

## Circulating Fetal DNA: Its Origin and Diagnostic Potential—A Review

D. W. Bianchi \*

Division of Genetics, Departments of Pediatrics, Obstetrics and Gynecology, Tufts-New England Medical Center and Tufts University School of Medicine, Box 394, 750 Washington Street, Boston, MA 02111, USA

Paper accepted 2 January 2004

**Objective:** In contrast to the traditional teaching that the placenta forms an impermeable barrier, multiple studies show that both intact fetal cells and cell-free nucleic acids circulate freely in maternal blood. Complications of pregnancy, such as pre-eclampsia, or fetal cytogenetic abnormalities, such as trisomy 21, increase transfusion of both intact fetal cells and cell-free fetal nucleic acids into the maternal circulation. The objective of our research is to show that abnormal fetomaternal trafficking of nucleic acids is associated with fetal and placental pathology, and that these observations may lead to novel non-invasive diagnostic and screening tests.

**Methods:** Real-time quantitative PCR amplification of *DYS1* is used to measure the levels of male fetal DNA in case-control sets of serum or plasma taken from pregnant women. In our laboratory, we use *DYS1*, a Y-chromosome specific gene, as a uniquely fetal DNA marker for the development of gestation-specific normal values and theoretical models.

**Results:** Women carrying fetuses with trisomies 21 or 13 (but not 18) have increased levels of fetal DNA in their fresh or archived serum and/or plasma samples. Women destined to develop pre-eclampsia have a characteristic bi-phasic elevation of cell-free fetal DNA that precedes clinical symptoms. Data obtained from a variety of clinical scenarios suggest that the placenta is the predominant source of the circulating fetal nucleic acids, although apoptotic haematopoietic cells may contribute to the pool as well.

**Conclusions:** Fetal cell-free DNA is elevated in a number of conditions associated with placental pathology. Widespread clinical implementation of fetal DNA as a screening tool awaits discovery of a reliable gender-independent marker, which may be DNA polymorphisms, epigenetic markers, or mRNA. Fetal cell-free nucleic acids have potential for non-invasive monitoring of placental pathology.

© 2004 IFPA and Elsevier Ltd. All rights reserved.

Placenta (2004), 25, Supplement A, Trophoblast Research, Vol. 18, S93–S101

### INTRODUCTION

In contrast to the traditional teaching that the placenta forms an impermeable barrier between the pregnant woman and her fetus, multiple studies show that both intact fetal cells and cell-free nucleic acids circulate freely within maternal blood. Because invasive techniques of prenatal diagnosis such as amniocentesis and chorionic villus sampling (CVS) are expensive and associated with risks to the mother and the fetus, alternative non-invasive methods are being actively explored. One approach, the isolation of *intact* fetal cells from the maternal circulation, has been extensively studied in our laboratory [1] and others. Advances in molecular biology have provided new and sensitive tools for the genetic analysis of fetal cells once isolated from the maternal blood. The major limitation in applying these techniques clinically, however, is the generally low number of fetal cells found in the maternal

circulation (approximately one fetal cell per ml of maternal blood) [2].

A new area of research developed following the discovery of large amounts of circulating *cell-free* tumour DNA in plasma and serum from cancer patients [3,4]. Reasoning that the rapidly growing fetus and placenta possessed tumour-like qualities, Lo et al. [5] first demonstrated the presence of male fetal DNA sequences in maternal plasma and serum. The results of subsequent studies by the same group showed surprisingly high mean concentrations of fetal DNA in maternal plasma DNA [6]. Significantly more fetal DNA is present in the cell-free *plasma* (or *serum*) of pregnant women as compared with the fetal DNA extracted from the cellular fraction of maternal blood [7]. Further investigations have focused on the clinical implications of this technique, specifically using real-time polymerase chain reaction (PCR) amplification assays for prenatal screening and diagnosis of pregnancy complications and genetic disorders. Compared with the analysis of fetal nucleated cells in the maternal blood, which in many cases requires the use of sophisticated fetal cell enrichment procedures, maternal plasma/serum DNA analysis has the advantage of being rapid, reliable, reproducible, and

Presented at the 9th International Federation of Placenta Associations' meeting, 27 September 2003, Mainz, Germany.

\* To whom correspondence should be addressed. Tel.: +1-617-636-1468; Fax: +1-617-636-1469; E-mail: Dbianchi@tufts-nemc.org

0143-4004/\$—see front matter

© 2004 IFPA and Elsevier Ltd. All rights reserved.

easily carried out for a large number of samples. The main limitation at present appears to be the availability of uniquely fetal gene sequences that identify and/or measure the presence of fetal DNA in both male and female fetuses.

An interesting aspect of this research is that the diagnostic applications for the detection and measurement of circulating cell-free fetal DNA have (to some extent) preceded an understanding of their fundamental biology. For example, the tissue sources of these nucleic acids, as well as their mechanisms of production and metabolism, are currently not definitively known. In this paper, I will review the available evidence to date that suggests that the predominant, although not exclusive, source of the circulating fetal cell-free DNA in the maternal circulation is the placenta. In the second part I will discuss the many diagnostic applications that arise from the measurement of fetal nucleic acids in the maternal circulation.

## **PATHOPHYSIOLOGY**

Working in a cancer centre, and aware of the similarities between the aggressively growing fetoplacental unit and a tumour, Lo et al. [5] initially asked the question whether fetal DNA is present in the plasma and serum of pregnant women. Using standard PCR amplification of Y-specific DNA sequences and agarose gel electrophoresis, they were able to detect fetal DNA in most plasma/serum samples of pregnant women bearing male fetuses. The subsequent development of the technique of real-time PCR, which uses the 5' to 3' exonuclease activity of the enzyme Taq polymerase, enabled these authors to measure the concentration of fetal DNA in maternal plasma [6]. The total amount of extracted DNA in maternal plasma/serum samples was measured by amplifying the ubiquitously present  $\beta$ -globin gene. The fractional concentration of fetal DNA in early pregnancy was 0.014–0.54 per cent (mean 0.13 per cent) in serum and 0.39–11.9 per cent (mean 3.4 per cent) in plasma. In late pregnancy there was a fractional concentration of fetal DNA of 0.032–3.97 per cent (mean 1.0 per cent) in serum and 2.33–11.4 per cent (mean 6.2 per cent) in plasma. The absolute concentration of fetal DNA in was similar in maternal plasma and serum. The main difference was the presence of a larger quantity of background maternal DNA in serum (released from the clot) compared with plasma. High concentrations of fetal DNA allowed for the detection of fetal *SRY* genes in maternal plasma or serum from every pregnant woman carrying a male fetus ( $n=12$ ). The data also demonstrated an increase in fetal DNA concentration as gestation advanced, with a remarkable sharp increase during the last 8 weeks of pregnancy.

The turnover of circulating fetal DNA was subsequently studied by investigating the clearance of circulating fetal DNA after delivery [8]. Most of the women studied (seven out of eight) had undetectable levels of circulating fetal DNA by 2 h postpartum. The mean half-life for fetal DNA was estimated to be 16.3 min (range 4–30 min). Assuming there are no abrupt

changes in circulating fetal DNA clearance associated with delivery, the authors speculated that to maintain a steady state, fetal DNA must be *continuously* liberated in large quantities into maternal circulation. Their calculations suggested that fetal DNA is liberated at a mean rate of  $2.24 \times 10^4$  copies/min into the maternal circulation. The rapid turnover implies that fetal DNA measurement provides an almost real-time picture of fetal DNA production and clearance, and, thus, may be useful for monitoring fetomaternal events having rapid dynamics.

To date, little is known about the molecular and biological characteristics of the fetal DNA present in maternal circulation. Due to the fact that the kinetics of real-time PCR result in a more efficient amplification of smaller amplicons, there is indirect evidence to suggest that the circulating fetal DNA fragments are small (<450 bp). Because the fetal DNA is not immediately metabolized and is so readily amplified from plasma or serum, it has also been suggested that the circulating DNA is protected within apoptotic bodies or attached to nucleosomes. Halicka et al. [9] demonstrated that there is separate packaging of DNA and RNA into different apoptotic bodies during apoptosis. Using acridine orange to stain nucleic acids, Bischoff and colleagues [10] flow-sorted nucleic acid positive material from the acellular fraction of plasma samples taken from five pregnant women at 12–16 weeks of gestation. They showed that apoptotic bodies are present in maternal plasma, and further demonstrated that Y chromosomal gene sequences could be amplified from these apoptotic bodies.

## **Tissue origin of fetal DNA in maternal circulation**

There has been much speculation as to the tissue source of the abundant quantities of circulating fetal DNA. Three possibilities will be discussed here: fetal haematopoietic cells, the placenta, and direct transfer of DNA molecules.

*Haematopoietic cells.* We initially considered fetal haematopoietic cells to be a reasonable candidate tissue source of the circulating fetal DNA due to our knowledge that a variety of fetal cell types circulate within maternal blood [1]. Our previous quantitative PCR studies, performed on the *intact* cellular fraction of maternal blood samples, demonstrated that there was approximately one nucleated fetal cell per ml of maternal whole blood in normal gestations [2]. These results contrasted with those of Lo et al. [5,6], who suggested that there was approximately 25 times that amount of cell-free DNA detectable in equivalent volumes of maternal plasma. Because plasma DNA has been demonstrated to be a marker of cell death [11], we hypothesized that the cell-free DNA may be liberated into the maternal circulation by the destruction of fetal cells that have crossed into the maternal circulation. To show that the intact fetal cells in the maternal circulation were undergoing apoptosis we performed terminal UTP nuclear

end labelling (TUNEL) staining on fetal nucleated erythrocytes (NRBCs) that had been flow-sorted using monoclonal antibody to the gamma chain of fetal haemoglobin, and conclusively identified as fetal because of chromosome-specific markers by interphase FISH analysis. Our results showed that 42.7 per cent of confirmed fetal NRBCs were undergoing apoptosis [12]. We hypothesized that cell-free fetal DNA may result from the interaction between apoptotic fetal cells and the maternal immune system. Similarly, Hristoskova et al. [13] demonstrated that over half of the erythroblasts in fetal and umbilical cord blood stained positively with the TUNEL assay. Using density gradient centrifugation, van Wijk and colleagues [14] showed that a large number of apoptotic fetal cell remnants were present in the plasma fraction. These data, when considered in aggregate, suggested that a large proportion of the fetal cells that had crossed the placenta were undergoing developmentally regulated apoptotic changes, and that the destruction of these cells might indeed account for part of the fetal DNA detected in the pregnant woman.

Intact fetal cells, however, are rare in maternal blood, and it is unlikely that this small number of fetal haematopoietic cells accounts for both the volume and turnover of cell-free DNA in the maternal circulation. Fetal cellular and nucleic acid trafficking are both increased in certain complications of pregnancy, such as pre-eclampsia and fetal aneuploidy, suggesting that a relationship between the two might exist [15]. To address the question whether apoptotic fetal erythroblasts were related to the amount of cell-free fetal DNA present in maternal plasma, Zhong et al. [16] studied samples from a variety of pregnant women with normal and abnormal pregnancies carrying male fetuses. In the same samples, they enumerated fetal cells in maternal whole blood by both fluorescent *in situ* hybridization studies using X and Y chromosome-specific probes and single-cell PCR, and then measured levels of cell-free DNA. No correlation between fetal cells and fetal DNA was seen in the normal pregnancies, pregnancies affected by or at risk for pre-eclampsia, or in the pregnancies affected by preterm labour. The most convincing data that fetal erythroblasts are not the source of fetal DNA was in the preterm labour group, in which there were significantly elevated fetal DNA levels without any incremental increase in fetal NRBCs.

We also addressed the question whether fetal haematopoietic cells were the source of circulating fetal DNA in a different way [17]. We hypothesized that after phlebotomy, apoptotic fetal cells would eventually disintegrate within the venipuncture tube and release their DNA. We also speculated that fetal cells, when exposed to maternal immune cells and cytokines, would be destroyed, causing further release of fetal DNA. Thus, we designed a study that evaluated sequential aliquots of plasma taken from the same original blood samples from women in their first and third trimesters of pregnancy. We measured the amount of Y chromosomal DNA present as the 'fetal' marker, and beta-globin DNA as the marker of 'total' (maternal plus fetal) DNA present. At both gestational ages there was no increase in fetal DNA in the original

maternal sample over a 24-h period [17]. This implies that the fetal DNA came from a source other than the haematopoietic cells within the phlebotomy tube.

*Placenta.* The placenta is a logical source of the circulating fetal DNA due to its size and abundant cellular activity. Multiple studies have shown that a relationship exists between the amount of cell-free fetal DNA in the maternal circulation and advancing gestational age [6,18,19]. In a recent study of 22 women who conceived using artificial reproductive technology, fetal *SRY* gene sequences were detected in serum samples from all ten women who carried at least one male fetus. Importantly, the *SRY* gene was found as early as day 18 following embryo transfer in a twin gestation, day 22 following embryo transfer in a singleton gestation, and by day 37 in all of the women studied [20]. The definitive fetoplacental circulation is not established until days 28–30 post-conception, and, in this study, by day 28, 80 per cent of the study subjects had detectable fetal DNA in their blood. This strongly implies that the source of the fetal DNA is not haematopoietic, and is most likely from trophoblast. Additional supporting data comes from studies in which fetal DNA and hCG concentrations were measured in the same maternal sample and strongly correlated with each other [21,22]. Perhaps the most convincing and direct evidence comes from the fact that placental-specific mRNA molecules are readily detectable in maternal plasma [23].

The kinetics of fetal DNA in the maternal circulation also suggests that the placenta is the predominant source of the DNA. The turnover of circulating fetal DNA was investigated by measuring the clearance of circulating fetal DNA after delivery [8]. Most of the women studied (7 out of 8) had undetectable levels of circulating fetal DNA by 2 h post-partum. Recently, however, Invernizzi et al. [24] suggested that cell-free fetal DNA could persist for months or years following delivery, raising the question once again as to whether the cell-free DNA was placental in origin. This report elicited several responses to the journal *Human Genetics*, all of which failed to reproduce Invernizzi et al.'s data [25–28]. The most likely interpretation of Invernizzi et al.'s results is that due to the absence of a recommended second centrifugation step in their DNA extraction technique [29] they are detecting male fetal *cellular* DNA that originates from fetal stem or progenitor cells or leukocytes that circulate for many years post-partum [30,31].

In a recent series of experiments, we studied cell-free fetal DNA levels in maternal plasma following elective first trimester termination of pregnancy [32]. Fetal DNA was detectable as early as 32 days of gestation and increased by a mean of 4.2 genome equivalents per ml of plasma per week of gestation. In 6 of 29 women who underwent medical termination with misoprostol we detected fetal DNA as long as 11 days following the medical termination procedure. We interpreted these results as demonstrating a sensitive way of detecting low-level retention of villi due to incomplete expulsion of placental remnants. Working in Italy, Smid and

colleagues [33] studied 53 twin gestations in which there was at least one male fetus. They showed that the amount of *SRY* gene sequence amplified correlates with the number of male fetuses. In the 24 gestations in which both fetuses were male, 17 had dichorionic and seven monochorionic placentation. There were no differences observed in the concentration of fetal DNA in maternal samples between the two different types of chorionicity. Thus, this group could not prove that the fetal DNA originates in placenta or membranes.

To clarify the origin of the cell-free fetal DNA in maternal plasma, Sekizawa et al. [34] analysed paired plasma samples from 15 women at the time of Caesarean section delivery and umbilical cord blood from their infants. The study subjects included five women with severe pre-eclampsia and ten women with normal pregnancies matched for gestational age. Seven different highly polymorphic short tandem repeat (STR) markers on chromosomes 13, 18, and 21 were used to distinguish fetal from maternal DNA. Approximately 70 per cent of samples were informative. The results showed that the median concentration of fetal DNA in maternal plasma was 14.3 per cent (range 2.3–60 per cent) and the median concentration of maternal DNA in umbilical plasma was 0.9 per cent (range 0.2–8.4 per cent), which was significantly different ( $P=0.007$ ), and implies unequal transfer of DNA. These authors speculated that the fetal DNA originates from apoptotic villous trophoblasts that border the intervillous space [34].

*Direct fetomaternal transfer of DNA molecules.* For the sake of completeness it is important to note that fetal DNA sequences have been detected in several other maternal body fluids, including amniotic fluid [35], maternal urine [36,37], maternal cerebrospinal fluid [38], and maternal peritoneal fluid [39]. The concentration of cell-free fetal DNA in the amniotic fluid compartment is approximately  $200\times$  the amount found in maternal plasma. This has led to some speculation regarding the possibility of a concentration gradient leading to the direct transfer of DNA molecules across placenta or membranes.

In all likelihood, the majority of the cell-free fetal DNA in the maternal circulation probably originates from the placenta with an additional (minor) contribution from haematopoietic cells and possibly from the fetus itself via direct transfer.

## CLINICAL APPLICATIONS

The ability to rapidly and reproducibly measure fetal cell-free DNA non-invasively has led to many novel clinical applications. In general, the clinical applications of fetal DNA measurement in maternal plasma and serum have focused on the *measurement* of fetal DNA sequences and the *detection* (present or absent) of uniquely fetal gene sequences [40]. Many different complications of pregnancy are associated with an increased amount of fetal DNA in the maternal circulation. Currently, however, a major limitation of the quantitation work is that it is limited to male fetuses, because they possess

a Y chromosome, which the pregnant woman lacks. Thus, Y chromosome-specific DNA sequences of fetal origin may be easily amplified and detected in the maternal serum or plasma. A variety of approaches have been taken to develop markers that would be equally useful in female fetuses. They include: autosomal DNA polymorphisms [41], fetal epigenetic markers [42], and measurement of mRNA transcripts from placentally expressed genes [43].

## Gender detection

Given the relatively easy identification of male DNA in maternal plasma and serum, one potential clinical application is in the non-invasive identification of male fetuses at risk for X-linked disorders. Honda et al. [18] suggested that fetal gender determination using DNA analysis of maternal serum could serve as a 'pre-test' to decide whether invasive testing should be performed on a fetus at risk to inherit an X-linked condition. Using conventional PCR, they demonstrated a 100 per cent sensitivity of detection of 40 male fetuses at 7 weeks of gestation, and using real-time PCR, they were able to achieve 100 per cent sensitivity by 5 weeks. They also suggested that fetal gender determination could also effectively be used to determine which pregnant women should receive dexamethasone administration as treatment for a potential fetus with congenital adrenal hyperplasia [44,45].

## Noninvasive diagnosis of fetal aneuploidy

Great interest exists in the non-invasive detection of fetal aneuploidy, primarily due to the fact that there is a procedure-related fetal loss rate associated with amniocentesis and chorionic villus sampling (CVS). Using the cellular fraction of maternal whole blood, our group initially observed a six-fold elevation of DNA from intact fetal cells in maternal blood when the fetus had trisomy 21 [2]. Thus, we questioned whether a parallel increase would also be observed using fetal DNA in maternal plasma. In a collaborating study between the Chinese University of Hong Kong and our own group, we measured the median circulating fetal DNA concentrations in fresh 2nd trimester plasma samples from pregnant women. In one centre, the fetal DNA levels from women carrying trisomy 21 ( $n=7$ ) and euploid male fetuses ( $n=19$ ) were 46.0 genome equivalents (GE)/ml and 23.3 GE/ml, respectively [46]. The corresponding numbers at the second centre were 48.2 GE/ml for trisomy 21 pregnancies ( $n=6$ ) and 16.3 GE/ml for euploid male pregnancies ( $n=18$ ). Overall, there appeared to be about twice as much fetal DNA present in the pregnant woman when the fetus had trisomy 21. Other groups have either validated [47] or were unable to show [48,49] that maternal plasma and serum fetal DNA levels are elevated in trisomy 21 fetuses.

All of the analyses for the Lo et al. [46] study were performed on fresh maternal plasma samples. We next asked if archived samples that had been initially obtained for the



standard 2nd trimester maternal serum screening for Down syndrome would contain enough fetal DNA of sufficient quality to permit real-time PCR amplification. We rationalized that if successful, it would allow us to utilize stored serum banks as a source of maternal samples, and it would permit simultaneous measurement of fetal DNA values as compared to the other analytes measured in the maternal serum screen. We identified frozen archived residual serum samples from eleven women known to be carrying fetuses with 47, XY, +21. Each case sample was paired with five control samples from women with a normal serum screening result and a euploid newborn (as determined by examination of newborn delivery record). Cases and controls were matched for gestational age and time in freezer storage. Fetal DNA concentration was amplified blindly by real time PCR using the *DYS1* sequence. Matched rank sum analysis and analysis of variance (ANOVA) were used for statistical analysis of results. The adjusted mean serum fetal DNA concentrations were 41.2 GE/ml in the Down syndrome cases, and 24.2 GE/ml in the euploid controls ( $P=0.002$ ) [50]. The mean observed rank of 5.0 in the Down syndrome group in the rank sum analysis was significantly higher than expected ( $P<0.005$ ). This study showed that in a rigorously controlled study, serum samples from women carrying fetuses with Down syndrome have a 1.7 fold higher level of circulating fetal DNA when compared to matched controls. Archived serum samples appear to be a useful source of clinical material for retrospective analyses, as long as duration of sample storage is controlled.

Another important clinical application is the use of fetal DNA measurements in the non-invasive diagnosis of other relatively common aneuploidies that result in the live birth of infants with anomalies and mental retardation, such as trisomies 13 and 18. Using the case-control model again, we obtained archived maternal serum samples from five women carrying male fetuses with trisomy 13 and five women carrying male fetuses with trisomy 18 [51]. Each case was matched for fetal gender, gestational age, and duration of freezer storage to four or five control serum samples presumed to be euploid after newborn medical record review. For the cases of trisomy 13, the unadjusted median serum fetal DNA concentrations were 97.5 GE/ml (range 29.2–187.0). For the cases of trisomy 18, the unadjusted median serum fetal DNA concentrations were 31.5 GE/ml (18.6–77.6), and the controls were 40.3 GE/ml (3.7–127.4). Fetal DNA levels in the cases of trisomy 13 were significantly elevated ( $P=0.016$ ) by ANOVA within each matched set. In contrast, DNA levels in the trisomy 18 cases were no different from controls ( $P=0.244$ ). The current standard 2nd trimester maternal serum screen does not identify fetuses at high risk for trisomy 13. Thus, cell-free fetal DNA measurement, if added to the standard maternal serum screen, could add value by indicating which fetuses are at risk for having trisomy 13 (provided that a gender-independent marker can be developed).

One of the advantages of using archived serum samples collected as part of a routine clinical maternal serum screening program is that the measurements for alpha-fetoprotein (AFP),

estriol (uE3), hCG, and inhibin A are already known. We hypothesized that it would be possible to treat cell-free fetal DNA as an independent serum marker, and that we could assess the potential contribution of cell-free fetal DNA to 2nd trimester multiple marker screening algorithms that screen for fetal Down syndrome. To test this hypothesis, we used the data from our previously published report [50] and added four new case-control sets, for a total of 15 samples from confirmed male Down syndrome pregnancies, and five control samples for each case matched for fetal gender, length of freezer storage time (within one month), and same completed week of gestation [52]. All samples studied were from pregnancies with confirmed male fetuses, but additional samples from women carrying female fetuses were included in the assay for quality control purposes. All samples were analysed blindly without knowledge of gender or case-control status. Median fetal DNA concentrations as a function of advancing gestational age were initially measured as GE/ml. The values in the control population were used to calculate a weighted log-linear regression plot [53]. All fetal DNA values were eventually expressed as multiples of the median (MoM), which is the way that the other serum proteins are reported in clinical algorithms. Interestingly, the distributions of the fetal DNA values in both the Down syndrome and control pregnancies followed a log-Gaussian pattern.

The detection rate of fetal Down syndrome was modelled for fetal DNA alone at different false positive rates, and compared with published data on the univariate performance of the four other 2nd trimester serum markers (AFP, uE3, hCG, inhibin A). Univariate performance of DNA was very similar to AFP, with a detection rate of 21 per cent at a false positive rate of 5 per cent. We then assessed the impact of adding fetal DNA to the current quadruple marker maternal serum screen. At a fixed false positive rate of 5 per cent, adding fetal DNA increased the detection rate of Down syndrome from 81 to 86 per cent. In our initial studies there was a wide distribution of fetal DNA levels in both cases and controls, which decreased the average separation between these two groups. We believe that with larger studies the screening performance of fetal DNA will improve, as was the case with AFP measurement. None the less, in our very first evaluation, we showed that fetal DNA analysis can modestly improve screening performance for Down syndrome above what is currently available in the 2nd trimester [52].

Another novel application of fetal DNA analysis is in the investigation of pregnancies at risk for aneuploidy due to a parental balanced translocation. Chen et al. [54] reported the detection of a paternally inherited fetal aneuploidy (3p trisomy and 7q36 deletion) that resulted from a paternal t(3;7) reciprocal translocation.

In our laboratory, using competitive genomic hybridization, we have recently amplified and analysed the abundant quantities of cell-free fetal DNA in amniotic fluid samples on commercially available DNA microarrays [55]. These arrays consist of overlapping genomic sequences that encompass all 24 human chromosomes, including areas affected by the

common microdeletion deletion syndromes such as Prader-Willi and Williams, as well as sub-telomere rearrangements that are involved in mental retardation syndromes. Our preliminary data indicate that the cell-free fetal DNA in amniotic fluid may have novel clinical application as an enhancement of the traditional metaphase 450-band karyotype. This so-called 'molecular karyotype' could routinely provide information on the presence of whole chromosome aneuploidy as well as more subtle deletions or unbalanced rearrangements.

### Detection of pregnancies at risk for pre-eclampsia

Many studies have demonstrated that there is a five-fold increase in the amount of fetal DNA in samples obtained from women with symptomatic pre-eclampsia [381 genome-equivalents (GE)/ml, compared with 76 GE/ml in maternal plasma] [56–60]. Fetal DNA levels are also elevated in women who will eventually develop pre-eclampsia [61]; thus, there is much interest in using measurement of cell-free fetal DNA as a predictive marker for the development of pre-eclampsia.

In collaboration with Dr Richard Levine of the National Institute of Child Health and Human Development, we measured fetal cell-free DNA levels in archived serum samples in a case-control study nested within the Calcium Prevention of Pre-eclampsia (CPEP) trial [62]. The subjects were healthy nulliparous women with complete outcome information, at least one serum sample obtained before 22 weeks of gestation, and a known male infant. For each woman who eventually developed pre-eclampsia, a normotensive case was selected, matched for centre of enrolment, gestational age at first serum collection, and freezer storage time. We analysed all serum samples available for 120 matched pairs (658 total serum samples). Fetal DNA levels were significantly elevated in cases vs controls as early as 17 weeks of gestation, long before the development of clinical symptoms of pre-eclampsia. Furthermore, in cases, a second phase of DNA elevation occurred approximately 3 weeks prior to the development of symptoms. We hypothesize that the initial elevation of DNA represents placental necrosis or apoptosis, whereas the later elevation may be due to subtle maternal end-organ dysfunction, resulting in impaired fetal DNA elimination. The two-stage elevation suggests the possibility of measurement of fetal DNA both to screen for pre-eclampsia and to indicate impending disease.

### Other complications of pregnancy

Increased levels of cell-free fetal DNA in maternal serum may also be a marker for pre-term delivery. In normal pregnancies, sequential measurements of fetal DNA in the serum/plasma of pregnant women demonstrate a gradual increase as gestation advances, then increasing sharply after 32 weeks' gestation [6]. This sharp rise may reflect changes that occur at the maternal-fetal interface [63]. The increase in maternal plasma fetal DNA

concentration may herald impending delivery. Leung et al. [64] provided data that support this hypothesis. Studying only women carrying male fetuses, they found significantly higher fetal DNA concentrations in women ( $n=20$ ) who had spontaneous pre-term deliveries that occurred between 26 and 34 weeks' gestation. Moreover, lower concentrations of fetal DNA were associated with successful tocolytic therapy.

Fetal DNA levels are also increased in the plasma of pregnant women with placenta previa [65] and hyperemesis gravidarum [21], but not in women whose fetuses have intrauterine growth restriction [66]. These clinical correlations also suggest that the source of the circulating nucleic acids is the placenta.

### Single gene disorders

Another clinical application is the non-invasive diagnosis of fetal Rhesus (Rh) (D) status. Knowledge of fetal Rh(D) genotype is useful for the management of Rh(D)-sensitized pregnant women whose partners are heterozygous for the Rh(D) gene because no further diagnostic or therapeutic procedures are necessary if the fetus is Rh(D) negative. To date, the feasibility of fetal Rh(D) genotyping from maternal plasma and serum has been reported in several studies [67–72]. The analysis of fetal DNA from maternal plasma is at present reproducible enough to become a routine diagnostic test for the non-invasive prenatal diagnosis of fetal Rh(D) genotype in Europe [73].

Myotonic dystrophy is an autosomal dominant disorder associated with the expansion of unstable CTG trinucleotide repeats in the 3' untranslated region of the DM kinase gene. In one study, paternally derived expanded alleles were detected in maternal plasma DNA [74]. In another report, a fetus with short limbs was suspected to have achondroplasia. DNA was extracted from maternal plasma and a segment of the fibroblast growth factor receptor gene (FGFR3) was amplified. A diagnostic G to A transition was demonstrated at position 1138 using restriction enzyme analysis of amplified products [75]. In at-risk pregnancies, the prenatal exclusion of cystic fibrosis, beta thalassemia, and congenital adrenal hyperplasia using the analysis of fetal DNA in maternal blood has also been described [76–78].

### FETAL CELL-FREE RNA IN THE MATERNAL CIRCULATION

A major limitation of using DNA in maternal blood is the fact that the mother and fetus share, on average, half of their genomic DNA sequences. Thus, it is only possible to detect uniquely fetal DNA sequences that are paternally inherited. There is great interest in the development of gender-independent nucleic acid markers, and RNA is a likely candidate. Following the demonstration of tumour-derived RNA sequences in the blood of cancer patients, Poon and

colleagues [79] demonstrated that fetal *ZFY* mRNA sequences could be detected in the maternal plasma of 22 per cent of women in early pregnancy and 63 per cent of women in later pregnancy. This work was performed using a two-step RT-PCR assay that was difficult to replicate. Subsequently an important paper was published demonstrating that mRNA in peripheral blood is remarkably stable [80]. The detection of circulating mRNA sequences was unexpected due to the known lability of RNA and the presence of ribonuclease in plasma. Tsui et al.'s data implied that mRNA is protected in the circulation by its likely association with particles, and suggested that fetal or placental mRNA sequences may be detected in maternal serum or plasma samples if processed appropriately. This group has also amplified transcripts from two placental-specific genes, human chorionic gonadotropin (hCG) and human placental lactogen (hPL) [23]. They recently addressed an initial clinical application for cell-free RNA in the prenatal diagnosis of pre-eclampsia by amplifying mRNA of the corticotropin-releasing hormone (CRH) locus [43]. Plasma CRH mRNA concentrations were greatly elevated in third trimester pregnant women with a clinical diagnosis of pre-eclampsia as compared with normotensive controls.

Measurement of specific fetal gene expression by analysis of circulating mRNA will likely have many future clinical applications in detection of placental pathology.

## ACKNOWLEDGEMENT

This work was supported by NO1-HD43204 and RO1-HD42053.

## REFERENCES

- [1] Bianchi DW. Fetal cells in the maternal circulation: feasibility for prenatal diagnosis. *Br J Haematol* 1999;105:574-83.
- [2] Bianchi DW, Williams JM, Sullivan LM, Hanson FW, Klinger KW, Shuber AP. PCR quantitation of fetal cells in maternal blood in normal and aneuploid pregnancies. *Am J Hum Genet* 1997;61:822-9.
- [3] Chen XQ, Stroun M, Magenat JL, Nicod LP, Kurt AM, Lyautey J et al. Microsatellite alterations in plasma DNA of small lung cancer patients. *Nat Med* 1996;2:972-4.
- [4] Nawroz H, Koch W, Anker P, Stroun M, Sidransky D. Microsatellite alterations in serum DNA of head and neck cancer patients. *Nat Med* 1996;2:972-4.
- [5] Lo YM, Corbetta N, Chamberlain PF, Rai V, Sargent IL, Redman CW et al. Presence of fetal DNA in maternal plasma and serum. *Lancet* 1997; 350:485-7.
- [6] Lo YM, Tein MS, Lau TK, Haines CJ, Leung TN, Poon PM et al. Quantitative analysis of fetal DNA in maternal plasma and serum: implications for noninvasive prenatal diagnosis. *Am J Hum Genet* 1998; 62:768-75.
- [7] Lo YM. Fetal DNA in maternal plasma: biology and diagnostic applications. *Clin Chem* 2000;46:1903-6.
- [8] Lo YM, Zhang J, Leung TN, Lau TK, Chang AM, Hjelm NM. Rapid clearance of fetal DNA from maternal plasma. *Am J Hum Genet* 1999; 64:218-24.
- [9] Halicka HD, Bedner E, Darzynkiewicz Z. Segregation of RNA and separate packaging of DNA and RNA in apoptotic bodies during apoptosis. *Exp Cell Res* 2000;260:248-56.
- [10] Bischoff FZ, Dang D, Horne C, Marquez-Do D, Brinkley WR, Lewis DE et al. Fetal DNA in maternal plasma circulates as apoptotic bodies: elucidation of the structural nature of fetal DNA for non-invasive prenatal genetic diagnosis. *Am J Hum Genet* 2003;73(Suppl):189.
- [11] Fournie GJ, Martres F, Pourrat JP, Alary C, Rumeau M. Plasma DNA as cell death marker in elderly patients. *Gerontology* 1993;39: 215-21.
- [12] Sekizawa A, Samura O, Zhen DK, Falco V, Farina A, Bianchi DW. Apoptosis in fetal nucleated erythrocytes circulating in maternal blood. *Prenat Diagn* 2000;20:886-9.
- [13] Hristoskova S, Holzgreve W, Hahn S. More than one-half of the erythroblasts in the fetal circulation and cord blood are TUNEL positive. *Clin Chem* 2001;47:1870-1.
- [14] Van Wijk IJ, deHoon AC, Jurhawan R, Tjoa ML, Griffioen S, Mulders MA et al. Detection of apoptotic fetal cells in plasma of pregnant women. *Clin Chem* 2000;46:729-31.
- [15] Bianchi DW, Lo YM. Fetomaternal cellular and plasma DNA trafficking: the Yin and the Yang. *Ann N Y Acad Sci* 2001;945:119-31.
- [16] Zhong XY, Holzgreve W, Hahn S. Cell-free fetal DNA in the maternal circulation does not stem from the transplacental passage of fetal erythroblasts. *Mol Hum Reprod* 2002;8:864-70.
- [17] Angert RM, LeShane ES, Lo YM, Chan LY, Delli-Bovi LC, Bianchi DW. Fetal cell-free plasma DNA concentrations in maternal blood are stable 24 hours after collection: analysis of first- and third-trimester samples. *Clin Chem* 2003;49:195-8.
- [18] Honda H, Miharu N, Ohashi Y, Samura O, Kinutani M, Hara T et al. Fetal gender determination in early pregnancy through qualitative and quantitative analysis of fetal DNA in maternal serum. *Hum Genet* 2002; 110:75-9.
- [19] Chan LY, Leung TN, Chan KC, Tai H-L, Lau TK, Wong EM et al. Serial analysis of fetal DNA concentrations in maternal plasma in late pregnancy. *Clin Chem* 2003;49:678-80.
- [20] Guibert J, Benachi A, Grebille AG, Ernault P, Zorn JR, Costa JM. Kinetics of SRY gene appearance in maternal serum: detection by real time PCR in early pregnancy after assisted reproductive technique. *Hum Reprod* 2003;18:1733-6.

## SUMMARY

The analysis of fetal cell-free DNA in maternal serum and plasma is an active area of research, as evidenced by the reference list below. Fetal nucleic acids are surprisingly stable in the maternal circulation, perhaps due to their protection in apoptotic bodies. Fetal cell-free DNA is abundantly produced and rapidly metabolized, providing a dynamic picture of events occurring during the current pregnancy. Physiologic and clinical information, when considered together, suggest that the majority of the circulating nucleic acids are derived from the placenta, with some contribution from the fetal haematopoietic system. The relative ease of measurement of fetal DNA levels in maternal plasma and serum has led to clinical applications in the detection of fetal aneuploidy, identification of pre-eclamptic pregnancies, and non-invasive diagnosis of fetal Rhesus D genotype and some single gene disorders. Novel approaches, such as using gender-independent polymorphisms, epigenetic markers, or circulating mRNA sequences, will eventually allow this technology to be applied to female fetuses. With the microarray technology that is already available it is not difficult to imagine that amplified fetal nucleic acids will ultimately permit a non-invasive fetal genome scan as part of routine prenatal screening.

- [21] Sekizawa A, Sugito Y, Iwasaki M, Watanabe A, Jimbo M, Hoshi S et al. Cell-free fetal DNA is increased in plasma of women with hyperemesis gravidarum. *Clin Chem* 2001;47:2164–5.
- [22] Ohashi Y, Miharu N, Honda H, Samura O, Ohama K. Correlation of fetal DNA and human chorionic gonadotropin concentrations in second-trimester maternal serum. *Clin Chem* 2002;48:386–8.
- [23] Ng EK, Tsui NB, Lau TK, Leung TN, Chiu RW, Panesar NS et al. mRNA of placental origin is readily detectable in maternal plasma. *Proc Natl Acad Sci U S A* 2003;100:4360–2.
- [24] Invernizzi P, Biondi ML, Battezzati PM, Perego F, Selmi C, Cecchini F et al. Presence of fetal DNA in maternal plasma decades after pregnancy. *Hum Genet* 2002;110:587–91.
- [25] Benachi A, Steffann J, Gautier E, Ernault P, Olivi M, Dumez Y et al. Fetal DNA in maternal serum: does it persist after pregnancy? *Hum Genet* 2003;113:76–9.
- [26] Johnson-Hopson CN, Artlett CM. Evidence against the long-term persistence of fetal DNA in maternal plasma after pregnancy. *Hum Genet* 2002;111:575.
- [27] Smid M, Galbiati S, Vassallo A, Gambini D, Ferrari A, Restagno G et al. No evidence of fetal DNA persistence in maternal plasma after pregnancy. *Hum Genet* 2003;112:617–8.
- [28] Invernizzi P, Battezzati PM, Podda M, Simoni G. Presence of fetal DNA in maternal plasma decades after pregnancy: further comments. *Hum Genet* 2002;111:576.
- [29] Chiu RW, Poon LL, Lau TK, Leung TN, Wong EM, Lo YM. Effects of blood-processing protocols on fetal and total DNA quantification in maternal plasma. *Clin Chem* 2001;47:1607–13.
- [30] Bianchi DW, Zickwolf GK, Weil GJ, Sylvester S, DeMaria MA. Male fetal progenitor cells persist in maternal blood for as long as 27 years postpartum. *Proc Natl Acad Sci U S A* 1996;93:705–8.
- [31] Evans PC, Lambert N, Maloney S, Furst DE, Moore JM, Nelson JL. Long-term fetal microchimerism in peripheral blood mononuclear cell subsets in healthy women and women with scleroderma. *Blood* 1999; 93:2033–7.
- [32] Wataganara T, Chen AY, LeShane ES, Sullivan LM, Borgatta L, Bianchi DW et al. Cell-free fetal DNA levels in maternal plasma after elective first trimester termination of pregnancy. *Fertil Steril* 2004 in press.
- [33] Smid M, Galbiati S, Vassallo A, Gambini D, Ferrari A, Viora E et al. Fetal DNA in maternal plasma in twin pregnancies. *Clin Chem* 2003b; 49:1526–8.
- [34] Sekizawa A, Yokokawa K, Sugito Y, Iwasaki M, Yukimoto Y, Ichizuka K et al. Evaluation of bidirectional transfer of plasma DNA through placenta. *Hum Genet* 2003;113:307–10.
- [35] Bianchi DW, LeShane ES, Cowan JM. Large amounts of cell-free fetal DNA are present in amniotic fluid. *Clin Chem* 2001;47:1867–9.
- [36] Botezatu I, Serdyuk O, Potapova G, Shelepov V, Alechina R, Molyaka Y et al. Genetic analysis of DNA extracted in urine: a new approach for detecting specific genomic DNA sequences from cells dying in an organism. *Clin Chem* 2000;46:1078–84.
- [37] Al-Yatama MK, Mustafa AS, Ali S, Abraham S, Khan Z, Khaja N. Detection of Y chromosome-specific DNA in the plasma and urine of pregnant women using nested polymerase chain reaction. *Prenat Diagn* 2001;21:399–402.
- [38] Angert RM, LeShane ES, Yarnell RW, Johnson KL, Bianchi DW. Cell-free fetal DNA in the cerebrospinal fluid of peripartum women. *Am J Obstet Gynecol* 2004 in press.
- [39] Cioni R, Bussani C, Scarselli B, Mello G, Mecacci F, Scarselli G. Detection of fetal DNA in the peritoneal cavity during pregnancy. *Eur J Obstet Gynecol Reprod Biol* 2003;107:210–1.
- [40] Pertl B, Bianchi DW. Fetal DNA in maternal plasma: emerging clinical applications. *Obstet Gynecol* 2001;98:483–90.
- [41] Pertl B, Sekizawa A, Samura O, Orescovic I, Rahaim PT, Bianchi DW. Detection of male and female fetal DNA in maternal plasma by multiplex fluorescent polymerase chain reaction amplification of short tandem repeats. *Hum Genet* 2000;106:45–9.
- [42] Poon LL, Leung TN, Lau TK, Chow KCK, Lo YM. Differential DNA methylation between fetus and mother as a strategy for detecting fetal DNA in maternal plasma. *Clin Chem* 2002;48:35–41.
- [43] Ng EK, Leung TN, Tsui NB, Lau TK, Panesar NS, Chiu RW et al. The concentration of circulating corticotrophin-releasing hormone mRNA in maternal plasma is increased in preeclampsia. *Clin Chem* 2003; 49:727–31.
- [44] Rijnders RJ, van der Schoot CE, Bossers B, de Vroede MA, Christaens CJ. Fetal sex determination from maternal plasma in pregnancies at risk for congenital adrenal hyperplasia. *Obstet Gynecol* 2001;98:374–8.
- [45] Bianchi DW. Prenatal exclusion of recessively inherited disorders: should maternal plasma analysis precede invasive techniques? *Clin Chem* 2002; 48:689–90.
- [46] Lo YM, Lau TK, Zhang J, Leung TN, Chang AM, Hjelm NM et al. Increased fetal DNA concentrations in the plasma of pregnant women carrying fetuses with trisomy 21. *Clin Chem* 1999;45:1747–51.
- [47] Zhong XY, Bürk MR, Troeger C, Jackson LR, Holzgreve W, Hahn S. Fetal DNA in maternal plasma is elevated in pregnancies with aneuploid fetuses. *Prenat Diagn* 2000;20:795–8.
- [48] Ohashi Y, Miharu N, Honda H, Samura O, Ohama K. Quantitation of fetal DNA in maternal serum in normal and aneuploid pregnancies. *Hum Genet* 2001;108:123–7.
- [49] Spencer K, de Kok JB, Swinkels DW. Increased total cell-free DNA in the serum of pregnant women carrying a fetus affected by trisomy 21. *Prenat Diagn* 2003;23:580–583.
- [50] Lee TL, LeShane ES, Messerlian GM, Canick JA, Farina A, Heber WW et al. Down syndrome and cell-free fetal DNA in archived maternal serum. *Am J Obstet Gynecol* 2002;187:1217–21.
- [51] Wataganara T, LeShane ES, Farina A, Messerlian GM, Lee T, Canick JA et al. Maternal serum cell-free fetal DNA levels are increased in cases of trisomy 13 but not trisomy 18. *Hum Genet* 2003;112:204–8.
- [52] Farina A, LeShane ES, Lambert-Messerlian GM, Canick JA, Lee T, Neveux LM et al. Evaluation of cell-free fetal DNA as a second-trimester maternal serum marker of Down syndrome pregnancy. *Clin Chem* 2003; 49:239–42.
- [53] Farina A, Caramelli E, Concu M, Sekizawa A, Ruggeri R, Bovicelli L et al. Testing normality of fetal DNA concentration in maternal plasma at 10–12 completed weeks' gestation: a preliminary approach to a new marker for genetic screening. *Prenat Diagn* 2002;22:148–52.
- [54] Chen CP, Chen SR, Wang W. Fetal DNA analyzed in plasma from a mother's three consecutive pregnancies to detect paternally inherited aneuploidy. *Clin Chem* 2001;47:937–9.
- [55] Larrabee PB, LeShane ES, Pestova E, Wilber K, Tantravahi U, Cowan JM et al. The prenatal molecular karyotype: DNA microarray analysis of cell-free fetal DNA (cffDNA) in amniotic fluid. *Am J Hum Genet* 2003; 73(Suppl):599.
- [56] Lo YM, Leung TN, Tein MS, Sargent IL, Zhang J, Lau TK et al. Quantitative abnormalities of fetal DNA in maternal serum in preeclampsia. *Clin Chem* 1999;45:184–8.
- [57] Holzgreve W, Ghezzi F, DiNaro E, Ganshirt D, Maymon E, Hahn S. Disturbed feto-maternal cell traffic in preeclampsia. *Obstet Gynecol* 1998;91:669–72.
- [58] Zhong XY, Laivuori H, Livingston JC, Ylikorkala O, Sibai BM, Holzgreve W et al. Elevation of both maternal and fetal extracellular circulating deoxyribonucleic acid concentrations in the plasma of pregnant women with preeclampsia. *Am J Obstet Gynecol* 2001; 184:414–9.
- [59] Swinkels DW, de Kok JB, Hendriks JC, Wiegerinck E, Zusterzeel PL, Steegers EA. Hemolysis, elevated liver enzymes, and low platelet count (HELLP) syndrome as a complication of preeclampsia in pregnant women increases the amount of cell-free fetal and maternal DNA in maternal plasma and serum. *Clin Chem* 2002;48:650–3.
- [60] Hahn S, Holzgreve W. Fetal cells and cell-free fetal DNA in maternal blood: new insight into pre-eclampsia. *Hum Reprod Update* 2002; 8:501–8.
- [61] Leung TN, Zhang J, Lau TK, Chan LY, Lo YM. Increased maternal plasma fetal DNA concentrations in women who eventually develop preeclampsia. *Clin Chem* 2001;47:137–9.
- [62] Levine RJ, Qian C, LeShane ES, Yu K, England L, Schisterman E et al. Two stage elevation of cell-free fetal DNA in maternal sera before onset of preeclampsia. *Am J Obstet Gynecol* 2002;187(Suppl):S55.
- [63] Bianchi DW. Fetal DNA in maternal plasma: the plot thickens and the placental barrier thins. *Am J Hum Genet* 1998;62:763–4.
- [64] Leung TN, Zhang J, Lau TK, Hjelm NM, Lo YMD. Maternal plasma fetal DNA as a marker for preterm labour. *Lancet* 1998;352:1904–5.
- [65] Sekizawa A, Jimbo M, Saito H, Iwasaki M, Sugito Y, Yukimoto Y et al. Increased cell-free fetal DNA in plasma of two women with invasive placenta. *Clin Chem* 2002;48:353–4.
- [66] Sekizawa A, Jimbo M, Saito H, Iwasaki M, Matsuoka R, Okai T et al. Cell-free fetal DNA in the plasma of pregnant women with severe fetal growth restriction. *Am J Obstet Gynecol* 2003;188:480–4.



- [67] Lo YMD, Hjelm NM, Fidler C, Sargent IL, Murphy MF, Chamberlain PF et al. Prenatal diagnosis of fetal RhD status by molecular analysis of maternal plasma. *N Engl J Med* 1998;339:1734–8.
- [68] Faas BHW, Beuling EA, Christiaens GCML, von dem Borne AEGKr, van der Schoot CE. Detection of fetal *RHD*-specific sequences in maternal plasma. *Lancet* 1998;352:1196.
- [69] Bischoff FZ, Nguyen DD, Marquéz-Do D, Moise KJ, Simpson JL, Elias S. Noninvasive determination of fetal RhD status using fetal DNA in maternal serum and PCR. *J Soc Gynecol Investig* 1999;6:64–9.
- [70] Zhong XY, Holzgreve W, Hahn S. Detection of fetal Rhesus D and sex using fetal DNA from maternal plasma by multiplex polymerase chain reaction. *BJOG* 2000;107:766–9.
- [71] Costa JM, Giovangrandi Y, Ernault P, Lohmann L, Nataf V, El Halali N et al. Fetal RhD genotyping in maternal serum during the first trimester of pregnancy. *Br J Haematol* 2002;119:255–60.
- [72] Turner MJ, Martin CM, O'Leary JJ. Detection of fetal Rhesus D gene in whole blood of women booking for routine antenatal care. *Eur J Obstet Gynecol Reprod Biol* 2003;108:29–32.
- [73] Finning KM, Martin PG, Soothill PW, Avent ND. Prediction of fetal D status from maternal plasma: introduction of a new noninvasive fetal RHD genotyping service. *Transfusion* 2002;42:1079–85.
- [74] Amicucci P, Gennarelli M, Novelli G, Dallapiccola B. Prenatal diagnosis of myotonic dystrophy using fetal DNA obtained from maternal plasma. *Clin Chem* 2000;46:301–2.
- [75] Saito H, Sekizawa A, Morimoto T, Suzuki M, Yanaihara T. Prenatal DNA diagnosis of a single-gene disorder from maternal plasma. *Lancet* 2000;356:1170.
- [76] González-González MC, García-Hoyos M, Trujillo MJ, Rodríguez de Alba M, Sánchez IL, Díaz-Rescasens J et al. Prenatal detection of a cystic fibrosis mutation in fetal DNA from maternal plasma. *Prenat Diagn* 2002;22:946–8.
- [77] Chiu RW, Lau TK, Cheung PT, Gong ZQ, Leung TN, Lo YM. Noninvasive prenatal exclusion of congenital adrenal hyperplasia by maternal plasma analysis: a feasibility study. *Clin Chem* 2002;48:778–80.
- [78] Chiu RW, Lau TK, Leung TN, Chow KC, Chui DH, Lo YM. Prenatal exclusion of beta thalassaemia major by examination of maternal plasma. *Lancet* 2002;360:998–1000.
- [79] Poon LL, Leung TN, Lau TK, Lo YM. Presence of fetal RNA in maternal plasma. *Clin Chem* 2000;46:1832–4.
- [80] Tsui NB, Ng EKO, Lo YM. Stability of endogenous and added RNA in blood specimens, serum, and plasma. *Clin Chem* 2002;48:1647–53.