Circulating nucleic acids in plasma and serum: applications in diagnostic techniques for noninvasive prenatal diagnosis

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Abstract: The analysis of fetal nucleic acids in maternal blood 13 years ago has led to the initiation of noninvasive methods for the early determination of fetal gender, rhesus D status, and a number of aneuploid disorders and hemoglobinopathies. Subsequently, a comparatively large quantity of fetal DNA and RNA has been demonstrated in amniotic fluid as well as small amounts in premature infant saliva. The DNA and RNA in amniotic fluid has permitted an analysis of core transcriptomes, whilst the DNA and RNA in saliva allows the early detection and treatment monitoring of fetal developmental problems. These aspects are discussed together with the methodology and limits of analysis for noninvasive prenatal diagnosis in predictive, preventive, and personalized medicine.

Keywords: fetal circulating DNA/RNA, amniotic fluid, saliva, aneuploidy, thalassemias

Introduction
The first demonstration of DNA in human blood more than 60 years ago was somewhat forgotten in the search for the structure of DNA and its identification as the gene. Thus, the presence of nucleic acids in the circulatory system was ignored until high DNA levels were demonstrated in the blood of patients with systemic lupus erythematosus as well as in cancer. Subsequent isolation of DNA from the blood of cancer patients showed it to be tumor cell derived. From such a platform were launched a series of studies analyzing nucleic acids isolated from plasma and serum of patients suffering from a wide spectrum of disorders.

Early studies on circulating nucleic acids demonstrated both DNA and RNA fragments. A review on the general biology of circulating nucleic acids in plasma and serum (CNAPS) has offered a wide range of possible sources for the DNA/RNA. However fetal nucleic acids in maternal blood are derived primarily from both the fetus and the placenta and, most likely, directly from the fetus in the case of amniotic fluid. Fetal DNA sequences tend to be short, implying that they are mainly derived by apoptosis rather than by necrosis. In addition, DNA and RNA sequences have been released from cells either by exocytosis as exosomes or as newly synthesized virions, complexes acting as intercellular messengers.

It is clear that CNAPS can be exploited as an approach for use in predictive, preventive, and personalized medicine. Consequently, studies on CNAPS have tended to fall into three categories; namely (1) fetal nucleic acid analyses and (2) clinical conditions, eg, oncology and diabetes, both areas being underpinned by (3) the study of the basic biology of CNAPS.
Prenatal screening and diagnosis has been established as a routine way of checking for fetal gender, fetal rhesus D (RhD), and fetal chromosomal abnormalities including aneuploidies, hemoglobinopathies, Tay-Sachs disease, sickle cell anemia, cystic fibrosis, and fragile X syndrome. In addition, birth defects and conditions such as spina bifida, cleft palate, and muscular dystrophy have also been screened. The main testing procedures include amniocentesis and chorionic villus sampling (CVS), together with ultrasonography, ultrasound, serum markers, and genetic screening. Each of these methods has a limited sensitivity, each test having a specific window of time for success. Thus, CVS cannot be performed before 9 weeks of gestation, while amniocentesis is normally offered between 15 and 20 weeks. Both methods carry a risk of fetal loss of <1.0%. Equally, ultrasonography cannot detect fetal gender during the first trimester. Thus, a minimally invasive assessment permitting the clarification of fetal gender, RhD, and abnormalities at an early stage of pregnancy was a desirable development.

The concept of the release of nucleic acids into the maternal circulating blood led Dennis Lo’s group to examine the possibility of the presence of fetal DNA/RNA in the maternal blood stream and to its separation and identification from maternal DNA. This has laid the basis for the exploitation of maternal peripheral blood as a source of fetal DNA and RNA for use as a noninvasive prenatal testing (NIPT) method for determining fetal gender, blood genotyping, aneuploid disorders, β-thalassemia, complete fetal genome sequencing, and the likelihood of pre-eclampsia onset. These aspects and the methodologies involved form the basis of the following review, together with a consideration of the ethics of the use of NIPT for early diagnosis of fetal disorders.

**Fetal DNA/RNA in maternal CNAPS, amniotic fluid, and premature infant saliva DNA in CNAPS**

Although fetal nucleated red blood (NRBC) cells have been demonstrated to be present in the maternal circulation, they have not yet proved a success for NIPT. However, using an erythroblast scoring system based on the morphological and hemoglobin-staining characteristics of these cells, together with an analysis of inherited DNA polymorphisms, has allowed a distinction to be made between NRBCs of maternal and fetal origin, independently of fetal gender.

Fetal cells in the maternal circulation also have been examined as possible sources of genetic information for noninvasive prenatal diagnosis. However, their small numbers present in maternal blood have limited their use in the routine genetic determination of fetal abnormalities. A subsequent multicenter clinical project aimed at noninvasive methods of prenatal diagnosis was developed (National Institute of Child Health and Human Development Fetal Cell Isolation Study – NIFTY). The initial objective was to assess fluorescence in situ hybridization (FISH) analysis of interphase nuclei of fetal cells from maternal blood as compared with metaphase karyotypes of fetal cells obtained by either amniocentesis or CVS. Although cell isolation and FISH analysis protocol differences between centers occurred, the blinded and pooled data led to a fetal gender detection rate of 41.4%. The detection rate for finding a minimum of one aneuploid cell for fetal aneuploidy was 74.4%. False positives for gender were 11.1% while that for aneuploidy were 0.6%–4.1%. This study was based on detection of a single fetal cell in nearly all cases, which together with the lack of a reliably determined false-positive rate for detecting aneuploidy, made strict improvements a necessity if this method should have a future use.

The alternative approach, of using fetal DNA/RNA present in the maternal blood, by Lo et al rested upon the identification of Y chromosome fragments, ie, the paternal male determining chromosome that would not be present in the mother’s X chromosome DNA. This approach was confirmed by Smid et al and Houfflin-Debarge et al. Employing a more sensitive real-time polymerase chain reaction (PCR) technique to amplify and measure the amounts of DNA, Lo et al were able to increase the sensitivity close to 100% for the identification of fetal-derived Y-chromosomal sequences from maternal plasma and serum. They also showed that the fetal DNA was present in high concentrations in maternal plasma, reaching a mean of 25.4 genome equivalents mL⁻¹ (range 3.3–69.4) in early pregnancy and 292.2 genome equivalents mL⁻¹ (range 76.9–769.0) in late pregnancy, ie, 3.40% (range 0.39%–11.90%) and 6.20% (range 2.33%–11.40%) of the total plasma DNA in early and late pregnancy, respectively. Thus, the absolute concentration of fetal DNA in maternal serum increased with gestational age, disappearing rapidly following birth, with a mean half-life of 16.3 minutes. Degraded fetal DNA has been detected in maternal urine, using massively parallel paired-end sequencing, becoming lost after birth.

Fetal DNA can be identified since it tends to be <300 base pairs (bp) in size and can be distinguished from maternal DNA, which is essentially >300 bp in size. The median percentages of plasma DNA with size > 201 bp were 57% in pregnant women and 14% in nonpregnant women, with median percentages of fetal DNA in maternal plasma of
sizes > 193 bp and >313 bp being 20% and 0%, respectively. The NIPT methods using CNAPS results in initial amounts of fetal DNA from 2 to 5 mL of maternal blood being very small, hence it is necessary to increase the amount of DNA – and RNA – if a successful analysis of fetal DNA/RNA is to be made in relation to diagnosis, prognosis, and treatment monitoring. This is usually effected by the various forms of PCR whereby thousands to millions of copies of a specific DNA sequence are generated from either a single or a few copies of a piece of DNA/RNA. One of the most effective approaches is that of quantitative real-time PCR (qRT-PCR) whereby the targeted DNA sequence is simultaneously amplified and quantified. RNA can also be amplified in this way using reverse-transcription PCR (RT-PCR).

Since the first studies of fetal DNA in CNAPS, technology has moved on apace with the development of second-generation sequencing tools or massively parallel sequencing. This encompasses several high-throughput approaches to DNA sequencing that became commercially available from about 2005. There are a number of such systems available using miniaturized and parallelized platforms for sequencing of 1–100 million short reads (50–400 bases). PCR is performed externally to the machines as part of the library preparation. However, within the machine, PCR of the DNA/RNA increases the amount of nucleic acid available for the sequencing step. Thus, both PCR and sequencing processes are performed in the same instrument, reducing the chances of contamination as well as increasing the speed and accuracy of analysis. This has permitted a larger-scale production of genomic sequences, including an increasing number of human genome sequences.28–31

Thus, the way has been opened for more rapid and detailed analyses of CNAPS in terms of diagnosis,32 eg, through the more ready identification of single nucleotide polymorphisms (SNPs).

Lo et al33 used paired-end massively parallel sequencing to study the genomic sequence and size distribution of fetal DNA in maternal plasma. They constructed a genome-wide genetic fetal map and a mutational profile of the fetus from maternal plasma DNA sequences, together with data concerning the paternal genotype and maternal haplotype. They suggested further that this minimally invasive process could be exploited for a prenatal, genome-wide scanning to determine fetal genetic disorders. The employment of an alternative approach of direct shotgun high-throughput put sequencing34,35 has permitted the full fetal genome to be derived by mapping and enumerating the fragments relative to the chromosome of origin. Counting the number of sequence tags mapped to each chromosome allows the detection of over- or underrepresentation of any chromosome in maternal plasma DNA contributed by an aneuploid fetus.34,35 A major advantage of this method is that it does not need the differentiation between fetal versus maternal DNA. Using large tag counts, the method can be applied to arbitrarily small fractions of fetal DNA. This approach for sequencing the full fetal genome was based upon the concept that within each pair of parental haplotypes, the transmitted haplotype is overrepresented relative to the nontransmitted one. Hence, measurement of the relative amount of parental haplotypes by counts of the number of alleles specific to each parental haplotype permits the inheritance of each parental haplotype to be deduced and, therefore, the building of the complete fetal genome. However, before using shotgun sequencing, the counting principle was applied directly to each allele in the fetal exome by performing exome capture on maternal plasma DNA. Thus, exome screening, of clinically relevant and deleterious alleles that were either paternally inherited or had arisen as de novo germ-line mutations, was determined. Such an analysis, combined with that of shotgun sequencing, offers a relatively comprehensive study of the fetal genome and so is a more reliable approach to NIPT.

DNA in amniotic fluid
Most studies of DNA have been made on plasma and serum. However, with the availability of amniotic fluid after its clinical diagnostic use, Hui and Bianchi were able to demonstrate that its cell free DNA content was present at concentrations higher (100–200-fold mL−1) than that observed in plasma. It is comprised essentially of fetal DNA and represents a separate pool of cell free DNA to that of the maternal plasma.36 The first application of amniotic fluid DNA involved comparative genomic hybridization (CGH) microarray analysis using GenoSensor Array 300, which permitted the identification of fetal sex and whole chromosome gains or losses, eg, trisomy 21 and monosomy X.37 Using CGH array analysis of amniotic fluid DNA from 13 fetuses with congenital abnormalities, Miura et al38 were able to correctly identify 12 fetuses with chromosomal losses or gains. A false-negative result occurred with a fetus having a balanced translocation, 45, XY, der(14;21)(q10;q10).

RNA
Male-specific mRNA was first described by Poon et al39 in maternal plasma from pregnant women carrying male fetuses by using a two-step RT-PCR assay. Y-chromosome-specific zinc finger protein (ZFy) mRNA and human leucocyte antigen
(HLA-G) mRNA were specifically identified, the total amounts of messenger RNA (mRNA) increasing with increasing time of pregnancy.

RNA present in plasma and serum is very stable,

although Reddi and Holland have demonstrated the presence of RNase in serum at an average amount of 104 units mL⁻¹. The RNAs tend to be found in a particulate form, with the RNA appearing to be protected by a membrane, eg, released from healthy cells in exosomes or together with lipoprotein complexes as viritosomes. However, RNA can be derived also by either apoptosis or necrosis.

The majority of studies have examined mRNAs from uniquely expressed placental genes, eg, human placental lactogen, β subunit of human placental chorionic gonadotropin, corticotrophin releasing hormone, and glia cell missing-1 mRNA. Such RNAs originate primarily from the syncytiotrophoblast rather than, as expected, from the maternal circulation.

RNA in amniotic fluid

Although most studies of RNA have been made on plasma and serum, as with DNA, cell free RNA has been shown to be present in amniotic fluid. The RNA data are of interest in that the cell free RNAs are derived almost exclusively from the embryo.

An in-silico study by Hui et al has identified 476 well annotated genes in the amniotic fluid from euploid mid-trimester amniotic fluid samples. Six physiological systems were represented in the amniotic fluid core transcriptome. These included the development and function of the musculoskeletal and nervous system plus embryonic and organismal development. A key canonical pathway identified was that of the mammalian target of rapamycin signaling. Of the 23 highly organ-specific transcripts identified, six were known to be highly expressed in the fetal brain. Although the obtention of amniotic fluid is an invasive procedure, this approach does permit the identification of fetal defects for which clinical management could commence in utero.

RNA in saliva

Many infants are born prematurely worldwide each year, resulting in very high annual health care costs, often due to severe medical sequelae. To develop NIPT biomarkers to detect disorders early so as to initiate treatment, Maron et al developed a method for the isolation and identification of RNAs from the saliva of premature infants. Using saliva RNA amplification and hybridization onto whole genomic microarrays together with bioinformatic analyses, they were able to demonstrate 9286 gene transcripts showing statistically significant gene expression changes across individuals over time, with 37.9% genes being downregulated and 62.1% genes upregulated. The gene expression changes were closely linked to developmental pathways. As might be expected, the downregulated expression was related to embryonic development, connective tissue, and hematological system development and function. However, significantly upregulated genes included those linked to behavior and the development of the nervous system, tissue, organ, and digestive system. This NIPT approach offers the screening of premature infants with a small volume of saliva (50–200 μL) that can be taken at successive times from any one infant, so allowing possible treatment to be instigated and monitored.

Fetal gender

Fetal gender determination has been performed on fetal DNA in maternal blood using either paternal derived fragments of the Y chromosome or paternal X-chromosome derived fragments of the amelogenin gene and multicopy DAZ sequence. The main method for gender determination is based upon the presence or absence of the Y-chromosome sequences DYS14, DAZ, and SRY, ie, they will only be present in male-bearing pregnancies. Tang et al have identified paternally inherited X-chromosomal microsatellite polymorphisms in the plasma of pregnant women carrying female fetuses. Similarly, Chen et al have demonstrated monosomy and disomy paternal 10q by two informative markers, D10S534 and D10S186 in maternal blood. These show the possibility for detection of female fetuses. However, most routine hospital and private clinic tests are based upon the presence or absence of Y-chromosome markers.

Rhesus status

A rhesus (Rh)-negative mother carrying an Rh-positive fetus may develop antibodies against the Rh antigen if fetal blood enters her bloodstream. If she subsequently becomes pregnant with an Rh-positive fetus, antibodies may cross the placental barrier resulting in hemolytic disease of the newborn. In a Caucasian population, there is deletion within the RHD gene, whilst in <80% of the African population, the phenotype is caused by a nonfunctional copy of the RHD gene, RHDpsi, the RHD pseudogene.

Fetal DNA can also be used for fetal blood group genotyping with the Rh status of the fetus being determined. The earliest studies came from Lo et al using fluorescence PCR and an RHD sequence, and Bischoff et al also using a 99 bp DNA fragment specific for the RHD gene and a
113 bp fragment specific for the RHCE gene as control. However, the detection level was somewhat low. Detection levels were significantly improved when Finning et al\textsuperscript{57} used qRT-PCR with the R, HD exons 4, 5, and 10. Such a technique, with an accuracy of \textgreater 99\%, was used in a study of eight females to detect Rh status when incorporating SRY and eight biallelic polymorphisms as internal positive control to avoid the inadequacies of the PCR. There are strong indications for the successful identification of other blood types including Rhc, Rhe, K, and FY.\textsuperscript{57,58} Finning et al\textsuperscript{59} have introduced a word of caution in using this approach since, for example, only a 95.7\% accuracy was achieved in separating Rh-negative and Rh-positive fetuses in a group of 1997 pregnant women. These workers also considered that high throughput RHD genotyping of fetuses in all RHD-negative women could substantially reduce the unnecessary administration of anti-Rhd immunoglobulin to Rhd-negative pregnant women carrying an Rhd-negative fetus. More recently, using quantitative PCR on plasma DNA for the detection of Rh exons 5 and 7, Cardo et al\textsuperscript{60} reported 93\% specificity and 100\% sensitivity, with a 97\% diagnostic accuracy, from a cohort of 111 Rhd-negative, first-trimester, pregnant women. High levels of detection of Rhd-negative carriers were made by Sedrak et al\textsuperscript{61} using RT-PCR, with 93.5\% and 91.1\% sensitivity and diagnostic accuracy, respectively, in the first trimester and increasing to 100\% and 97.78\%, respectively, in the second trimester. Bombard et al\textsuperscript{62} studied two cohorts: (1) with a serotype reference (11–13 weeks gestation) and (2) with a reference source (6–30 weeks gestation) using matrix-assisted laser desorption/ionization/time-of-flight mass spectrometry. Transforming growth interacting factor (TGIF)-like X/Y; AJ427749; (referred to as TGIF) is a human-specific DNA homology block mapping to Yp11.2/Xq21.3 and represents the major shared region between the X and Y chromosomes.\textsuperscript{63} Only samples showing the presence of a well defined spectral peak for TGIF were utilized to determine the presence or absence of RHD exons 4, 5, and 7 as well as a 37-bp insertion of exon 4 indicating the psi-pseudogene. In addition, the study included three Y-chromosome sequences, SRY, DBY, and TTTY2. The presence of all three Rhd exonspecific markers allowed classification as Rhd positive. The sample was called Rhd negative when either one or none of the three exon sequences was detected, whilst the presence of only two sequences was inconclusive. The occurrence of the psi-insertion exon 4 insertion indicated the psi (+)/RHD variant. The serotype RhD reference cohort showed correct classification for 201/207 patients with a test accuracy and a sensitivity and specificity for prediction of RhD serotype of 97.1\%, 97.2\%, and 96.8\%, respectively. The genotype RHD reference cohort showed correct classification in 198/199 patients to yield a test accuracy, specificity, and sensitivity for prediction of RhD genotype of 99.5\%, 98.3\%, and 100.0\%, respectively.

**Aneuploidy**

After the first use of NIPT, attempts were made to address the identification of aneuploid situations, and in particular, trisomy 21 (Down’s syndrome). If this was successful, it was presumed that the methodology could also be applied to other trisomies, eg, 18 (Edwards syndrome) and 13 (Patau syndrome). Short tandem repeats (STRs) are useful in the determination of the male or female origin of the extra chromosome and, if maternal in origin, its derivation from either meiosis I or meiosis II.\textsuperscript{64} The analysis of the DNA isolated from both peripheral blood and amniotic fluid was compared with results from karyotyping. The STRs employed for diagnosis were D21S11, D21S1413 for trisomy 21, D18S51 for trisomy 18, and D13S631 and D13S258 for trisomy 13. There was a good correlation between the trisomies identified by karyotyping and STRs. Other chromosome 21 STRs used in such studies include S100/BR/BP, D21S167/F/R/P, and IGFIF/R/P.\textsuperscript{65,66} Additional approaches have been reviewed by Lo and Chiu\textsuperscript{67} and by Go et al.\textsuperscript{68} Currently, there are a few remaining as potential NIPT tests. One concerns the exploitation of differentially methylated DNA immuno-precipitation (MeDIP) in conjunction with qRT-PCR.\textsuperscript{69,70} For each chromosome analyzed, these workers identified \textgreater 2000 regions of differential methylation between maternal whole blood and placental DNA including a subset of differentially methylated regions. Moreover, 56\%–83\% of the regions located to nongenic regions, with only 1\%–11\% overlapping with CpG (cytosine and guanine phosphodiester bonds) islands. Using this approach for trisomy 21, correct diagnosis was achieved for 14/14 trisomy 21 cases and 26 controls (normal).

However, the more robust approach appears to lay with the use of high-throughput shotgun sequencing technology. Thus Fan et al,\textsuperscript{71} studying 18 normal and aneuploid pregnancies, were able to identify nine cases of trisomy 21, two of trisomy 18, and one of trisomy 13, with detection being made as early as the 14th week of gestation. This was achieved through sequencing an average of about 10 million 25-bp sequence tags per blood sample. About half of the reads mapped specifically to the human genome to cover about 4\% of the complete genome. An average of circa 154,000 sequence tags mapped to chromosome 13, circa 135,000 to chromosome.
18, and circa 65,700 to chromosome 21. Although few cases were studied, the approach looks very positive.

An alternative approach was adopted by Chiu et al\textsuperscript{71} in which assays were made for trisomy 21 by combining samples from more than one donor to improve accuracy and also to speed up the rate of analysis of clinical samples. Thus, a unique synthetic DNA “barcode” of 6 bp (an index) was attached to one end of each plasma DNA molecule per donor to act as a signature for each maternal plasma sample. Hence, eight different indices will be needed for each of the eight test or control samples being massively parallel co-sequenced (8-plex sequencing). The experiment compared the pooling of either eight or two maternal plasma DNA preparations (8-plex or 2-plex sequencing). Measurement of the percentage of plasma DNA derived from chromosome 21 was more precise using the 2-plex protocol rather than the 8-plex protocol, with seven times more plasma DNA molecules being identified in the former case. Some trisomy 21 fetuses remained unidentified by the 8-plex protocol but were detected using the 2-plex protocol. The number of plasma DNA molecules analyzed per sample seems to improve the sensitivity of the sequencing. Although trisomy 18 and trisomy 13 were considered by this method, the approach for them appeared to need modifying. The multiplexing approach was also used by Ehrich et al,\textsuperscript{72} with 39 trisomy 21 samples correctly classified and one sample misclassified as trisomy 21 from an original 449 samples assayed. Sensitivity of 100% and specificity of 99.7% were claimed.

More recently, a multicenter study, the MELISSA (MatERNal BLood IS Source to Accurately diagnose fetal aneuploidy) study, was made by Bianchi et al\textsuperscript{73} on 532 samples. They reported successful identification of 89/89 trisomy 21 cases, 35/36 trisomy 18 cases, and 11/14 trisomy 13 cases, with an additional two cases of trisomy 20 (defects in various organs and intrauterine growth retardation) and trisomy 16 (most common chromosomal cause of miscarriages). The overall study was much broader than those described above, including correct analyses for XXX, XXY, and XYY, as well as mosaicism for three cases of trisomy 21, 1 case of trisomy 18, 3 cases of translocation trisomy, and 2/7 cases of monosomy X. This study shows not only support for the abovementioned trisomy analyses but the possibilities of extending NIPT to a broader range of abnormalities. In a study on twin pregnancies,\textsuperscript{74} massively parallel shotgun sequencing was performed upon 25 high-risk pregnancies in a blinded test, where 17 were euploid, five discordant, and two concordant for Down’s syndrome. One was also discordant for trisomy 13, whilst there were two euploid triplet pregnancies. All pregnancies were correctly diagnosed with seven trisomy 21, one trisomy 13, and 15 normal twin and two normal triplet pregnancies, ie, there were no false-positives. This confirms the ability to use DNA sequencing of maternal plasma to identify Down’s syndrome and other trisomies in multiple gestations.

In two papers by Palomaki et al,\textsuperscript{75,76} trisomy 21, 18, and 13 were studied in a blinded, nested case-control study of a cohort of 4664 pregnancies at high risk for Down’s syndrome. A laboratory-developed test based upon next generation sequencing in which there was an adjustment of chromosome counts for guanine-cytosine base content was used. For trisomy 21 in 212 Down’s syndrome and 1484 matched euploid pregnancies, the detection level was 209/212 (98.6%), with 3/1471 (0.02%) false-positives. The 13 failed tests (0.8) were all euploid. In the case of trisomy 18 (62 pregnancies) and 13 (12 pregnancies), the detection levels were 59/59 and 11/12, respectively, with false-positive levels being 0.28% and 0.97%, respectively. In a study of trisomy 18 and 21, Norton et al\textsuperscript{77} used assays previously described\textsuperscript{78,79} that employ ligation of locus-specific oligonucleotides to yield sequencing templates from only specific genomic loci. This resulted in a reduction in the DNA sequencing required. All 81 trisomy 21 cases were considered high risk for trisomy 21, with one false-positive from 2888 normal cases, yielding a sensitivity of 100% and a 0.03% false-positive level. A total of 37/38 trisomy 18 cases were considered high risk, with two false-positives from 2888 normal pregnancies, giving a sensitivity of 97.4% and a 0.07% false-positive level.

**Hemoglobinopathies**

**β-thalassemia**

β-thalassemia is a common autosomal recessive single-gene disorder causing severe anemia through mutations in the β-globulin gene. One of these mutations, namely, the deletion of four nucleotides (-CTTT) at codons 41/42, was described\textsuperscript{80} in the case of two pregnancies. Noninvasively, the fetal genotype was completely concordant with conventional analysis and β-thalassemia major was excluded in the case of two pregnancies. Prenatal detection of β-thalassemia is also feasible with fetal DNA isolated from the maternal blood using an allele-specific based RT-PCR method.\textsuperscript{81,82} A successful analysis was made with eleven paternally inherited SNPs having a high degree of heterozygosity from the β-globulin gene for the diagnosis of β-thalassemia. Papassava et al\textsuperscript{83} have also successfully developed a NIPT method for β-thalassemia using Solexa high throughput sequencing technology, which
is based on the combined use of the microchip technology and a single nucleotide base extension method. Using four SNPs of the β-globin gene, the paternally inherited alleles of the fetus were detected in eight out of ten maternal plasma samples, confirming the CVS analysis.

α0-thalassemia
An alternative approach has been adopted by Yan et al\textsuperscript{84} for the less studied α0-thalassemia based upon an approach exploited by Ho et al.\textsuperscript{55,86} Nondeleted paternally inherited fetal alleles in cases of Hb Bart’s syndrome were identified using microsatellite markers within deletion (−SEA) breakpoints that involved detecting paternal SNPs with deletion breakpoints for NIPT exclusion of monozygotic α0-thalassemia. For α0-thalassemia, Yan et al\textsuperscript{84} considered 16 SNPs, three of which were novel. Using g. 31921 T>C as a marker, examination of 65 potential cases showed a correct identification of either the presence (n = 33) or absence (n = 30) of the normal paternal allele, with two cases unresolved due to blood transport incidents.

Other single-gene disorders
Fetal nucleic acid methods have been devised to tackle a number of other disorders involving single-gene mutations. However, often the method has been devised and tested on only a very small number of cases (Table 1).

Pre-eclampsia
Fetal nucleic acids may also be exploited in the early identification of pregnant women who risk developing pre-eclampsia with SRY (that triggers male embryonic development) and DYS (DNA Y-chromosome segment) sequences as markers (reviewed in Sifakis et al\textsuperscript{97}). Using the DYS marker, Sifakis et al\textsuperscript{95} found higher average amounts (95.5 genome equivalents mL\textsuperscript{-1}) in early pre-eclampsia than late pre-eclampsia (50.8 genome equivalents mL\textsuperscript{-1}). Higher levels of β-globulin and SRY genes were also found in pregnant mothers who developed pre-eclampsia and intrauterine growth retardation.\textsuperscript{89} A different approach was used by Chim et al\textsuperscript{97} who showed that the hypermethylated DNA was derived from the maternal blood cells, whilst the hypomethylated form was of fetal origin. Normally, the hypomethylated form is cleared from pregnant women’s blood, but about a sixfold increase occurred in the case of pre-eclampsia. However, the SRY gene and DYS markers are strictly of Y-chromosomal origin, and so only male fetus carriers can be diagnosed. RNA also may be used as an early marker of pre-eclampsia.\textsuperscript{98} The fetus-specific circulating mRNA for corticotrophin-releasing hormone increases tenfold, such increases relating to the severity of the pre-eclampsia. Significant downregulation of miRNA 325 has also been reported for pregnancies complicated by pre-eclampsia (median: 0.069 versus 0.101; \(P < 0.003\)). Lázár et al\textsuperscript{99} suggested that miRNA 325 expression could indicate a possible role in protein expression changes in pre-eclampsia.

Ethical considerations
Currently, ethical considerations concern only present-day NIPT methods.\textsuperscript{100} However, the fetal genome can be sequenced\textsuperscript{32,34} and SNPs identified as has already occurred for the human genome with a possible SNP every 100–300 bp, ie, 10–30 million potential SNPs per genome. Given the identification of SNPs prior to birth and subsequent genome sequencing of individuals, fetal diagnostic processes can be employed more generally in predictive, preventive, and personalized medicine. Determining fetal gender and Rh is of less concern, but with increasingly sensitive methodology being developed, the wide selection of possible disorders identified, eg, through SNPs, will increase the ethical problems. The presence or absence of particular SNPs or DNA sequences that might lead to the development of a clinical disorder does not necessarily mean that the disorder will develop, so leading to uncertainties. How will such medical records be stored and how safe will they be? Who will have access to such information? Who will pay for such analyses and the upkeep of data bases, especially in the absence of a national health service. Can insurance companies demand access to such information, and if yes, how will they exploit the information? Such questions need urgent debate.

Conclusion
The exciting developments in prenatal diagnosis already permit the analyses of fetal gender, Rh factor, and some fetal clinical disorders at early stages of fetal development, as well as the onset of pre-eclampsia. A number of these tests

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<tr>
<th>Disease</th>
<th>Gene/mutation</th>
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<tr>
<td>Achondroplasia</td>
<td>G1138A of FGFR3 gene</td>
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are already available through national health services and the private sector. Increasing sophistication of the analytical methods, both those available and in development, should ensure an extension of fetal NIPT.

However, with different approaches on offer, it is important that there is quality control and assurance in order for both the routine success of the procedures and reassurance for the parents involved. A start has been made by some working groups, eg, Legler et al. In addition, in the USA, the main accreditation organizations for molecular laboratories standards and guidelines for molecular testing are the US Food and Drug Administration, Clinical Laboratory Improvement Amendments, the American College of Medical Genetics, and the College of American Pathologists. In Europe, EuroGentest and the SAFE network (Special Advances in Fetal and neonatal Evaluation network of excellence) [http://www.safeno.org/cocoon/safeg.html] have taken on this task. In the UK, this aspect is covered by RAPID (Reliable, Accurate Prenatal non-Invasive Diagnosis) and the United Kingdom National External Quality Assessment Service.

Nevertheless, there is still much to be accomplished in the field that is now up to speed in the search for NIPT markers.

**Disclosure**

The author reports no conflict of interest in this work.

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