagnostic efficiency was observed in a follow-up study performed by the same group. The study included 153 pregnancies in the first trimester including normal
and trisomy 21 cases. The results demonstrated 100% sensitivity and 89.7% specificity [108]. This approach has also been applied in pregnancies with trisomy 18 (Table 1) [109].

**Digital PCR-based approach**

Digital PCR has been used for fetal DNA quantification by direct analysis of DNA extracted from maternal plasma. The proof-of-principle of the approach has been described by two independent groups that used placental DNA to estimate the percentages of fetal DNA present in the maternal circulation [110,111]. Digital PCR differs from other quantitative approaches such as real-time PCR as it allows the reaction to proceed to its plateau instead of the exponential phase of the PCR reaction. The interpretation of the digital PCR result is a ‘yes/no’, which corresponds to the presence or absence of input template [112]. Two different approaches have been developed, the digital RNA-SNP strategy and the digital relative chromosome dosage (RCD) approach. The former strategy involves the determination of the allele imbalance of a SNP in fetal-specific PLAC4 mRNA, whereas in digital RCD the total copy number of chromosome 21 of a sample is compared with the total copy number of a reference chromosome (overrepresentation of chromosome 21 would indicate trisomy 21) [110].

**Epigenetic-genetic chromosome-dosage approach**

An alternative novel approach towards NIPD of chromosomal aneuploidies involves the combination of epigenetic and genetic markers of fetal origin present in maternal plasma. The group who investigated this approach identified a number of DMRs between fetal and maternal DNA using sodium bisulfite conversion. They then selected the fetal-specific hypermethylated marker HLCS (the gene encoding holocarboxylase synthetase) located on chromosome 21, and a fetal-specific marker located on chromosome Y (encoding zinc finger protein, Y-linked, ZFY). The RCD approach was followed using digital PCR by comparing the dosage of the epigenetic marker HLCS with the copy numbers of ZFY. The results obtained from 24 normal cases and five trisomy 21 cases have shown that all but one normal case were correctly classified [79]. Improvements to the above approach presented by the same group included the development of a new assay using an autosomal marker on chromosome 14 instead of
*ZFY* as the genetic marker for comparison [78]. The method was validated in 33 normal cases and 14 trisomy 21 cases. One of the normal cases and one of the trisomy 21 cases were misclassified. The samples were collected at 12 to 18 weeks of gestation. Although the improved method was not restricted to male fetuses, it required the presence of informative SNPs within the genetic marker. Furthermore, this approach required the use of microfluidic digital PCR, which is expensive and not easily available to diagnostic laboratories (Table 1). This method also requires to be validated by independent studies.

**Challenges for clinical implementation**

The new era of NIPD for aneuploidies has opened new possibilities for the implementation of these technologies into clinical practice in the near future. Biotechnology companies that are partly or wholly dedicated to the development of NIPD tests have initiated large-scale clinical studies towards their implementation. In October 2011 one of the companies dedicated to the development of prenatal diagnostic tests announced the launch of its first NIPD test for trisomy 21, which is available in 20 major metropolitan regions across the United States (SEQUENOM Inc., San Diego, CA, USA) [113]. Their test (MaterniT21 test) is a LDT that analyzes circulating cell-free DNA extracted from a maternal blood sample using next-generation MPSS analysis. The test detects an increased representation of chromosome 21 material, which is associated with trisomy 21 [91].

A second biotechnology company (Aria Diagnostics Inc., San Jose, CA, USA) [114] has also developed an NGS-based approach [95]. According to the company, the approach requires one-tenth of the sequencing depth of the MPSS approach, as employed in the MaterniT21 test. In addition, 750 samples can be analyzed in one sequencing run, compared with the four to eight that have been tested with shotgun sequencing methods.

Another company (Verinata Health Inc., Redwood City, CA, USA) [115] has recently announced the launch of their NIPD test (Verifi prenatal test). The company has conducted a large validation study of 532 pregnancies including 89 trisomy 21 cases, 36 trisomy 18 cases, 14 trisomy 13 cases and 16 monosomy X cases using massively parallel sequencing. The results demonstrated 100% specificity and sensitivities of 100%, 97.2%, 78.6% and 93.8%, respectively. The pregnancies tested were in the range of 8 to 22 weeks of gestation [116]. This study is the first to demonstrate the diagnostic efficiency of NGS in other aneuploidies in addition to trisomy 21.

NGS approaches have the potential to be implemented in clinical practice not only for NIPD of trisomy 21 cases and other aneuploidies, but also for other chromosomal abnormalities, since they can be used for whole genome investigation in a single experiment. On the other hand, NGS approaches are time-consuming, laborious and technically challenging. Furthermore, they require a highly technological infrastructure, which is expensive and therefore not easily implemented in diagnostic laboratories (Table 1).

Another approach based on MeDIP real-time qPCR [99] is currently being developed as a commercially available test by another biotechnology company (NIPD Genetics Ltd, Nicosia, Cyprus) [117]. This is an antibody-based approach and relies on the sensitivity and specificity of the antibody to capture only methylated sites and not unmethylated sites. Therefore, it requires thorough quality control prior to use. Furthermore, this method remains to be validated by independent studies. Nevertheless, the approach is relatively fast compared with NGS approaches (Table 1).

An additional novel approach is the application of digital PCR for NIPD of aneuploidies. The proof-of-principle of this assay was described by two independent research groups, as mentioned earlier [110,111]. However, only one group has used this assay on maternal plasma samples for NIPD of trisomy 21 through the epigenetic-genetic chromosome-dosage approach [78,79]. The approach is being developed with a biotechnology company (SEQUENOM Inc.) [113]. The pilot study performed demonstrated that this approach has the potential to be implemented for NIPD of trisomy 21. Nevertheless, the results from a larger validation study still remain to be seen. Digital PCR technology is very promising since it allows accurate quantification of copy numbers in contrast to other quantification methods such as real-time PCR. However, it requires the development of microfluidic devices with several thousand reaction chambers in order to make it less laborious and time-consuming compared with manually performed conventional PCR. These devices are expensive and not available to the vast majority of diagnostic laboratories, and therefore this approach will not be easily implemented in clinical practice (Table 1).

The identification of fetal-specific mRNA molecules in maternal plasma and the development of non-invasive diagnostic assays has also been proposed. As discussed earlier, a team used the fetal-specific *PLAC4* mRNA transcript for NIPD of trisomy 21 using MS [106,108]. Although this approach demonstrates high diagnostic efficiency, it can only be applied to pregnancies with informative SNPs within *PLAC4* mRNA - this is rarely achieved since it requires a large panel of SNPs to be tested. Furthermore, this approach is laborious and limited by the stability of mRNA molecules, and requires specialized infrastructure that is not available to the majority of diagnostic laboratories. This method remains to be validated by independent studies (Table 1).
The final selection of the test or tests that will likely be used for routine clinical diagnosis will rely on identifying the method that will provide the most convincing evidence of validity. Large-scale validation studies will be needed to provide a more accurate estimation of the diagnostic efficiency of each of the above methods. Furthermore, the selected test would need to be suitable for easy implementation in diagnostic laboratories worldwide. The test should require as little expensive equipment and reagents as possible, and should be as simple and as fast as possible since in the clinical diagnostic setting the speed of the test is one of the most critical factors, especially in prenatal diagnosis. Moreover, the selected test or tests would need to fulfill national or international criteria. Evaluation of the tests could, for example, be done using the quality assessment tool of diagnostic accuracy studies (QUADAS) guidelines [118], which consists of a 14-item checklist encompassing the most common sources of bias and variation observed in diagnostic accuracy.

Conclusions and future directions

NIPD is of substantial medical importance as it targets the development of safer and more effective methods to avoid the risk of fetal loss associated with currently used invasive methods. After 30 years of research, NIPD of aneuploidies is becoming a reality. Novel methods such as NGS approaches, the MeDIP real-time qPCR-based approach, mRNA detection methods and digital PCR-based methods have facilitated progress towards the implementation of NIPD of trisomy 21 in clinical practice. Nevertheless, current approaches have limitations and restrictions that hinder early and easy implementation. Such limitations are often associated with the cost of the infrastructure required for the assay to be applied, such as in the application of NGS approaches and digital PCR. NGS approaches and mRNA detection methods are also laborious and require prior knowledge. Furthermore, the successful implementation of the MeDIP real-time qPCR-based approach depends on the quality and efficiency of the antibody used and requires thorough quality control before use.

So far, the most validated approach that has been tested in a large number of samples by independent groups is the NGS approach. This approach is expected to be the first to be introduced into clinical practice, since several biotechnology companies have already started providing services [113-115]. However, due to the limitations discussed earlier, such as the high cost and the time required, the method will require further improvements before it can be implemented in diagnostic laboratories.

The technologies developed so far refer mainly to NIPD of trisomy 21. Nevertheless, the development of NIPD for other common aneuploidies such as those associated with chromosomes 13 and 18 is very close. NGS-based approaches have already been investigated in pilot studies to address the issue [92,94]. In addition, the MeDIP real-time qPCR-based approach has already taken into account the need for the development of NIPD of aneuploidies associated with chromosomes 13, 18, X and Y through the identification of DMRs located on these chromosomes [98]. Developers predict that NIPD will be applied to monogenic diseases and complete fetal genome mapping within the next 10 years [119]. Furthermore, we speculate that NIPD will replace the existing invasive methods within the next 5 years, eliminating the risk of fetal loss.

Abbreviations

cfDNA, cell-free fetal DNA; CVS, chorionic villi sampling; DANSR, digital analysis of selected regions; DMR, differentially methylated region; FACS, fluorescence-activated cell sorting; fFDA, free fetal DNA; FISH, fluorescence in situ hybridization; LDT, laboratory-developed test; MACS, magnetic-activated cell sorting; MeDIP, methylated DNA immunoprecipitation; MPSS, massively parallel shotgun sequencing; MS, mass spectroscopy; NGS, next-generation sequencing; NIPD, non-invasive prenatal diagnosis; NRBC, nucleated red blood cell; PCR, polymerase chain reaction; qPCR, quantitative polymerase chain reaction; RCD, relative chromosome dosage; SNP, single nucleotide polymorphism.

Competing interests

The authors have filed a PCT patent application for the NIPD MeDIP real-time qPCR based approach (PCT Patent Application No. PCT/IB2011/0000217). The MeDIP real-time qPCR study performed by our team was funded by the SAFE Network of Excellence European Commission Funded 6th Framework Package Project Number: LSHB-CT-2004-503243, the Cyprus Institute of Neurology and Genetics and NIPD Genetics Ltd.

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