**Human Reproduction Update, Vol.11, No.1 pp. 59–67, 2005** doi:10.1093/humupd/dmh053
Advance Access publication November 29, 2004.

# Cell-free fetal DNA in maternal blood: kinetics, source and structure

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**The kinetics and structure of cell-free fetal DNA in maternal plasma is currently under investigation. Plasma fetal DNA seems quite stable albeit cleared rapidly following birth, suggesting continuous fetal DNA release into the maternal circulation during pregnancy. However, to understand better the kinetics of circulating DNA, studies to determine the biological (structural) form in which fetal and maternal DNA exist and the mechanisms underlying variation in plasma are warranted to ensure quantitative diagnostic reliability. It is likely that circulating fetal DNA is released from fetal and/or placental cells undergoing apoptosis. Thus, the majority of fetal DNA is proposed to circulate in membrane-bound vesicles (apoptotic bodies). This review summarizes the latest reports in this field.**

*Key words*: apoptosis/apoptotic bodies/fetal DNA in maternal plasma/non-invasive prenatal diagnosis/quantitative PCR

## Introduction

Prenatal genetic diagnosis has traditionally required invasive procedures such as amniocentesis or chorionic villous sampling (CVS); however, each carries a small but finite risk of fetal loss and injury. Maternal serum analyte screening and ultrasound can identify individuals at risk for fetal aneuploidy (predominantly trisomy 21), but like other non-invasive screening methods, they are hampered by non-optimal sensitivities and high false-positive (procedure) rates. For several years, we and others have focused on the isolation of intact fetal cells from maternal blood, a non-invasive method that can yield definitive results. Universal presence of fetal cells in maternal blood is now accepted, but their occurrence is rare and requires complex enrichment and identification strategies. There exist one to six fetal cells per millilitre of blood from normal pregnant women (Hamada *et al.*, 1993; Krabchi *et al.*, 2001). The largest study concerning efficacy is the multicentre National Institute of Child Health and Development (NICHD) fetal cell study group in which we and other groups collaborated. Of 2744 maternal samples (Bianchi *et al.*, 2002), fetal male cells were correctly identified in 41.4% when the fetus was euploid ($n = 1292$). Among confirmed aneuploid cases, the detection rate was higher: 74.4%. Although further improvement of existing enrichment and isolation protocols is warranted, progress remains hampered by both rarity of fetal cells and the lack of fetal-specific cell markers. More recently, we and other researchers have also focused on non-cellular fetal DNA in the plasma fraction of maternal blood for quantification and analysis of locus-specific sequences. In this review we emphasize advancements related to evaluation of the dynamic changes and biological nature of circulating nucleic acids in maternal plasma.

### Existence of circulating nucleic acids in plasma of cancer patients

Nucleic acids (DNA and RNA) in plasma were first observed >50 years ago. In the early 1970s increased quantities of DNA were verified in the plasma of cancer patients (Leon *et al.*, 1977). In the late 1980s and 1990s several groups demonstrated that plasma DNA derived from cancer patients displayed tumour-specific characteristics, including decreased strand stability, *Ras* and *p53* mutations, mircrosatellite alterations, abnormal promoter hypermethylation of selected genes, mitochondrial DNA mutations and tumour-related viral DNA (Stroun *et al.*, 1989; Sorenson *et al.*, 1994; Vasioukhin *et al.*, 1994; Chen *et al.*, 1996; Nawroz *et al.*, 1996; Anker *et al.*, 1999; Chan *et al.*, 2002). Tumour-specific DNA for a wide range of malignancies has been found: haematological, colorectal, pancreatic, skin, head-and-neck, lung, breast, kidney, ovarian, nasopharyngeal, liver, bladder, gastric, prostate and cervix. In aggregate, the above data show that tumour-derived DNA in plasma is ubiquitous in affected patients, and likely the result of a common biological process such as apoptosis. Investigations into the size of

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these plasma DNA fragments from cancer patients has revealed that the majority show lengths in multiples of nucleosomal DNA, a characteristic of apoptotic DNA fragmentation (Giacona *et al.*, 1998; Jahr *et al.*, 2001).

If a cancer shows specific viral DNA sequences or tumour suppressor and/or oncogene mutant sequences, PCR-specific strategies can be developed. However, for most cancers (and most Mendelian disorders), clinical application awaits optimization of methods to isolate, quantify and characterize the tumour-specific DNA compared to the patient's normal DNA, which is also present in plasma. Therefore, understanding the molecular structure and dynamics of DNA in plasma of normal individuals will be necessary to achieve further advancement in this field.

## Presence of cell-free fetal nuceic acids in maternal blood

### *Circulating cell-free fetal DNA in maternal plasma and serum*

As studies of tumour-derived DNA detection in plasma of cancer patients were being pursued, Lo *et al.* (1997) demonstrated fetal DNA in plasma and serum from healthy pregnant women. Using quantitative real-time PCR, surprisingly high mean concentrations (6.2% of total plasma DNA) of fetal DNA were found in maternal plasma in early and late pregnancy (Lo *et al.*, 1998b). In plasma, fetal DNA reached a mean of 25.4 genome equivalents (GEq)/ml (range 3.3–69.4) in early pregnancy and 292.2 GEq/ml (range 76.9–769) in late pregnancy. Mean concentration was less (3.4% of total serum DNA) but still substantive in maternal serum. As assessed by the numbers of copies of SRY (a single-copy Y chromosome-specific sequence), the ratio of fetal to maternal DNA was 775–970-fold greater in the plasma than amount of DNA derived from intact fetal cells would indicate.

Interestingly, Jimenez *et al.* (2003) demonstrated detection of fetal DNA in the maternal serum of rhesus monkeys with apparently similar kinetics related to gestational age and postnatal clearance characteristics observed in humans. The availability of such an animal model system should prove very useful in addressing further questions relating to origin, mechanism and nature of fetal DNA in maternal circulation.

### *Fetal/placental-derived RNA in maternal plasma*

In plasma from cancer patients, RNA is also shown to be present (Kopreski *et al.*, 1999; Lo *et al.*, 1999a; Chen *et al.*, 2000; Silva *et al.*, 2001). These molecules are likely packaged in apoptotic bodies and, hence, rendered more stable compared to 'free RNA' (Hasselmann *et al.*, 2001; Anker *et al.*, 2002; Tsui *et al.*, 2002; Ng *et al.*, 2003). Thus, it is not surprising that stable fetal RNA is also present in maternal plasma (Poon *et al.*, 2000b). Ng *et al.* (2003a) detected maternal plasma mRNA transcripts exclusively expressed from the placenta. Two placenta-expressed genes (human placental lactogen, hPL; β subunit of hCG) were detected in each of 10 maternal samples. This study provides direct evidence that RNA is stable in whole blood prior to processing and clearance following delivery. Investigations involving another plasma-expressed gene, corticotrophin-releasing hormone (CRH), have shown increased CRH mRNA in plasma of women with pre-eclampsia ($n = 12$; mean 1070 copies/ml) compared with normal gestational age-matched controls ($n = 10$; 102 copies/ml) (Ng *et al.*, 2003).

## Temporal changes in circulating cell-free DNA

### *Total DNA*

Given establishment of fetal DNA in maternal plasma, attention turned to kinetics of the phenomenon. The concentration of total DNA in plasma of healthy (non-pregnant) adults is in the range of 10–100 ng or $10^3$–$10^4$ GEq/ml Wu *et al.* (2002). Total plasma DNA (fetal and maternal) levels are significantly higher during pregnancy (Lo *et al.*, 1998b; Lo, 2000) as well as cancer (Wu *et al.*, 2002; Taback *et al.*, 2004). The explanation of increased concentration of total DNA during pregnancy is unclear. DNA from fetal sources comprises only a small portion (5–7%) of total circulating DNA. Moreover, fetal DNA in maternal plasma seems quite stable (Angert *et al.*, 2003) albeit cleared at an extremely rapid rate following birth (Lo *et al.*, 1999d). Thus, a continuous supply of fetal DNA is emitted into the maternal circulation. Such findings suggest that during pregnancy, cellular turnover of maternal cells is enhanced. Although such quantitative changes may serve as a means for distinguishing between euploid and abnormal pregnancies, clinical application will require a better understanding of the cause associated with variability among normal specimens.

### *Fetal DNA*

As expected, detection of relatively low levels of fetal DNA sequences (as compared to maternal DNA levels) is dependent on the sensitivity of the assay as well as the amount of target fetal sequences. Several reports have confirmed that gestational age correlates positively with amount of fetal DNA in plasma; thus, higher detection rates are reported with increased gestation. Lo *et al.* (1998b) reported fetal concentrations to be low in the first trimester, rising in the second and third trimester. Ariga *et al.* (2001) combined real-time kinetic PCR with liquid oligomer hybridization with $^{32}$P-labelled probes to quantify Y chromosome-specific sequences throughout pregnancy. In 20 women confirmed to have a male fetus and followed from the first to third trimester, fetal DNA concentrations increased from 10.1 to 130.5 copies per 0.5 ml maternal plasma. Rijnders *et al.* (2003) studied pregnant women after assisted reproduction and reported detection of fetal DNA as early as 5 weeks and 2 days gestation in one of two patients; however, detection reached 100% by 9 weeks gestation. It is likely that earlier studies (i.e. Thomas *et al.*, 1995) demonstrating 100% Y-sequence detection using maternal whole blood between 4 and 7 weeks were actually measuring cell-free fetal DNA and not intact cells. Although there appears to be considerable variability among subjects in the quantity and timing of fetal DNA's initial presence in maternal circulation, the overall trend of increased quantity of this DNA with increasing gestational age is consistent.

During the last 8 weeks of pregnancy there is a sharp increase of fetal DNA in maternal plasma (Lo *et al.*, 1998). This might be related to gradual breakdown of the maternal–fetal interface/placental barrier (Bianchi, 2000). To address this, Chan *et al.* (2003) performed serial analysis of fetal DNA concentrations in late pregnancy, showing a positive correlation with gestational



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age in the third trimester. During the late third trimester, they observed a mean increase of 29.3% of fetal DNA each week. Thus, they provide normative values for comparative studies involving pregnancy related pathological conditions such as preterm labour and pre-eclampsia.

Ohashi *et al.* (2002) report a correlation of fetal DNA and hCG concentrations using second trimester maternal serum samples. Farina *et al.* (2002) demonstrated a significant correlation between early gestational age (10–12 weeks) and total fetal DNA concentrations among 63 euploid pregnancies that was normally distributed. In addition, several studies to assess the sensitivity and specificity of fetal DNA in first and second trimester maternal plasma have also been reported, with relatively large sample sizes (Costa *et al.*, 2001; Sekizawa *et al.*, 2001; Zhong *et al.*, 2001a; Honda *et al.*, 2002). Real-time quantitative PCR was employed based on Y-specific sequences in pregnancies carrying male fetuses. Overall, 95–100% sensitivity with 100% specificity was observed. Sensitivity of detection in first trimester (7–12 weeks) euploid samples was less efficient (70–95%), likely reflecting lower amounts of fetal DNA.

## In what form does fetal DNA exist? Apoptotic bodies as likely vehicle

### *Apoptosis as ubiquitous source of circulating DNA*

Three sources of circulating DNA can be plausibly hypothesized: (i) dying cells (necrotic or apoptotic); (ii) active secretion of DNA; (iii) terminal differentiation. Apoptosis (programmed cell death) is the most common form of cell death, continuing through life from early stages of embryogenesis to death. Because $10^{11}$–$10^{12}$ cells divide daily and the same amount should be lost to maintain tissue homeostasis, ~1–10 g of DNA can be expected to be degraded each day in the human (Rudin *et al.*, 1997). Given the high turnover, it is not surprising that some DNA escapes final cleavage/degradation and thus appears in the plasma. The concentration of DNA in plasma is in the range of 10–100 ng or $10^3$–$10^4$ GE/ml (Jen *et al.*, 2000a; Wu *et al.*, 2002). During pregnancy this DNA could be fetal and/or placental in origin.

### *Apoptosis giving rise to fetal DNA: distinguishable size fragments*

The biochemical hallmark of apoptosis is fragmentation of genomic DNA, an irreversible event that commits the cell to die. This occurs before changes in plasma membrane permeability (prelytic DNA fragmentation) (Barrett *et al.*, 2001; Martelli *et al.*, 2001). In many systems, this DNA fragmentation has been shown to result from activation of endogenous $Ca^{2+}$- and $Mg^{2+}$-dependent nuclear endonuclease. This enzyme selectively cleaves DNA at sites located between nucleosomal units (linker DNA) generating mono- and oligonucleosomal DNA fragments. The process of DNA cleavage is not random but likely under regulatory control. Thus, certain DNA sequences are relatively more susceptible to cleavage, generating predictable size fragments. Given that the normal mechanisms of apoptosis may be saturated during pregnancy or that an entirely different mechanism of apoptosis is involved, fetal and/or placental cells undergoing apoptosis may contain DNA that is distinguishable from maternal DNA in plasma based on molecular characteristics. Indeed, a recent report by Chan *et al.* (2004) investigated the size distribution of plasma DNA among pregnant and non-pregnant women. They have shown that plasma DNA (based on amplicon sizes of the leptin gene) in maternal samples is significantly longer than DNA in plasma of non-pregnant women. In addition, among maternal samples, maternal-derived DNA (based on leptin gene sequences) was longer than fetal-derived (based on SRY) DNA. In their study of 31 pregnant women, median percentage of plasma DNA with size >201 bp was 57% compared to 14% among non-pregnant women ($n = 34$). Of the fetal-derived DNA, 20% displayed sizes >193 bp but not exceeding 313 bp. These studies support the hypothesis that fetal DNA may be distinguishable from maternal DNA, enabling development of fetal DNA enrichment strategies.

### *Fetal DNA likely exists in the form of apoptotic bodies*

Irrespective of the source, various forms of circulating DNA in plasma can be suggested: shed cells, apoptotic bodies, nucleosomes, other nucleoproteins, and free DNA. Halicka *et al.* (2000) described formation of apoptotic bodies during apoptosis of different cell types induced by various treatments. These authors reported that apoptosis is not a random or chaotic process but under regulatory control. Thus, formation of apoptotic bodies is a specific stage of programmed cell death. Apoptotic bodies usually contain either DNA or RNA, but not both (Halicka *et al.*, 2000). Evidence that apoptotic bodies are the vehicle for cell-free DNA in plasma has been provided by detection of stabilized circulating RNA in patients with malignant melanoma (Kopreski *et al.*, 1999) and breast cancer (Chen *et al.*, 2000). The same holds for fetal DNA in normal pregnant women (Tsui *et al.*, 2002). Given that plasma is rich in RNase activity and that RNA is highly sensitive to nuclease attack, detection of stable RNA further suggests that this nucleic acid is protected likely in vesicles or apoptotic bodies.

### *Evidence that apoptotic bodies contain fetal DNA: Baylor results*

Although nucleosomes have been observed and quantified in plasma of cancer patients, little is known of the basic properties of apoptotic bodies which are known to contain nucleosomes. It is unclear how apoptotic bodies are distributed during blood fractionation into cellular and plasma fractions by centrifugation or ultrafiltration. Because we have such compelling evidence for the presence and utility of fetal DNA in maternal circulation for clinical diagnostic application, a better understanding of the nature of this potential source of fetal genetic material is warranted. To address some of these issues, we performed studies that enable more efficient recovery and concentration of circulating DNA from maternal plasma.

#### *Transmission electron microscopy*

Most isolation protocols target a particular form of DNA; thus, standard approaches will inevitably result in loss of non-targeted DNA (fetal and/or maternal). Thus, to maximize recovery of all possible forms of fetal DNA, we have used the Microcon centrifugal filter device (Millipore, USA). These filter units employ a low-binding, anisotropic, hydrophilic regenerated cellulose membrane that allows high yield recovery rates (>95% of the sample) with concentration factors as high as 100-fold. Using



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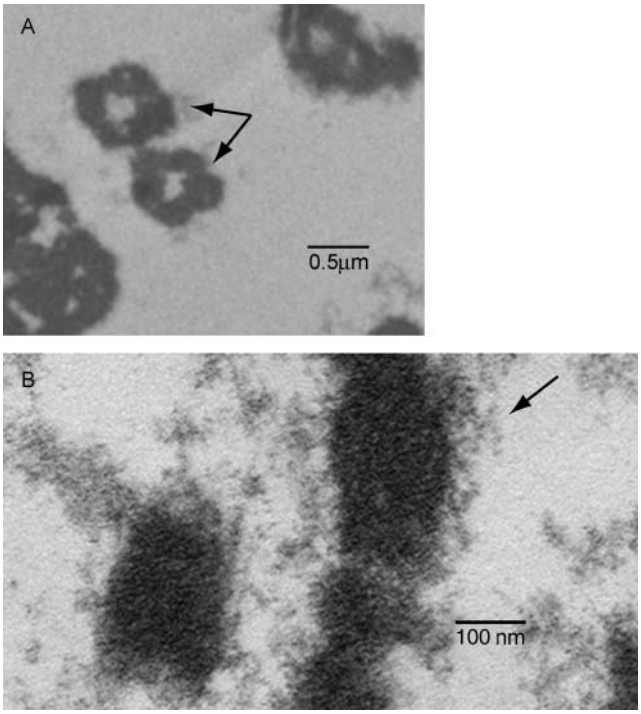


**Figure 1.** Transmission electron microscopic analysis of maternal plasma pellets. Images display electron micrographs of maternal plasma pellets at two magnifications. (**A**) Arrows identify the presence of nucleosomes among various structures that are likely ruptured vesicles (apoptotic bodies). (**B**) Higher magnification illustrates presence of chromatin (arrow).

these filter units enables collection of all nucleic acids $>10$ bp in size in a plasma sample. By concentrating the plasma DNA content, suitable size plasma pellets were prepared and subjected to electron microscopic analysis that demonstrated for the first time presence of substantial quantities of nucleosomes in plasma of pregnant women. In Figure 1, the images display transmission electron micrographs of maternal plasma pellets at two magnifications. In Figure 1A, arrows identify the presence of small spherical bodies that appear quite symmetrical among various other structures that are likely ruptured vesicles, such as apoptotic bodies. These structures are nucleosomes (DNA bound to histones), which had likely been engulfed by apoptotic bodies. Because the initial step in our plasma concentration protocol involves a 16 000 *g* centrifugation to force the suspension through the filter, it is not surprising that such membrane-bound components would rupture and release their components in the plasma pellet. At higher magnification (Figure 1B), these structures clearly contain and are attached to chromatin (arrow). This chromatin is abundant, with varying size and level of condensation, consistent with an apoptotic pathway. This finding supports the hypothesis that fetal DNA may exist in distinct forms and thus be subject to enrichment.

*Real-time quantitative PCR*

In a preliminary study, maternal plasma ($n = 28$; 15 male, 13 female) was separated by centrifugation (800 *g*) from 12–16 week gestations. Acridine Orange (AO), a nucleic acid stain, was used to label recovered plasma, followed by flow cytometric separation of the AO-positive non-cellular fraction. Sorted plasma was analysed by real-time PCR to quantify DYS1 (fetal) and GAPDH sequences. Microscopic analysis revealed the presence of apoptotic bodies and nucleosomes in plasma following separation. Real-time PCR quantification demonstrated significant enrichment with mean detection of 28% fetal sequences in sorted compared to 2.8% in non-sorted specimens. Thus, based on these observations, fetal DNA enrichment is feasible and can enhance fetal sequence detection.

## Technical advances in assaying cell-free fetal DNA

***Recovery of plasma DNA***

More efficient or selective methods of plasma fetal DNA isolation should improve fetal DNA sequence detection in first trimester cases. The amount of DNA isolated from plasma is dependent on the specific method of DNA isolation employed (Houfflin-Debarge *et al.*, 2000; Jen *et al.*, 2000b). Centrifugation speed is critical for blood separation and recovery of fetal DNA from plasma (Lui *et al.*, 2002). Chiu *et al.* (2001) showed that inefficient processing could lead to residual cells in the plasma and interfere with accuracy in quantification of fetal sequences. This likely explains the discrepancies between groups reporting on whether intact fetal cells are actually present in maternal plasma (Poon *et al.*, 2000a; Bayrak-Toydemir *et al.*, 2003; Bischoff *et al.*, 2003b). Because intact fetal cells in maternal plasma are likely apoptotic (Kolialexi *et al.*, 2001), their localization to the plasma fraction after centrifugation is likely the indirect result of these cells floating from the mononuclear cell layer due to reduced cellular density.

To address some of the obvious variables associated with plasma DNA isolation and utilization, the five participating centres of the NICHD fetal cell study group developed a standard protocol for inter-laboratory comparison of fetal DNA detection (Johnson *et al.*, 2004). This collaborative effort examined variables impacting data quality, and determined sensitivity and specificity for detection of the Y chromosome. These studies conclusively showed not only that fetal DNA, as judged by the Y chromosome, is consistently present in maternal plasma, but defined the range of fetal DNA during the weeks of gestation relevant for prenatal diagnosis. Overall results among the five centres were based on matched maternal plasma specimens (total of 35 known male and 28 known female fetuses). Efficiency of amplification of known quantities of standard DNA was consistent between all centers. Surprisingly, we found that detecting the fetal allele, and thus sensitivity and negative predictive power, was disproportionately impacted by total DNA recovery. The quantity of male DNA (SRY sequences) amplified from maternal plasma when the fetus was male ranged from 51 to 228 GEq/ml, whereas control DNA (GAPDH sequences) ranged from 5939 to 12 397 GEq/ml in these cases. Sensitivity among centres varied from 31.4 to 97.1% with specificity of 92.8 to 100%. Given that the amount of DNA recovered correlates positively with sensitivity, variable sensitivities are predictable. This result puts a premium on efficient DNA purification.

***Sampling of isolated plasma DNA***

The quantity of background (circulating maternal) DNA can influence the detection rate of low copy number sequences



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(Stenman *et al.*, 2001). When fetal DNA is present in very low copy numbers (e.g. 1–3 GEq/ml), real-time PCR is likely at the limit of its assay sensitivity. Thus, vicissitudes of sampling affect detection rate and quantitative measurements. Detection of the target DNA may therefore be influenced not only by the amount of material used per test but by the number of fractions tested. As a result, an increased number of replicates is likely necessary to ensure reliable results (Hromadnikova *et al.*, 2003).

#### *Stabilization and transportability of specimens prior to DNA isolation*

It is not surprising that intact cell lysis due to time delay prior to blood processing or treatment conditions (i.e. centrifugation speed) would result in increased concentrations of maternal background DNA, thus causing dilution of relatively low levels of circulating fetal DNA (Dukes *et al.*, 2004). Dhallan *et al.* (2004) propose treatment of blood samples with formaldehyde. This seems plausible in preventing lysis of intact cells (maternal and fetal). Data support this hypothesis with increased percentage of fetal DNA detected in seven of 10 matched formaldehyde-treated (mean 20.2% fetal) versus untreated (mean 7.7% fetal) cases. If fetal DNA exists in the form of apoptotic bodies, then treatment of maternal blood samples with formaldehyde is also likely to assist in the stabilization of this DNA. Formaldehyde-treated plasma DNA may be more resilient to the adverse affects caused by delay in processing time, centrifugation speeds, and storage conditions (i.e. −80 versus −20°C temperature) prior to DNA extraction. Lam *et al.* (2004) also report improved recovery of plasma DNA and analysis when blood is collected in EDTA rather than heparin or citrate if processing is to be delayed by >6 h after blood draw.

Transportability of specimens soon after venipuncture has been considered. This potential problem is likely to be obviated by technical advances as well. For example, our group reported success in detection of fetal sequences using dried maternal blood spots and real-time PCR (Bischoff *et al.*, 2003a). Fetal Y-specific (DYS1) sequences were detected in all 19 (100%; 4.20–24.68 GEq/ml of blood) maternal blood specimens from women carrying male fetuses. Feasibility of detecting fetal sequences in maternal blood spots dried onto filter paper allows for more efficient collection and transport of specimens, thus enabling cell-free DNA to be incorporated into non-invasive screening regimes on a wide scale.

## Cell-free fetal DNA for detecting aneuploidy

#### *Trisomies 13, 18 and 21*

Cell-free fetal DNA can play a role in non-invasive prenatal genetic diagnosis of aneuploidy by helping to identify pregnancies at sufficient risk for trisomy 21 or 18 such that an invasive diagnostic procedure should be offered for definitive diagnosis. The fetal DNA is likely derived from genes located throughout the fetal genome, not just fetal chromosome 21. Using real-time PCR to quantify Y-specific sequences, Lo *et al.* (1999b) demonstrated a 2-fold increase in fetal DNA levels for trisomy 21, compared to euploid cases. Subsequent studies have supported these observations on trisomy 21, although increase is not observed in trisomy 18 (Zhong *et al.*, 2000a). This suggests that different fetal growth and placental pathologies may result in different levels of fetal DNA.

Interestingly, Hromadnikova *et al.* (2002) were not able to detect increased fetal DNA levels or differences in the fetal: maternal DNA ratio in maternal plasma of patients with affected trisomy 21 fetuses compared to normal controls. Although Spencer *et al.* (2003) could not detect increased levels of fetal cell-free DNA in serum of ten trisomy 21-affected women, total DNA (fetal and maternal) was observed to be increased. Based on real-time quantification of ubiquitous albumin gene sequences, the median level of total DNA was significantly greater in women with trisomy 21 (36 152 GEq/ml) compared to controls (5832 GEq/ml). Given that maternal cell lysis increases the overall quantity of DNA in serum, analysis using maternal serum rather than plasma in this study likely explains the inability to detect increased fetal DNA levels in trisomy 21.

Wataganara *et al.* (2003) found maternal serum fetal DNA levels to be elevated in cases of trisomy 13 (29.2–187.0 GEq/ml) but not trisomy 18 (18.6–77.6 GEq/ml). The level of fetal DNA detected was quite variable in the two groups as well as in controls (3.7–127.4 GEq/ml). Archival maternal serum samples displayed a 1.8-fold increase in the amount of fetal DNA levels in trisomy positive pregnancies compared to gestational age-matched controls.

Although methods to improve consistency of fetal DNA quantities are likely to be achieved with more efficient plasma/serum DNA isolation, these findings point to the possible use of either fetal DNA or total DNA (fetal and maternal) as an additional aneuploidy screening analyte. Farina *et al.* (2003) evaluated the use of circulating fetal DNA as a second trimester maternal serum marker of Down's syndrome. The median fetal DNA concentration was 1.7-fold greater in Down's syndrome cases than in controls. Used singularly as a non-invasive marker for Down's syndrome, fetal DNA gives a 21% detection rate with a set 5% false-positive rate. If combined with the quadruple marker screen, non-invasive aneuploidy detection would improve to 86% at a fixed 5% false-positive rate.

## Cell-free fetal DNA to identify pregnancy complications

#### *Increased fetal DNA concentrations*

Circulating fetal DNA has also been targeted as a marker for assessing feto-maternal well-being. Increased fetal DNA concentrations have been reported for several pregnancy-related complications (Table I). The best studied is pre-eclampsia,

**Table I.** Pregnancy-related disorders shown to be associated with increased fetal DNA concentrations in maternal plasma

| Pregnancy-related disorders | References |
|---|---|
| Pre-eclampsia | Lo *et al.*, 1999; Zhong *et al.*, 2001 |
| Preterm labour | Leung *et al.*, 1998 |
| Invasive placentation | Sekizawa *et al.*, 2002 |
| Hyperemesis gravidarum | Sekizawa *et al.*, 2001; Sugito *et al.*, 2003 |
| Intrauterine growth restriction (IUGR) | Caramelli *et al.*, 2003 |
| Feto-maternal haemorrhage | Lau *et al.*, 2000 |
| Polyhydramnious | Zhong *et al.*, 2000 |



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a multisystem disorder characterized by hypertension (high blood pressure) and proteinuria (the presence of protein in urine). An example of a pedestrian complication of pregnancy, pre-eclampsia, has long been a leading cause of maternal mortality in the USA and UK. Although well-established diagnostic criteria exist (Roberts *et al.*, 1993; Redman *et al.*, 1999), these are specific to onset of clinical symptoms relatively late in pregnancy. In pre-eclampsia, placentas show failure of trophoblast cells to invade and remodel the maternal environment (Redman *et al.*, 2001). Failure to remodel the maternal spiral arteries restricts the flow of blood to the fetus and thus contributes to onset of pre-eclampsia. Among possible explanations for the failure of trophoblasts to function properly, the most commonly speculated is an increased rate of apoptosis. Alternatively, motility and/or invasiveness of trophoblasts may be altered. Irrespective, there is growing evidence to support increased placental apoptosis, although the underlying biological basis remains unclear.

In pre-eclampsia, a significant increase in the number of copies of fetal DNA (Y-sequences) was observed at the time the disease was manifested (Lo *et al.*, 1999c; Leung *et al.*, 2001; Zhong *et al.*, 2001b, 2002b). Elevated concentrations of fetal DNA in plasma of pre-eclamptic women has been shown to appear prior to the onset of clinical symptoms as well as correlate with severity of this pathological condition (Leung *et al.*, 2001; Swinkels *et al.*, 2002; Zhong *et al.*, 2002b). Interestingly, Byrne *et al.* (2003) reported that fetal DNA concentrations were not correlated with severity of pre-eclampsia when whole blood analysis was performed. This further suggests that cell-free fetal DNA in plasma of these patients is derived from placental or fetal cells undergoing apoptosis rather than from intact circulating fetal cells. Such findings are consistent with previous reports demonstrating a lack of correlation between circulating intact fetal cells and cell-free fetal DNA (Bischoff *et al.*, 2002; Zhong *et al.*, 2002a).

### *Impaired clearance of fetal DNA*

Fetal DNA clearance is also impaired in pre-clampsia (Lau *et al.*, 2002). That abnormal clearance has not been observed in trisomy 21 suggests that the underlying mechanism and/or form of fetal DNA in maternal plasma may differ between the two conditions. Some investigators hypothesize that the increase of circulating fetal DNA in pre-eclampsia may be due to some form of cell injury and placental breakdown with release of cellular 'micro-debris' into the circulation (Holdenrieder *et al.*, 2001; Reister *et al.*, 2001 Stroun *et al.*, 2000). We speculate whether these micro-debris particles are likely apoptotic bodies or nucleosomes. More recently, Levine *et al.* (2004) reported that fetal DNA in cases of pre-eclampsia is elevated in two stages, at 17–28 weeks (36 GEq/ml) and then 29–41 weeks (176 GEq/ml) as compared to normal controls (16 and 75 GEq/ml respectively), reflecting placental necrosis and apoptosis in the first stage and impaired DNA clearance in the second.

### *Detection of paternally inherited alleles using maternal plasma*

#### *Single gene sequences*

Initial studies characterizing fetal DNA in maternal circulation used PCR to detect Y-chromosome sequences corresponding to male DNA, presumably derived from the fetus. Because of high amounts of maternal (background) DNA, approaches have focused on cases where the allele of interest would not be present in the maternal genome (e.g. paternal inheritance of a dominant disease). PCR-based assays for fetal DNA have been developed for a number of single gene disorders. In particular, Rh determination during pregnancies in which a rhesus-negative mother is at risk for a rhesus-positive child has been validated (Faas *et al.*, 1998; Bischoff *et al.*, 1999). In our retrospective study of 20 frozen serum samples from sensitized RhD-negative pregnant women confirmed to have RhD-positive fetuses, we demonstrated positive fetal RhD detection in 70% of cases using conventional fluorescent PCR for simultaneous amplification of the RhD and RhCE (control) genes (Bischoff *et al.*, 1999). Failure to detect RhD sequences in 100% of cases was most likely due to either analysis using serum samples (lysis of maternal cells further dilutes fetal DNA), DNA degradation as a result of freezing and thawing serum specimens or inefficient DNA isolation. Using improved methods of plasma DNA isolation and more sensitive sequence detection assays, namely real-time PCR, highly accurate (98–100%) fetal RhD genotyping can be achieved (Lo *et al.*, 1998a; Zhong *et al.*, 2000b; Finning *et al.*, 2002). Indeed, the high detection and accuracy rates have resulted in the implementation of this non-invasive test at the International Blood Group Reference Laboratory (IBGRL) (Finning *et al.*, 2002). Several other single gene disorders have also been tested for feasibility in detection or exclusion of an affected fetus based on DNA sequences isolated from maternal plasma. Table II summarizes these disorders; however, each reflects analysis of only one or very few cases.

#### *Epigenetic modifications*

Inability of PCR to distinguish readily between maternally inherited fetal DNA and native maternal DNA is clearly a diagnostic impediment. A comparable equivalent to Y-specific DNA that could serve as a facile internal control to verify presence of fetal DNA in the sample being assessed is lacking. However, studies of epigenetic changes resulting in distinguishable patterns of DNA methylation between fetal and maternal DNA provide promise in the development of unique gene or global fetal-specific sequence detection assays (Poon *et al.*, 2002). Similarly, placental-specific transcripts in maternal plasma provide an alternative approach for quantification assays for detection of fetal trisomies or pregnancy complications.

## Conclusion

Overall, apoptosis seems to be the most plausible source of fetal DNA in maternal circulation, accounting for quantitative

**Table II.** Single gene mutations detected using cell-free fetal DNA in maternal plasma

| Single gene disorder | References |
|---|---|
| Achondroplasia | Saito *et al.*, 2000 |
| Myotonic dystrophy | Amicucci *et al.*, 2000 |
| Congenital adrenal hyperplasia | Chiu *et al.*, 2002b |
| Beta-thalassaemia | Chiu *et al.*, 2002a,c |
| Cystic fibrosis | Gonzalez-Gonzalez *et al.*, 2002 |
| Huntington disease | Gonzalez-Gonzalez *et al.*, 2003a,b |



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increase of this DNA with gestational age and rapid clearance following delivery. Irrespective of the source, various forms of circulating DNA in plasma can be suggested: shed cells, apoptotic bodies, nucleosomes, other nucleoproteins, and free DNA. Thus, an important goal in this field should be to identify the predominant form of fetal DNA in maternal plasma among normal pregnancies. Identification of the forms of circulating fetal DNA can lead to selection or creation of more effective methods of enrichment and DNA isolation from plasma for improved clinical applications.

## Acknowledgements

We thank Dr William Brinkley for expertise in transmission election microscopy. We also thank Dianne Dang and Belinda Felder for assistance in manuscript preparation. This work was supported in part by NIH/NICHD contract N01-HD-43203.

## References

Amicucci P, Gennarelli M, Novelli G and Dallapiccola B (2000) Prenatal diagnosis of myotonic dystrophy using fetal DNA obtained from maternal plasma. Clin Chem 46,301–302.

Angert RM, LeShane ES, Lo YM, Chan LY, Delli-Bovi LC and Bianchi DW (2003) Fetal cell-free plasma DNA concentrations in maternal blood are stable 24 hours after collection: analysis of first- and third-trimester samples. Clin Chem 49,195–198.

Anker P and Stroun M (2002) Progress in the knowledge of circulating nucleic acids: plasma RNA is particle-associated. Can it become a general detection marker for a cancer blood test? Clin Chem 48, 1210–1211.

Anker P, Mulcahy H, Chen XQ and Stroun M (1999) Detection of circulating tumour DNA in the blood (plasma/serum) of cancer patients. Cancer Metastasis Rev 18,65–73.

Ariga H, Ohto H, Busch MP, Imamura S, Watson R, Reed W and Lee TH (2001) Kinetics of fetal cellular and cell-free DNA in the maternal circulation during and after pregnancy: implications for noninvasive prenatal diagnosis. Transfusion 41,1524–1530.

Barrett KL, Willingham JM, Garvin AJ and Willingham MC (2001) Advances in cytochemical methods for detection of apoptosis. J Histochem Cytochem 49,821–832.

Bayrak-Toydemir P, Pergament E and Fiddler M (2003) Are fetal cells in maternal plasma really there? We think they are. J Hum Genet 48, 665–667.

Bianchi DW (2000) Fetal cells in the mother: from genetic diagnosis to diseases associated with fetal cell microchimerism. Eur J Obstet Gynecol Reprod Biol 92,103–108.

Bianchi DW, Simpson JL, Jackson LG, Elias S, Holzgreve W, Evans MI, Dukes KA, Sullivan LM, Klinger KW, Bischoff FZ et al. (2002) Fetal gender and aneuploidy detection using fetal cells in maternal blood: analysis of NIFTY I data National Institute of Child Health and Development Fetal Cell Isolation Study. Prenat Diagn 22,609–615.

Bischoff FZ, Nguyen DD, Marquez-Do D, Moise KJ, Jr, Simpson JL and Elias S (1999) Noninvasive determination of fetal RhD status using fetal DNA in maternal serum and PCR. J Soc Gynecol Invest 6,64–69.

Bischoff FZ, Sinacori MK, Dang DD, Marquez-Do D, Horne C, Lewis DE and Simpson JL (2002) Cell-free fetal DNA and intact fetal cells in maternal blood circulation: implications for first and second trimester non-invasive prenatal diagnosis. Hum Reprod Update 8,493–500.

Bischoff FZ, Dang DX, Marquez-Do D, Martinez D, Horne C, Lewis DE and Simpson JL (2003a) Detecting fetal DNA from dried maternal blood spots: another step towards broad scale non-invasive prenatal genetic screening and feasible testing. Reprod Biomed Online 6,349–351.

Bischoff FZ, Hahn S, Johnson KL, Simpson JL, Bianchi DW, Lewis DE, Weber WD, Klinger K, Elias S, Jackson LG, Evans MI, Holzgreve W and de la CF (2003b) Intact fetal cells in maternal plasma: are they really there? Lancet 361,139–140.

Byrne BM, Crowley A, Taulo F, Anthony J, O'Leary JJ and O'Herlihy C (2003) Fetal DNA quantitation in peripheral blood is not useful as a marker of disease severity in women with preeclampsia. Hypertens Pregn 22,157–164.

Caramelli E, Rizzo N, Councu M, Simonazzi G, Carinci P, Bondavalli C, Bovicelli L and Farina A. (2003) Cell-free fetal DNA concentration in plasma of patients with abnormal uterine artery Doppler waveform and intrauterine growth restriction–a pilot study. Prenat Diagn 23, 367–371.

Chan KC and Lo YM (2002) Circulating nucleic acids as a tumor marker. Histol Histopathol 17,937–943.

Chan KC, Zhang J, Hui AB, Wong N, Lau TK, Leung TN, Lo KW, Huang DW and Lo YM (2004) Size distributions of maternal and fetal DNA in maternal plasma. Clin Chem 50,88–92.

Chan LY, Leung TN, Chan KC, Tai HL, Lau TK, Wong EM and Lo YM (2003) Serial analysis of fetal DNA concentrations in maternal plasma in late pregnancy. Clin Chem 49,678–680.

Chen XQ, Stroun M, Magnenat JL, Nicod LP, Kurt AM, Lyautey J, Lederrey C and Anker P (1996) Microsatellite alterations in plasma DNA of small cell lung cancer patients. Nat Med 2,1033–1035.

Chen XQ, Bonnefoi H, Pelte MF, Lyautey J, Lederrey C, Movarekhi S, Schaeffer P, Mulcahy HE, Meyer P, Stroun M and Anker P (2000) Telomerase RNA as a detection marker in the serum of breast cancer patients. Clin Cancer Res 6,3823–3826.

Chiu RW, Poon LL, Lau TK, Leung TN, Wong EM and Lo YM (2001) Effects of blood-processing protocols on fetal and total DNA quantification in maternal plasma. Clin Chem 47,1607–1613.

Chiu RW, Lau TK, Cheung PT, Gong ZQ, Leung TN and Lo YM (2002a) Noninvasive prenatal exclusion of congenital adrenal hyperplasia by maternal plasma analysis: a feasibility study. Clin Chem 48,778–780.

Chiu RW, Lau TK, Cheung PT, Gong ZQ, Leung TN and Lo YM (2002b) Noninvasive prenatal exclusion of congenital adrenal hyperplasia by maternal plasma analysis: a feasibility study. Clin Chem 48,778–780.

Chiu RW, Lau TK, Leung TN, Chow KC, Chui DH and Lo YM (2002c) Prenatal exclusion of beta thalassaemia major by examination of maternal plasma. Lancet 360,998–1000.

Costa JM, Benachi A, Gautier E, Jouannic JM, Ernault P and Dumez Y (2001) First-trimester fetal sex determination in maternal serum using real-time PCR. Prenat Diagn 21,1070–1074.

Dhallan R, Au WC, Mattagajasingh S, Emche S, Bayliss P, Damewood M, Cronin M, Chou V and Mohr M (2004) Methods to increase the percentage of free fetal DNA recovered from the maternal circulation. J Am Med Assoc 291,1114–1119.

Dukes KA, Sullivan LM, Lewis D, Johnson KL, Bianchi DW, Simpson JL, Holzgreve W, Hahn S, Bischoff FZ and Jackson LG (2004) The effect of the elapsed time between blood draw and processing on the recovery of fetal cells from maternal blood. J Soc Gynecol Invest 11,154–165.

Faas BH, Beuling EA, Christiaens GC, dem Borne AE and Van der Schoot CE (1998) Detection of fetal RHD-specific sequences in maternal plasma. Lancet 352,1196.

Farina A, Caramelli E, Concu M, Sekizawa A, Ruggeri R, Bovicelli L, Rizzo N and Carinci P (2002) 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 22, 148–152.

Farina A, LeShane ES, Lambert-Messerlian GM, Canick JA, Lee T, Neveux LM, Palomaki GE and Bianchi DW (2003) Evaluation of cell-free fetal DNA as a second-trimester maternal serum marker of Down syndrome pregnancy. Clin Chem 49,239–242.

Finning KM, Martin PG, Soothill PW and Avent ND (2002) Prediction of fetal D status from maternal plasma: introduction of a new noninvasive fetal RHD genotyping service. Transfusion 42,1079–1085.

Giacona MB, Ruben GC, Iczkowski KA, Roos TB, Porter DM and Sorenson GD (1998) Cell-free DNA in human blood plasma: length measurements in patients with pancreatic cancer and healthy controls. Pancreas 17, 89–97.

Gonzalez-Gonzalez MC, Garcia-Hoyos M, Trujillo MJ, Rodriguez dA, Lorda-Sanchez I, Diaz-Recasens J, Gallardo E, Ayuso C and Ramos C (2002) Prenatal detection of a cystic fibrosis mutation in fetal DNA from maternal plasma. Prenat Diagn 22,946–948.

Gonzalez-Gonzalez MC, Trujillo MJ, Rodriguez dA, Garcia-Hoyos M, Lorda-Sanchez I, Diaz-Recasens J, Ayuso C and Ramos C (2003a) Huntington disease-unaffected fetus diagnosed from maternal plasma using QF-PCR. Prenat Diagn 23,232–234.

Gonzalez-Gonzalez MC, Trujillo MJ, Rodriguez dA and Ramos C (2003b) Early Huntington disease prenatal diagnosis by maternal semiquantitative fluorescent-PCR. Neurology 60,1214–1215.

Halicka HD, Bedner E and Darzynkiewicz Z (2000) Segregation of RNA and separate packaging of DNA and RNA in apoptotic bodies during apoptosis. Exp Cell Res 260,248–256.



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Hamada H, Arinami T, Kubo T, Hamaguchi H and Iwasaki H (1993) Fetal nucleated cells in maternal peripheral blood: frequency and relationship to gestational age. Hum Genet 91,427–432.

Hasselmann DO, Rappl G, Tilgen W and Reinhold U (2001) Extracellular tyrosinase mRNA within apoptotic bodies is protected from degradation in human serum. Clin Chem 47,1488–1489.

Holdenrieder S, Stieber P, Bodenmuller H, Busch M, von Pawel J, Schalhorn A, Nagel D and Seidel D (2001) Circulating nucleosomes in serum. Ann, NY Acad Sci 945,93–102.

Honda H, Miharu N, Ohashi Y, Samura O, Kinutani M, Hara T and Ohama K (2002) Fetal gender determination in early pregnancy through qualitative and quantitative analysis of fetal DNA in maternal serum. Hum Genet 110,75–79.

Houfflin-Debarge V, O'Donnell H, Overton T, Bennett PR and Fisk NM (2000) High sensitivity of fetal DNA in plasma compared to serum and nucleated cells using unnested PCR in maternal blood. Fetal Diagn Ther 15,102–107.

Hromadnikova I, Houbova B, Hridelova D, Voslarova S, Calda P, Nekolarova K, Kofer J, Stejskal D, Doucha J, Cinek O and Vavrirec J (2002) Quantitative analysis of DNA levels in maternal plasma in normal and Down syndrome pregnancies. BMC Pregnancy Childbirth 2(1):4.

Hromadnikova I, Houbova B, Hridelova D, Voslarova S, Kofer J, Komrska V and Habart D (2003) Replicate real-time PCR testing of DNA in maternal plasma increases the sensitivity of non-invasive fetal sex determination. Prenat Diagn 23,235–238.

Jahr S, Hentze H, Englisch S, Hardt D, Fackelmayer FO, Hesch RD and Knippers R (2001) DNA fragments in the blood plasma of cancer patients: quantitations and evidence for their origin from apoptotic and necrotic cells. Cancer Res 61,1659–1665.

Jen J, Wu L and Sidransky D (2000a) An overview on the isolation and analysis of circulating tumor DNA in plasma and serum. Ann, NY Acad Sci 906,8–12.

Jen J, Wu L and Sidransky D (2000b) An overview on the isolation and analysis of circulating tumor DNA in plasma and serum. Ann NY Acad Sci 906,8–12.

Jimenez DF and Tarantal AF (2003) Quantitative analysis of male fetal DNA in maternal serum of gravid rhesus monkeys (Macaca mulatta). Pediatr Res 53,18–23.

Johnson KL, Dukes KA, Vidaver J, LeShane ES, Ramirez I, Weber WD, Bischoff FZ, Hahn S, Sharma A, Dang DX, Hire LM et al. (2004) Interlaboratory comparison of fetal male DNA detection from common maternal plasma samples by real-time PCR. Clin Chem 50(3):516–521.

Kolialexi A, Tsangaris GT, Mavrou A, Antsaklis A, Tzortzatou F, Touliatou V and Metaxotou C (2001) Use of annexin V antibody to identify apoptotic cells during pregnancy. Ann NY Acad Sci 945,145–150.

Kopreski MS, Benko FA, Kwak LW and Gocke CD (1999) Detection of tumor messenger RNA in the serum of patients with malignant melanoma. Clin Cancer Res 5,1961–1965.

Krabchi K, Gros-Louis F, Yan J, Bronsard M, Masse J, Forest JC and Drouin R (2001) Quantification of all fetal nucleated cells in maternal blood between the 18th and 22nd weeks of pregnancy using molecular cytogenetic techniques. Clin Genet 60,145–150.

Lam NY, Rainer TH, Chiu RW and Lo YM (2004) EDTA is a better anticoagulant than heparin or citrate for delayed blood processing for plasma DNA analysis. Clin Chem 50,256–257.

Lau TK, Lo KW, Chan LY, Leung TY and Lo YM. (2000) Cell-free fetal deoxyribonucleic acid in maternal circulation as a marker of fetal-maternal hemorrhage in patients undergoing external cephalic version near term. Am J Obstet Gynecol 183, 712–716.

Lau TW, Leung TN, Chan LY, Lau TK, Chan KC, Tam WH and Lo YM (2002) Fetal DNA clearance from maternal plasma is impaired in pre-eclampsia. Clin Chem 48,2141–2146.

Leon SA, Shapiro B, Sklaroff DM and Yaros MJ (1977) Free DNA in the serum of cancer patients and the effect of therapy. Cancer Res 37, 646–650.

Leung TN, Zhang J, Lau TK, Hjelm NM and Lo YM (1998) Maternal plasma fetal DNA as a marker for preterm labour. Lancet, 352, 1904–1905.

Leung TN, Zhang J, Lau TK, Chan LY and Lo YM (2001) Increased maternal plasma fetal DNA concentrations in women who eventually develop preeclampsia. Clin Chem 47,137–139.

Levine RJ, Qian C, Leshane ES, Yu KF, England LJ, Schisterman EF, Wataganara T, Romero R and Bianchi DW (2004) Two-stage elevation of cell-free fetal DNA in maternal sera before onset of preeclampsia. Am J Obstet Gynecol 190,707–713.

Lo YM (2000) Fetal DNA in maternal plasma. Ann NY Acad Sci 906, 141–147.

Lo YM, Leung TN, Tein MS, Sargent IL, Zhang J, Lau TK, Haines CJ and Redman CW (1999c) Quantitative abnormalities of fetal DNA in maternal serum in preeclampsia. Clin Chem 45,184–188.

Lo YM, Tein MS, Lau TK, Haines CJ, Leung TN, Poon PM, Wainscoat JS, Johnson PJ, Chang AM and Hjelm NM (1998b) Quantitative analysis of fetal DNA in maternal plasma and serum: implication for noninvasive prenatal diagnosis. Am J Hum Genet 62,768–775.

Lo YM, Corbetta N, Chamberlain PF, Rai V, Sargent IL, Redman CW and Wainscoat JS (1997) Presence of fetal DNA in maternal plasma and serum. Lancet 350,485–487.

Lo YM, Hjelm NM, Fidler C, Sargent IL, Murphy MF, Chamberlain PF, Poon PM, Redman CW and Wainscoat JS (1998a) Prenatal diagnosis of fetal RhD status by molecular analysis of maternal plasma. N Engl J Med 339,1734–1738.

Lo YM, Tein MS, Lau TK, Haines CJ, Leung TN, Poon PM, Wainscoat JS, Johnson PJ, Chang AM and Hjelm NM (1998b) Quantitative analysis of fetal DNA in maternal plasma and serum: implications for noninvasive prenatal diagnosis. Am J Hum Genet 62,768–775.

Lo KW, Lo YM, Leung SF, Tsang YS, Chan LY, Johnson PJ, Hjelm NM, Lee JC and Huang DP (1999a) Analysis of cell-free Epstein-Barr virus associated RNA in the plasma of patients with nasopharyngeal carcinoma. Clin Chem 45,1292–1294.

Lo YM, Lau TK, Zhang J, Leung TN, Chang AM, Hjelm NM, Elmes RS and Bianchi DW (1999b) Increased fetal DNA concentrations in the plasma of pregnant women carrying fetuses with trisomy 21. Clin Chem 45,1747–1751.

Lo YM, Leung TN, Tein MS, Sargent IL, Zhang J, Lau TK, Haines CJ and Redman CW (1999c) Quantitative abnormalities of fetal DNA in maternal serum in preeclampsia. Clin Chem 45,184–188.

Lo YM, Zhang J, Leung TN, Lau TK, Chang AM and Hjelm NM (1999d) Rapid clearance of fetal DNA from maternal plasma. Am J Hum Genet 64,218–224.

Lui YY and Dennis YM (2002) Circulating DNA in plasma and serum: biology preanalytical issues and diagnostic applications. Clin Chem Lab Med 40,962–968.

Martelli AM, Zweyer M, Ochs RL, Tazzari PL, Tabellini G, Narducci P and Bortul R (2001) Nuclear apoptotic changes: an overview. J Cell Biochem 82,634–646.

Nawroz H, Koch W, Anker P, Stroun M and Sidransky D (1996) Microsatellite alterations in serum DNA of head and neck cancer patients. Nat Med 2,1035–1037.

Ng EK, Tsui NB, Lau TK, Leung TN, Chiu RW, Panesar NS, Lit LC, Chan KW and Lo YM (2003) mRNA of placental origin is readily detectable in maternal plasma. Proc Natl Acad Sci USA 100, 4748–4753.

Ohashi Y, Miharu N, Honda H, Samura O and Ohama K (2002) Correlation of fetal DNA and human chorionic gonadotropin concentrations in second-trimester maternal serum. Clin Chem 48,386–388.

Poon LL, Leung TN, Lau TK and Lo YM (2000a) Prenatal detection of fetal Down's syndrome from maternal plasma. Lancet 356,1819–1820.

Poon LL, Leung TN, Lau TK and Lo YM (2000b) Presence of fetal RNA in maternal plasma. Clin Chem 46,1832–1834.

Poon LL, Leung TN, Lau TK, Chow KC and Lo YM (2002) Differential DNA methylation between fetus and mother as a strategy for detecting fetal DNA in maternal plasma. Clin Chem 48,35–41.

Redman CW and Sargent IL (2001) The pathogenesis of pre-eclampsia. Gynecol Obstet Fertil 29,518–522.

Redman CW, Sacks GP and Sargent IL (1999) Preeclampsia: an excessive maternal inflammatory response to pregnancy. Am J Obstet Gynecol 180,499–506.

Reister F, Frank HG, Kingdom JC, Heyl W, Kaufmann P, Rath W and Huppertz B (2001) Macrophage-induced apoptosis limits endovascular trophoblast invasion in the uterine wall of preeclamptic women. Lab Invest 81,1143–1152.

Rijnders RJ, Van der Luijt RB, Peters ED, Goeree JK, Van der Schoot CE, Ploos Van Amstel JK and Christiaens GC (2003) Earliest gestational age for fetal sexing in cell-free maternal plasma. Prenat Diagn 23, 1042–1044.

Roberts JM and Redman CW (1993) Pre-eclampsia: more than pregnancy-induced hypertension. Lancet 341,1447–1451.



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Rudin CM and Thompson CB (1997) Apoptosis and disease: regulation and clinical relevance of programmed cell death. Annu Rev Med 48, 267–281.

Saito H, Sekizawa A, Morimoto T, Suzuki M and Yanaihara T (2000) Prenatal DNA diagnosis of a single-gene disorder from maternal plasma. Lancet 356(9236),1170.

Sekizawa A, Kondo T, Iwasaki M, Watanabe A, Jimbo M, Saito H and Okai T (2001) Accuracy of fetal gender determination by analysis of DNA in maternal plasma. Clin Chem 47,1856–1858.

Sekizawa A, Jimbo M, Saito H, Iwasaki M, Sugito Y, Yukimoto Y, Otsuka J and Okai T (2002) Increased cell-free fetal DNA in plasma of two women with invasive placenta. Clin Chem 48,353–354.

Silva NH, Pimenta G, Pulcheri WA, Fournier MV, Spector N and Costa Carvalho MG (2001) Detection of messenger RNA in leukocytes or plasma of patients with chronic myeloid leukemia. Oncol Rep 8, 693–696.

Sorenson GD, Pribish DM, Valone FH, Memoli VA, Bzik DJ and Yao SL (1994) Soluble normal and mutated DNA sequences from single-copy genes in human blood. Cancer Epidemiol Biomarkers Prev 3,67–71.

Spencer K, de Kok JB and Swinkels DW (2003) Increased total cell-free DNA in the serum of pregnant women carrying a fetus affected by trisomy 21. Prenat Diagn 23,580–583.

Stenman J and Orpana A (2001) Accuracy in amplification. Nat Biotechnol 19,1011–1012.

Stroun M, Anker P, Maurice P, Lyautey J, Lederrey C and Beljanski M (1989) Neoplastic characteristics of the DNA found in the plasma of cancer patients. Oncology 46,318–322.

Stroun M, Maurice P, Vasioukhin V, Lyautey J, Lederrey C, Lefort F, Rossier A, Chen XQ and Anker P (2000) The origin and mechanism of circulating DNA. Ann NY Acad Sci 906,161–168.

Sugito Y, Sekizawa A, Farina A, Yukimoto Y, Saito H, Iwasaki M, Rizzo N and Okai T (2003) Relationship between severity of hyperemesis gravidarum and fetal DNA concentration in maternal plasma. Clin Chem 49,1667–1669.

Swinkels DW, de Kok JB, Hendriks JC, Wiegerinck E, Zusterzeel PL and Steegers EA (2002) 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 48,650–653.

Taback B, O'Day SJ and Hoon DS (2004) Quantification of Circulating DNA in the Plasma and Serum of Cancer Patients. Ann NY Acad Sci 1022,17–24.

Thomas MR, Tutschek B, Frost A, Rodeck CH, Yazdani N, Craft I and Williamson R (1995) The time of appearance and disappearance of fetal DNA from the maternal circulation. Prenat Diagn 15,641–646.

Tsui NB, Ng EK and Lo YM (2002) Stability of endogenous and added RNA in blood specimens serum and plasma. Clin Chem 48,1647–1653.

Vasioukhin V, Anker P, Maurice P, Lyautey J, Lederrey C and Stroun M (1994) Point mutations of the N-ras gene in the blood plasma DNA of patients with myelodysplastic syndrome or acute myelogenous leukaemia. Br J Haematol 86,774–779.

Wataganara T, LeShane ES, Farina A, Messerlian GM, Lee T, Canick JA and Bianchi DW (2003) Maternal serum cell-free fetal DNA levels are increased in cases of trisomy 13 but not trisomy 18. Hum Genet 112, 204–208.

Wu TL, Zhang D, Chia JH, Tsao KH, Sun CF and Wu JT (2002) Cell-free DNA: measurement in various carcinomas and establishment of normal reference range. Clin Chim Acta 321,77–87.

Zhong XY, Burk MR, Troeger C, Jackson LR, Holzgreve W and Hahn S (2000a) Fetal DNA in maternal plasma is elevated in pregnancies with aneuploid fetuses. Prenat Diagn 20,795–798.

Zhong XY, Holzgreve W and Hahn S (2001b) Circulatory fetal and maternal DNA in pregnancies at risk and those affeceted by preeclampsia. Ann N Y Acad Sci 945,138–140.

Zhong XY, Holzgreve W and Hahn S (2000b) Detection of fetal Rhesus D and sex using fetal DNA from maternal plasma by multiplex polymerase chain reaction. Br J Obstet Gynecol 107,766–769.

Zhong XY, Holzgreve W, Li JC, Aydinli K and Hahn S (2000c) High levels of fetal erythroblasts and fetal extracellular DNA in the peripheral blood of a pregnant woman with idiopathic polyhydramnios: case report. Prenat Diagn 20,838–841.

Zhong XY, Hahn S and Holzgreve W (2001a) Prenatal identification of fetal genetic traits. Lancet 357,310–311.

Zhong XY, Laivuori H, Livingston JC, Ylikorkala O, Sibai BM, Holzgreve W and Hahn S (2001b) Elevation of both maternal and fetal extracellular circulating deoxyribonucleic acid concentrations in the plasma of pregnant women with preeclampsia. Am J Obstet Gynecol 184, 414–419.

Zhong XY, Holzgreve W and Hahn S (2002a) Cell-free fetal DNA in the maternal circulation does not stem from the transplacental passage of fetal erythroblasts. Mol Hum Reprod 8,864–870.

Zhong XY, Holzgreve W and Hahn S (2002b) The levels of circulatory cell free fetal DNA in maternal plasma are elevated prior to the onset of preeclampsia. Hypertens Pregn 21,77–83.

*Submitted on July 23, 2004; resubmitted on October 25, 2004; accepted on October 28, 2004*



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