Biochemical and Clinical Diagnostic Aspects of Circulating Nucleic Acids

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Part I: Introduction of biochemical and clinical diagnostic aspects of circulating nucleic acids

Summary

Mandel and Métais first reported the discovery of extracellular nucleic acids in human blood plasma in 1948 [1]. However, little attention was drawn to the findings until 1966, when Tan et al demonstrated that the high levels of circulating DNA present in patients with system lupus erythematosus (SLE) [2]. Further studies showed that increased concentrations of DNA in the plasma could be detected in cancer patients [3]. In 1989, Stroun et al suggested that the circulating DNA in cancer patients contains the characteristics of tumor DNA [4]. This important suggestion was shown to be correct in the following studies [5, 6]. Those studies showed that plasma DNA could be used for tumor detection and monitoring.

In 1997 Lo et al demonstrated the presence of fetal DNA in the plasma of pregnant women by the detection of Y-chromosomal sequences [7]. Quantitative analysis has shown that relative concentration of fetal DNA in maternal plasma is much higher than that of fetal DNA in the cellular fraction. The finding suggested that fetal DNA in maternal plasma would be a very valuable material for noninvasive prenatal diagnosis. This approach was first successfully applied to detect Y-chromosome specific sequences from women who were carrying a male fetus. Using quantitative real-time PCR assay, abnormally high concentrations of circulating fetal DNA in maternal plasma has been observed from those women who were suffering from preeclampsia [8,9], preterm labor [10], idiopathic polyhydramnios [11] , as well as those who were carrying a aneuploid fetus [12, 13]. These results showed that measuring the circulating fetal DNA could serve as a screening tool for pregnancy-associated disorders. Later study showed that fetal gender determination using cell free DNA in maternal circulation could be used as a pre-

test for inherited X-linked condition [14]. The first sex-independent marker is the paternally inherited RhD gene and this detection is already in use for clinical diagnosis of fetal RhD genotype in Europe [15,16]. Using polymorphic DNA markers, abnormal paternally inherited fetal DNA, such as chromosomal translocations [17] and trinucleotide repeated expansions of the dystrophia myotonica protein kinase (DMPK) gene [18], has correctly been determined from maternal plasma. Subsequently, fetal paternally inherited single gene mutations in maternal circulation have been detected by PCR combined restriction enzyme assay [19-22] (paper2). Recently, such mutations have successfully been detected by mass spectrometry-based assay [23]. Even though many researchers have been trying to understand the biochemical and molecular aspects of circulating fetal DNA, to date, those issues have not been completely understood. However, much progress has been made. For example, studies indicated that placenta is the main tissue origin of circulating fetal DNA [24]. Recently, Chan et al, and we too, have demonstrated that the fetal DNA in maternal circulation exists as small fragments [25], (paper1). Our results showed that the paternally inherited DNA polymorphisms were more easily detected in the enriched fetal DNA from maternal plasma. Furthermore, the presence or absence of paternally inherited single gene mutations can be detected from such size-selected circulating fetal DNA (paper 3). This enrichment of circulating fetal DNA could overcome the limitation caused by high background maternal DNA.

Recently, donor-derived DNA, as well as tumor-specific gene could be detected in the urine of kidney transplant recipients and some kinds of cancer patients, respectively [26], (paper5). Analysis of the urinary DNA indicated that it might serve as a monitoring and prognostic marker for cancer treatment or kidney graft rejection.

Biochemical aspects of circulating nucleic acids

Although the presence of circulating DNA in individuals has been a well-established phenomenon, its molecular and biological aspects are still in an early phase of investigation. It is very important to study these issues and to seek the answers to some fundamental questions regarding the nature of circulating nucleic acids.

1. Origin of plasma DNA

Some studies have been done to try to understand the sources of plasma DNA. In the following, two possible mechanisms will be discussed. In the first one, apoptosis has been considered to play an important role in the release of DNA into the bloodstream. Apoptosis is the most common form of cell death throughout the whole of life [27, 28]. During cell apoptosis, a set of caspases is activated, leading to the cleavage of the chromatin into mutilple oligo and mononucleosomes. Nucleosomes in human plasma have been detected by immunoassay [29,30]. The fragmentation of genomic DNA due to the nucleosomal cleavage is a major characteristics of apoptosis and such a DNA laddering can be observed by agarose gel electrophoresis [31, 32]. We used Southern blot analysis of total circulatory plasma DNA from healthy individuals and pregnant women with highly repetitive Alu sequence. Our results confirmed that circulating DNA presents typical apoptotic characteristics, displaying a ladder obtained by nucleosomal cleavage (paper 1).

The second mechanism considered was that some types of nucleated cells shed DNA into circulation. The early report that described the release of DNA from activated lymphocytes was by Rogers JC and colleagues [33]. They found that the cultured

lymphocytes in the presence of phytohemagglutinin or antigen excreted DNA into the medium. That DNA is released as a consequences of terminal differentiation should also be considered since final stages of differentiation of erythrocytes, keratinocytes, and lens crystalline cells are accompanied by chromatin cleavage and extrusion of nuclear material from the cells [34- 36]. A recent report provides evidence for the haematopoitic origin of cell free DNA in healthy individuals [37]. Lui et al quantified Y-chromosomal sequences in the plasma and serum of patients receiving sex-mismatched bone-marrow transplants, and found that circulating DNA was predominantly of donor origin. We also observed that a proportion of the circulatory DNA has a very large molecular size, larger than 10 kb by southern blot analysis (paper 1). We suppose that these large circulatory DNA species could be derived from the erythropoietic system because the DNA isolated from terminally differentiating erythroblasts exhibited similar characteristics. (unpublished data from our group).

Furthermore, necrosis might also play a role in the generation of plasma DNA. Jahr and colleagues used established murine models for the induction of liver cell necrosis [38]. After 4 hours induction, the levels of plasma DNA were dramatically increased. However, necrotic cell death is limited in a healthy body. Thus, necrotic cell death cannot be responsible for a significant part of plasma DNA in healthy individuals. During pregnancy, whether necrosis takes place or how much the plasma DNA levels would be affected are unknown.

2. Plasma DNA Chimerism

Plasma DNA chimerism is a novel concept since the foreign genetic material, for instance tumor-derived DNA or fetal-derived DNA, has been discovered in the circulation of some individuals. This concept is adapted from the originally well-established concept of cellular chimerism, in which cells with genetic differences can be found in the host circulation [39, 40].

To date, several types of plasma DNA chimerism have been found in human circulation. Besides those mentioned above, namely tumor-derived DNA and fetal-derived DNA, donor-derived DNA that exists in the plasma from the liver or kidney transplantation patients was reported in 1998 [41]. Another type of plasma DNA chimerism is virus-derived DNA in human plasma. Viral DNA has been shown to be detectable in the circulation of patients suffering from nasopharyngeal carcinoma (NPC) [42], lymphoma [43], head and neck tumors [44] and cervical carcinoma [45]. The circulating Epstein-Barr virus (EBV) DNA is well established as a tumor marker in the clinical management of NPC patients [46]. Recently, the circulating *plasmodium falciparum* DNA has been for the first time detected in the plasma of patients suffering from malaria [47].

The findings of plasma DNA chimerism are extremely important in clinical applications since it can be used as diagnostic, monitoring and prognostic markers.

The concept of DNA chimerism has further been developed to look for urinary DNA in the urine of bladder cancer or kidney transplant patients [48,49], (paper 5). The analysis of urinary DNA has indicated that it may serve as a non-invasive marker for monitoring and predicting the treatment and process of cancer or kidney graft rejection.

3. Fetal DNA in maternal plasma

Although intact fetal cells have been found in maternal circulation for several decades, the rarity (1 in 10^6) and the sophisticated procedures limited their utility in clinic. The discovery of fetal DNA in maternal circulation has opened the avenue for non-invasive prenatal diagnosis since fetal DNA is more abundant than fetal cells, and the diagnosis can be easily and rapidly carried out.

3.1 Origin of fetal DNA in maternal plasma

No studies have conclusively addressed this question. Much evidence has shown that the placenta may be a major source of the fetal genetic material released into maternal circulation [24]. The levels of circulating fetal DNA increased in the plasma of women with advanced gestation age [50, 51] or preeclampsia [8, 9]. Correspondingly, it has been observed that placenta apoptosis increases significantly as pregnancy progresses and in preeclampsia [52], suggesting that the presence of circulating fetal DNA is the result of cell death in the placenta. In the study of women who underwent assisted reproduction, the fetal DNA was found in maternal serum even before fetal circulation was established. This strongly implies that the source of circulating fetal DNA is most likely from trophoblasts [53].

Haematopoietic cells were considered to be a reasonable candidate for the source of circulating fetal DNA because a variety of fetal cell types circulate in maternal blood [54]. Some studies have shown that a large number of fetal nucleated erythrocytes in maternal circulation undergo apoptosis [55, 56]. It is hypothesized that the circulating fetal DNA may result from the interaction between apoptotic cells and the maternal

immune system. However, since intact fetal cells are rare in maternal blood (0.0035-0.008%) [54], it is unlikely that this small number of fetal cells accounts for such a high level of fetal DNA in maternal circulation (2.33-11.4%) [51].

In addition, other potential sources have also been proposed. For example, the direct feto-maternal transfer of DNA molecules via placenta or membranes [57].

3.2 The size distribution of fetal DNA in maternal plasma

We used southern blot analysis with highly repeated Alu probe to hybridize total plasma DNA (paper 1). Our results have shown that the size distribution pattern of total maternal plasma DNA presented apoptotic characteristics. To study the size distribution of fetal DNA in maternal circulation, we used a combination of size-separation on agarose gel electrohporesis and highly sensitive real time PCR assay to analyze the plasma DNA molecules. We surprisingly found that the fetal DNA comprised small fragments of a size of less than 300bp. On the other hand, most maternally derived molecules were considerably larger than 500bp.

Our results are remarkably similar to those reported by Chan et al. They used two panels of quantitative PCR assays [25]. One amplified the leptin genes, which represent the size distribution of total maternal DNA, with the amplicon ranging in sizes from 105 to 798 bp. The other amplified the SRY genes, which represent the size distribution of fetal DNA in maternal plasma, with the amplicon ranging in sizes from 107 to 524 bp. Their results showed that a median of more than 90% of the fetal-derived DNA molecules was less than 313bp in length.

Chan et al and our results support the hypothesis that fetal DNA is derived from the placenta, whereas the vast proportion of maternal circulating DNA is of hemopoietic origin [24, 37].

It is known that the concentrations of circulating fetal DNA in healthy maternal plasma has a mean of 3.4% and 6.2% of the total plasma DNA in early and late pregnancy, respectively [51]. The discovery of the size distribution of circulating fetal DNA provides the possibility to enrich fetal DNA from maternal plasma. After such enrichment, the concentration of circulating fetal DNA has a mean of 28.4% and 68.7% of the total plasma DNA in early and late pregnancy, respectively (paper 1).

3.3 Clearance of fetal DNA from maternal plasma:

Clearance of fetal DNA from maternal plasma after delivery in healthy pregnant women has been shown to be very rapid and much more rapid than the clearance of fetal nucleated cells [58, 59]. Lo et al showed that the mean half-life for fetal DNA was estimated to be 16.3 min (range 4-30min) [60]. Most of the women studied had undetectable levels of circulating fetal DNA by 2 hour postpartum.

Potential mechanisms for clearance of circulating fetal DNA include plasma nuclease, hepatic clearance, and degradation via its interaction with maternal cells.

To study the role of plasma nuclease in the clearance of fetal DNA from maternal plasma, Lo et al digested the maternal blood samples with plasma nuclease at 37°C for 2 hours [60]. Their results showed that three samples had plasma fetal DNA concentration >90% of pre-incubation values. The remaining seven subjects' samples had concentrations within a range of 31-74% of the values before incubation. These data indicated that

plasma nuclease plays only a partial role in the removal of circulating fetal DNA. However, since it was an *in vitro* study, it could not completely explain the predominance *in vivo*.

Another possible mechanism for clearance of circulating fetal DNA is that circulating fetal DNA is removed by detoxification organs, such as the liver. Studies in experimental animals have shown that circulating DNA is rapidly removed by the liver [61]. Gauthier et al investigated the clearance of the circulating mononucleosomes in mice [62]. Their study showed that 71.0 to 84.7% of nucleosomes removed from circulation were localized in the liver.

Alternatively, the maternally immunologic system, for instance, spleen, and lymphocytes in maternal circulation, is involved in the removal of circulating DNA. Because the fetal DNA exists in maternal circulation as a foreign material, it is reasonable that maternal immunological system takes out the " trash". It is known that degenerating apoptotic and necrotic cells *in vivo* are efficiently taken up by macrophages [63].

4. Conflicting findings concerning fetal DNA in maternal urine

Could circulating fetal DNA cross the kidney barrier and be secreted into maternal urine? Botezatu and colleagues first time reported that highly repetitive male-specific DNA sequences (DYZ1) could be detected in the urine of pregnant women who were carrying a male fetus (7-10 weeks) by the use of a nest-PCR analysis [64]. They could detect urinary fetal DNA in 8 of 10 samples. And no false positive results were reported. Their studies further indicated that the DNA present in the urine had a size of less than 200bp.

The second report, by Al-Yatama et al, examined urine samples from 80 pregnant women between 7 and 40 week's gestation [65]. They were able to detect Y chromosome-specific sequences in 38% of the urine samples from women carrying a male fetus. There was no significant difference in the detection between different trimesters in women bearing a male fetus. However, for the 25 women bearing a female fetus, three (12%) and one (4%) showed false-positive results in plasma and urine, respectively.

In our experiments we were not able to reproduce these results, even though we examined specific pregnancy-related disorders, in which condition the levels of circulatory fetal DNA are significantly elevated and renal function is known to be affected (paper 4). (see page 46-49)

Botezatu et al's studies also reported that purified DNA (0.1-0.5μg/animal) was injected into mice and that approximately 0.06% of the injected DNA was detected in the urine of the animals [64]. However, those observations were based on the clearance of the purified DNA in animal models, whereas we know that plasma DNA is usually associated with protein (for example, Histone). Moreover, the concentration of injected DNA in mice was higher than that of fetal DNA in maternal plasma. Those studies could not explain the phenomenon of the clearance of fetal DNA from maternal circulation, occurring in pregnant women's bodies.

5. Urinary DNA as a marker for renal transplantation

Urinary DNA chimerism has been described in kidney transplant recipients in that donor-derived cell-free DNA was detected in the recipient's urine [49, 66, 67]. Zhang et al detected the donor-derived DNA from the urine of females who received male kidney

transplantation by real time PCR assay with Y-chromosomal specific sequences [49]. The results of quantitative analysis indicated that urinary DNA might serve as a new maker to monitor kidney transplant engraftment because the concentrations of urinary DNA increased under conditions of graft rejection and decreased to basal values after immunosuppressive treatment.

A caveat of these studies was that they relied on sex-disparate donor-recipient conditions: because the PCR assays used were specific for the Y chromosome, cell-free DNA from the donor kidney could be detected only in the urine of female recipients who had received a male kidney.

To address the question of whether kidney donor-derived DNA sequences could be detected in the urine of transplant recipients by using sex-independent markers, we tested for the presence of donor-specific STR loci and donor-derived GSTM1 (glutathione S-transferase M1) gene in the urine for cases in which the donor and recipient were either of the same sex or the donor was female and the recipient was male (paper 5). Our results indicated that mircosatellite markers and DNA polymorphisms would be potentially alternative markers for the quantification of urinary DNA in kidney transplant recipients (see page 49-51).

Clinical diagnostic aspects of circulating nucleic acids

Since fetal DNA has been found in maternal circulation, its clinical applications have mainly focused on the quantification of fetal DNA sequences. Its increase is related to many pregnancy-associated disorders. However, there is a limitation since only pregnancies with male fetuses can be detected because the method relies on the detection of Y chromosomal specific sequences, which are absent from maternal plasma. Other paternally inherited fetal gene sequences, such as the RhD gene, DNA polymorphisms and single gene mutations, have been subsequently reported.

1. Circulating fetal DNA in screening

The quantification of circulating fetal DNA can be used as a tool for screening pregnancy-related disorders. A current focus is its use as a maternal plasma marker in early pregnancy for fetus chromosomal aneuploidies. Several groups have observed that the fetal DNA levels in the plasma of pregnant women carrying a fetus affected by trisomy 21 were higher, compared with unaffected pregnancies by the use of real-time PCR analysis [12, 68]. The levels of circulating fetal DNA were also reported to increase in pregnancies complicated by pre-eclampsia and polyhydramnios [8,11,69,70]. Increased levels of circulating fetal DNA in maternal plasma may also be a marker for pre-term delivery [10]. However, this approach, which depends on quantification of the fetal Y chromosome-specific sequences, cannot distinguish one pregnancy-related disorder from another. Moreover, only pregnancies with male fetuses can be analyzed.

Furthermore, fetal gender determination can also be used as a “pre-test” to determine whether invasive prenatal diagnosis should be performed on a fetus having a risk of an X-

linked recessive disorder [14]. If the fetus is shown to be female, any invasive prenatal diagnosis is unnecessary, thus, avoiding risk of fetal loss.

2. Detection of paternally inherited RhD gene

The first successful detection of non-Y chromosomal fetal-derived gene sequences in maternal circulation was the Rhesus D gene [15]. The Rh blood group system is the most polymorphic of the human blood groups and of wide interest in clinical medicine because of its incompatibilities, such as hemolytic disease of the new-born (HDN) and autoimmune disease. Approximately 15% of Caucasian pregnancies are still potentially at the risk of severe HDN [71].

To date, the feasibility of fetal Rhesus D genotyping from maternal plasma and serum has been reported in several studies [15,16,72,73]. 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 Rhesus D genotyping, especially in Europe [16].

The prediction of paternal RhD genotyping is very useful for counseling a couple in future pregnancy since there is only 50% of chance that the pregnancy is affected if the father has heterozygous RhD gene. We precisely determined paternal Rhesus D zygotsity by real-time PCR assay (paper 6).

3. Detection of paternally inherited DNA polymorphisms:

The success in the detection of fetal Y-chromosomal and RhD DNA sequences in maternal plasma opens up the possibility that this approach may also be used to detect other paternally inherited DNA sequences in maternal plasma. Tang et al reported the

detection of paternally inherited X chromosomal microsatellite polymorphisms from maternal plasma (74). Their data indicated that in cases in which the fetus possessed an allele that was not present in the mother, this approach could achieve sensitivity of 5/10 (50%) at the second trimester and of 14/15 (93%) at the third trimester of gestation. Similar data also have been reported by Pertl et al [75]. They used multiplex fluorescent PCR to detect fetal-specific alleles in the maternal plasma samples. 12 samples were collected close to term, 4 of which having experienced infrequent, light contractions. Their results showed that the paternally inherited fetal alleles were detected in 84% of informative short tandem repeats and missed in 16%. Compared with TaqMan real time PCR, the low sensitivity is due to the fact that since the microsatellite PCR system amplified both fetal and maternal sequences, the latter masks the amplification of the paternally inherited allele. However, those samples which were detected were older than the second trimester of gestational age. Thus, this approach is not clinically useful for early prenatal diagnosis.

Recently we discovered that the majority of fetal DNA fragments in maternal plasma is of a small size of less than 300bp (paper 1). This discovery indicated that circulating fetal DNA could be selectively enriched by size separation. We examined paternally inherited DNA polymorphisms from such size-fractionated circulating DNA by using highly polymorphic STR sequences on chromosome 21. Our results indicated that paternally inherited fetal DNA polymorphisms were more easily detected from size-selected circulating DNA in contrast to detection from total circulating DNA. These results suggest that this non-invasive approach could potentially be applied to detect fetal aneupoildies.

4. Detection of Paternally inherited single gene mutations*:*

The first detection of a paternally inherited disease –causing mutation in maternal plasma was reported by Amicucci et al who were able to detect a trinucleotide repeat expansion in the dystrophia myotonic protein kinase (DMPK) gene in maternal plasma DNA [76]. After that, several reports have showed that the paternally inherited single gene mutations in maternal plasma could be detected by using PCR or nested PCR combined with restriction enzyme digestion. Saito et al reported the detection of a single point mutation in the plasma of a woman carrying a fetus suspected of having achondroplasia [19]. Gonzalez-Gonzalez et al detected a cystic fibrosis mutation in fetal DNA from maternal plasma [21]. Fucharoen et al detected fetal hemoglobin E gene mutation in the plasma of Thai pregnant women [20]. However, the method of PCR combined restriction digestion is relatively insensitive. Moreover, all the reports above were based on one or only a few cases of clinical samples.

Chiu et al employed mutation-specific real time PCR analysis to exclude β-thalassemia major caused by a four base deletion on the β-globin gene [77]. However, the deletion of the 4 bases of codon41/42 mutation (-CTTT) makes the detection possible by this simple strategy. They also tried to detect other 3 single-point mutations by the same method. However, reliable discrimination of the fetal mutant from the background maternal DNA was not achieved (their unpublished observations). More recently, Ding and Chiu et al developed a mass spectrometry-based system for the detection of the presence or absence of the paternal β-globin gene mutations in circulating nucleic acids [23]. Even though this method is more accurate and sensitive, the results are based on a very small population of only 5 pertinent cases. Furthermore, the method requires very sophisticated and

expensive equipment that is not readily available to the vast majority of diagnostic or research laboratories.

Recently, we discovered that the masked fetal polymorphic loci were easily detected from the size-selected circulating fetal DNA. We were curious as to whether such a selection was suitable for the detection of paternally inherited fetal single gene mutations. We examined a fetal point mutation in the fibroblast growth factor receptor 3 gene (FGFR3), which causes Achondroplasia, and paternally inherited β-globin gene mutations, which cause β-thalassemia, from the size-selected circulating fetal DNA (papers 2,3). Our study indicated that fetal genetic traits involving point mutations can be detected from the analysis of size-fractionated circulating fetal DNA having a size of less than approximately 300bp.

5. β- thalassemia

The thalassemia are, worldwide, the commonest monogenetic diseases in Man, causing a major public health problem, especially in the Mediterranean area, the Middle East, the Indian subcontinent, tropical Africa and in a line stretching from southern China through Thailand and the Malay peninsula to the island populations of the Pacific. They are also common in countries in which there has been immigration from these high-frequency populations.

β-thalassemia is caused by mutations in the β-globin gene. To date, over 200 mutations of β-thalassemia have been described. The following figure illustrates the global distribution of the β-thalassemia mutations [78] (Figure 1).

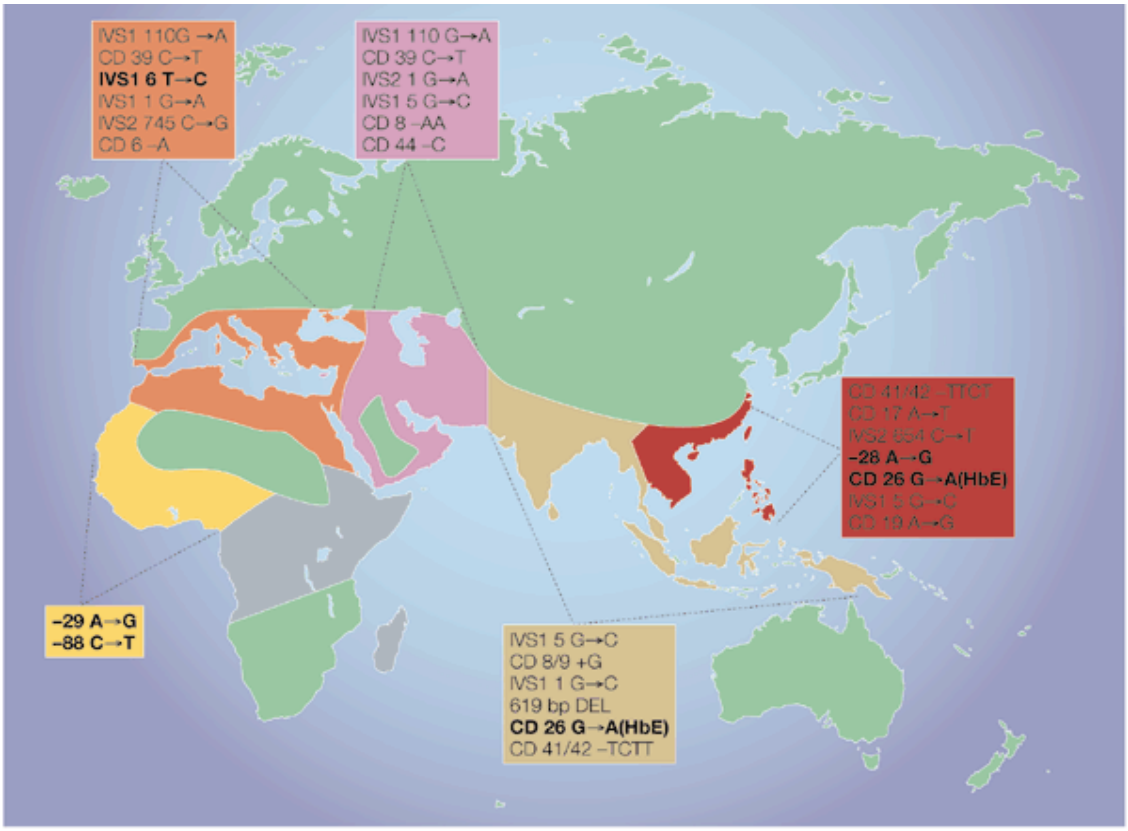

Fig 1.The global distribution of the β-thalassaemia mutations

β-globin gene is a structural gene found in a cluster with the other β like genes on the short arm of chromosome 11and it has 3 exons and 2 introns [79]. Most mutations that cause of β-thalassemia are due to point mutations in functionally important regions of the β-globin gene, others include deletions or addition of nucleotides. The following figure illustrates the classes of mutations that underlie β-thalassemia [78]. These abnormalities of β-globin gene lead to a defect in the synthesis of one or more of the globin polypeptide chains of hemoglobin. As a result, the erythrocytes are characterized by decreased intracellular hemoglobin content (hypochromia) and small size (microcytosis).

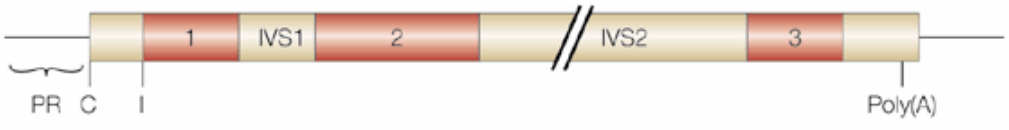

Fig 2. 1,2,3 are exons indicated in red. IVS1, IVS2 are intron. PR, promoter; C, CAP site; I, initiation codon; NS, nonsense; FS, frameshift; SPL, splicing.

Considering that at least 150,000 lethally affected homozygous thalassemia are born annually, prenatal diagnosis of β-thalassemia is extremely important, especially for some areas where a high incidence of β-thalassemia trait is present; for instance, in certain Greek islands and some villages of Sardinia, the incidence reaches 20 to 30% [79]. Moreover, the cost and difficulties of required treatment (especially blood transfusions and the complications arising from these) will be a major burden even in the Western countries, but particularly in the developing countries in which the disease is endemic.

An important step in the prevention of cases of severe β-thalassemia is the exclusion of homozygous and compound heterozygous pregnancy. A couple having two affected heterozygotes should be aware that they have a 25% chance of having a severely affected homozygous or compound heterozygous child.

At present, clinical prenatal diagnosis of β-thalassemia is mainly based on molecular diagnosis. The DNA-based methods were mentioned in the following table [80]. The source of DNA may be amniotic fluid cells, chorionic villi or fetal blood sampling. These invasive methods cause at least 1% of abnormal pregnancies. Development of noninvasive prenatal diagnosis is urgent needed.

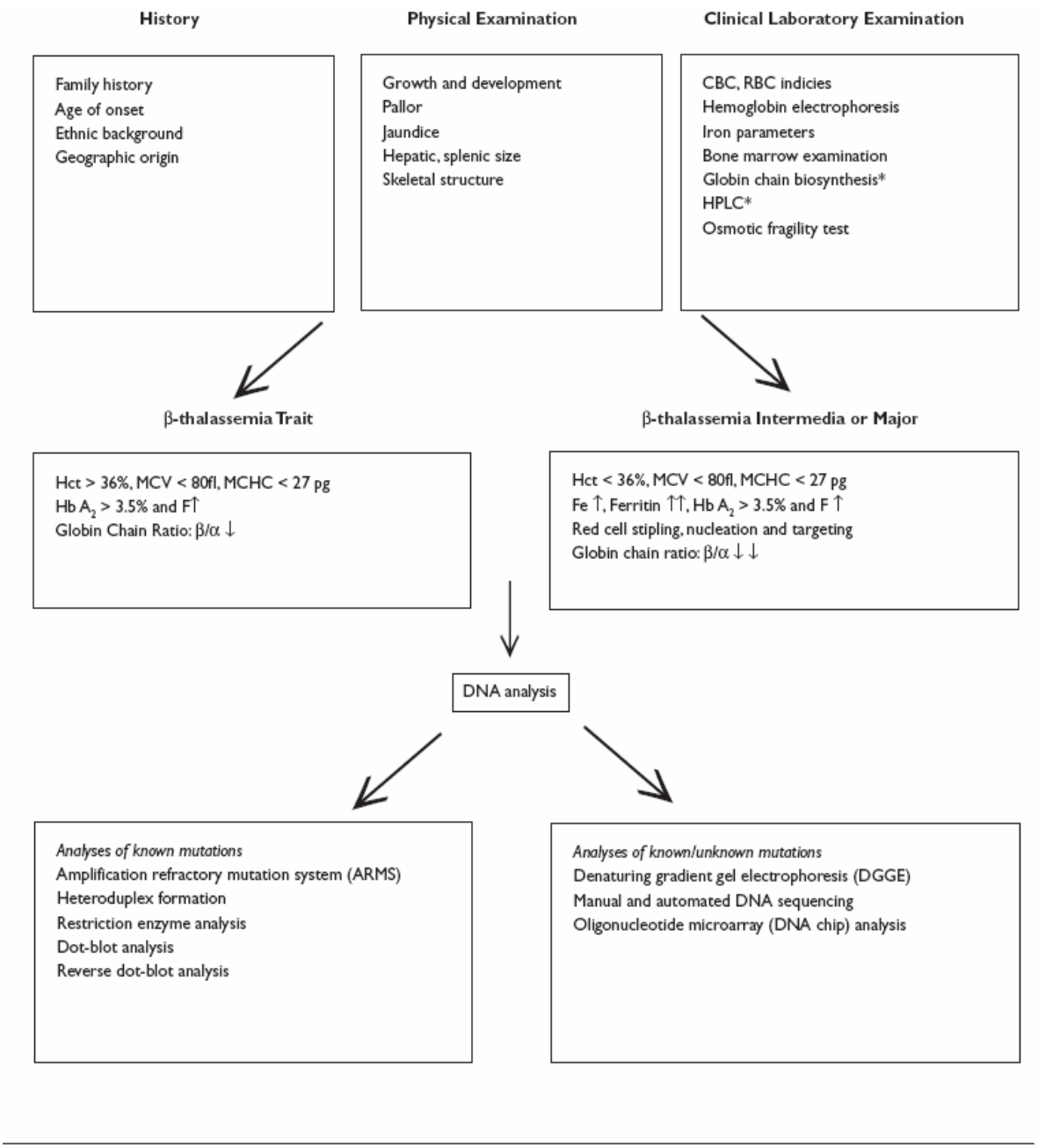

Table 1: The methods of diagnosis of β-thalassemia

Two non-invasive sources could be considered for prenatal diagnosis. One is the isolation of fetal cells, specifically erythroblasts, from the blood of pregnant women. Di Naro et al used a novel step density gradient for the enrichment of fetal erythroblasts from maternal

blood, and adapted PCR analysis of individually isolated fetal cells for prenatal diagnosis of the fetal β-globin genotype in pregnancies at risk for β-thalassemia [81]. They analyzed 4 samples, in each of these cases, five cells were isolated and the fetal genotype was correctly determined. Cheung et al in 1996 successfully identified the fetal genotype in two pregnancies at risk for sickle cell anaemia and β-thalassemia by analysis of fetal cells in maternal blood using the similar strategies [82].

Compared with the analysis of fetal nucleated cells in the maternal blood, maternal plasma DNA analysis has the advantage of being more abundant, and can be rapidly and reliably carried out for a large number of samples. By examining paternally inherited fetal gene mutations, the risk for a compound heterozygous pregnancy can be excluded. So far several strategies have been developed for prenatal exclusion of β-thalassemia major by using maternal plasma DNA, as discussed above (see pages 20-21).

In brief, using maternal plasma DNA for non-invasive prenatal diagnosis of β-thalassemia is very promising for the future. Suitable procedures, which are rapid, accurate, simple and easy to be popularized, are being sought, especially for developing and under-developed countries.

6. Achondroplasia

Achondroplasia (ACH), the most common genetic form of dwarfism, is inherited as an autonomic dominant trait with 100% penetrance. The estimated frequency of ACH is 1 in 26,000, with at least 80% of cases being sporadic [83-85].

The clinical features of Achondroplasia include rhizomelic dwarfism, relative marocephaly, exaggerated lumbar lordosis, and other typical skeletal abnormalities [85].

With linkage analysis, Achondroplasia gene was genetically mapped to 4p16.3, distal to Hunting's disease (HD) [86, 87]. Further studies showed Achondroplasia was caused by the mutations in the fibroblast growth factor receptor 3gene (FGFR3). There are 2 single gene changes in FGFR3 that can explain the disease. More than 98% of all Achondroplasia patients have the same missense mutation in the transmembrane domain of FGFR3, a G-A transition at nucleotide 1138. Most remaining mutations are a G-C transversion at the same nucleotide [88]. Both mutations result in the substitution of an arginine for a glysine at position 380 (G380R) of the mature protein.

Achondroplasia can be diagnosed by characteristic clinical and radiographic findings in most affected individuals. In individuals who may be too young to diagnose with certainty or in individuals with atypical findings, molecular genetic testing can be used to detect a mutation in the FGFR3 gene.

Because Achondroplasia arises as a spontaneous mutation, absolute prevention is not possible. However, genetic counseling is helpful for " high-risk pregnancy", in which one or both parents have achondroplasia. Then the fetus has a 50% risk of having abonormality if one of the parents has achondroplasia. When both parents have Achondroplasia, the chance of their offspring of having Achondroplasia is 75%, and of having homozygous Achondroplasia is 25%.

For a high-risk pregnancy, routine prenatal ultrasound examination may identify short fetal limbs. Usually such ultrasonographic findings are not apparent until the third trimester. Thus, fetal genotyping is necessary. The detection of fetal FGFR3 gene mutation from maternal circulation is a non-invasive early diagnosis for Achrondronplasia.

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Part II: Summary of publications

Aim:

The aim of the present study was to understand the biochemical aspects of plasma DNA and cell-free fetal DNA, as well as to investigate the size distribution of fetal DNA in maternal circulation. The clinical applications for non-invasive prenatal diagnosis using size selection of fetal DNA from maternal plasma, especially in the aspect of detection of paternally inherited single gene mutations, were explored.

The study was also explored to investigate the clinical applications of urinary DNA for prenatal diagnosis, as well as on seeking sex-independent markers in urinary DNA for monitoring kidney transplant engraftment.

Knowledge of paternal RhD zygosity is important for consulting RhD negative pregnant women about the risk of HDN (hemolytic disease of the newborn). The aim of this study was mainly focused on the detection paternal RhD zygosity by real-time PCR assay.

The size of fetal DNA in maternal plasma and detection of paternally inherited point mutations by size selection of fetal DNA in maternal plasma (papers 1, 2, 3)

The studies of the plasma DNA from cancer patients have indicated that the plasma DNA displays apoptotic characteristics [30, 31]. We were curious as to whether maternal plasma DNA also displayed such features. We used Southern blot analysis of total circulating plasma DNA with the ubiquitous, highly repetitive Alu sequence. The results showed that oligonucleosomal fragments, which are the major characteristics of apoptosis, could be detected. The smallest size was approximately 180 bp, accompanied by DNA fragments two, three, or four times this size. There were also high molecular weight DNA fragments, larger than 10kb detected (Figure3). Such a DNA pattern was also present in plasma samples from non-pregnant female blood and from cord blood.

Our results supported the hypothesis that plasma DNA derives from apoptosis, in that we could readily discern oligonucleosomally cleaved fragments by Southern blot analysis. Furthermore, the large circulatory DNA species (>10kb) could be derived from the erythropoietic system because the DNA isolated from terminally differentiating erythroblasts exhibited similar characteristics (unpublished data from our group).

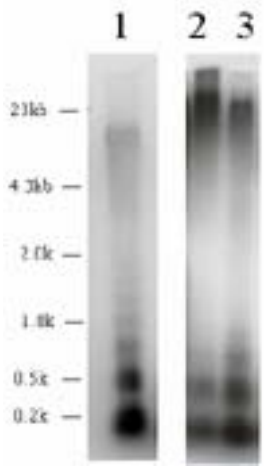

Figure 3: Sounthern blot analysis of plasma DNA.

1.plasma from cord blood

2.plasma from pregnant woman (GA: 13wks).

3.plasma from non-pregnant female control

The next question that we asked was, what is the size distribution of circulating fetal DNA in maternal plasma? For this analysis, we size-separated circulating DNA from pregnant women carrying a male fetus on agarose gel electrophoresis. Real-time PCR was carried out for the analysis of the proportions of fetal DNA and maternal DNA by quantification of the SRY gene on the Y-chromosome and of the GAPDH gene in size-fractionated fragments, respectively. Surprisingly, we found that most of the circulating fetal DNA consisted of fragments of less than 300bp, very little or no fetal DNA having a molecular size of more than 0.5kb. On the other hand, maternally-derived sequences were considerably larger than 0.5kb. Our results showed that in the early pregnancy, the percentage of enriched fetal DNA was 11.6-56.6 % (mean 28.4%) compared to 0.032-11.9 % (mean 3.4%) of non-enriched, whereas in the third trimester, the percentage of enriched fetal DNA was 22.2-87.1% (mean 68.7 %) compared to 2.33-11.4% (mean 6.2%) of non-enriched [51] (Figure4). Thus, circulating fetal DNA from maternal plasma can be enriched by size-selection of fragments.

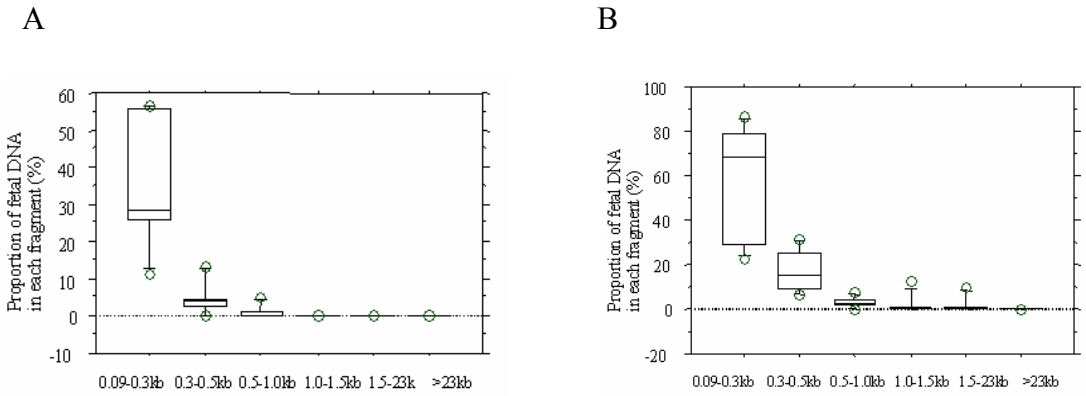

Figure 4 . Size distribution of circulating fetal DNA in maternal plasma. A) 6 samples were taken at early pregnancy (median gestational age: 13 +2 wks); B) 8 samples were taken at third trimester close to term (median gestational age: 34+4 wks).

By size-selection of circulating fetal DNA, we examined whether paternally inherited DNA polymorphisms could be detected in the maternal plasma samples. We used highly polymorphic STR sequences on chromosome 21 for the analysis. Our results showed that the paternally inherited STR allele was barely detectable in the total plasma DNA but was clearly present in the DNA fraction with a fragment size of less than 300bp both in samples from early pregnancies (mean gestational age: 13+3wks) and from third trimester (mean gestational age: 34+4wks) (Figure 5). This feature is very important because the analysis of such highly polymorphic markers can be very useful for the determination of fetal aneuploidies.

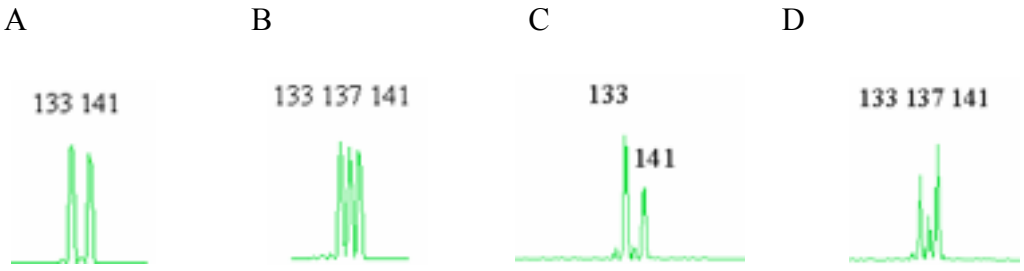

Figure 5. The example of STR analysis for a plasma sample from a mother carrying a fetus with trisomy 21 (gestational age: 13+6wks, STR marker: D21S1432). A) Maternal genotype; B) Fetal genotype; C) Analysis of total plasma DNA, paternally inherited allele 137bp not detectable; D) Analysis of plasma DNA with a size of less than 300bp, paternally inherited allele easily detected.

Another important aspect of our discovery is that it aids in the examination of paternally inherited single gene mutations, because these analyses should no longer be hindered by a large excess of maternal DNA sequences in the circulation.

In this regard, we first examined paternally-inherited FGFR3 (Fibroblast Growth Factor Receptor 3) mutation (G-A), which causes Achondroplasia, from maternal plasma [88]. Due to the FGFR3 mutation at 1138 creates a unique SfcI restriction site, we used the touchdown PCR to amplify the enriched fetal DNA, followed by digestion of the amplicon with SfcI enzyme. The digested fragments were visualized on 6% polyacrylamide gel with SYBR Green staining. Our results indicated that this approach permits a more precise detection of the fetal mutation allele, compared with conventional analysis of total plasma circulating DNA (Figure 6).

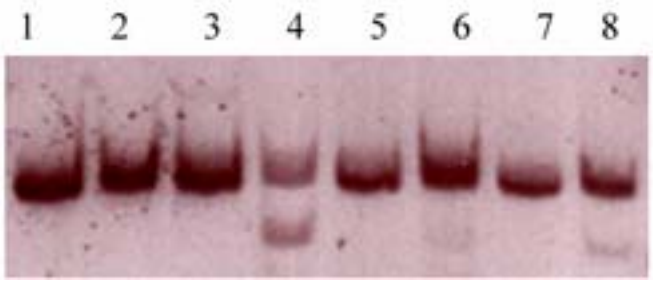

Figure 6. Restriction analysis of the PCR products on 6.0% polyacrylamid gel containing SYBRGreen. 1: Maternal DNA (undigested). 2: Maternal DNA (digested with SfcI). 3: Paternal DNA (undigested). 4: Paternal DNA (digested with SfcI). 5: Total circulatory DNA in maternal plasma (undigested). 6: Total circulatory DNA in maternal plasma (digested with SfcI). 7: Size-fractionated circulatory DNA in maternal plasma (undigested). 8: Size-fractionated circulatory DNA in maternal plasma (digested with SfcI).

We next examined the paternally inherited fetal point mutations in the β-globin gene, which cause β-thalassemia, from maternal plasma. This examination can exclude the risk for compound heterozygous pregnancies. Four common β-thalassemia point mutations: IVSI-1, IVSI-6, IVSI-110 and codon39, were detected from 32 clinical samples taken at

10-12 weeks of pregnancy (mean gestational age: 10+5wks). These samples were detected in a blind test and chosen on the basis that the father was a carrier for one of the 4 mutations described above, and the mother had been genotyped to carry another β-globin gene mutation. Circulating fetal DNA was enriched by size-fractionation and subjected to PCR with a Peptide nucleic acid (PNA) clamping. PNA sequences for maternal allele blocks the amplification of the normal maternal sequences. Thus, only mutant allele was amplified. The paternal mutant allele was then detected by allele-specific real time PCR, which was monitoring with SYBR Green Dye. We used a ΔCT system, whereby the amplification of the normal wild-type allele (CT_N) was subtracted from that of the mutant allele (CT_M), to discriminate the mutant allele from the normal allele. By the use of this $\Delta CT_{(M-N)}$ analysis, we observed a clear cutoff area distinguishing the mutant DNA and wild type DNA (paper 3, Figure 7). This analysis also showed that we would be able to detect the mutant allele in conditions when it only presented 6% of the total DNA in the sample.

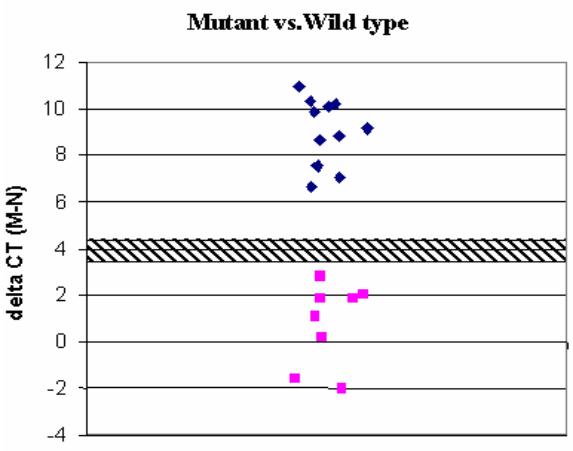

Fig 7. Example from Codon 39 mutation. Clear discrimination of the wild-type allele from the mutant allele, diluted in wild type DNA. Wild-type alleles are indicated by (◆) and mutant alleles diluted in wild type are indicated by (■).

For the clinical samples, the results were confirmed by CVS (chorionic villus sampling) test. One sample was excluded due to a lack of diagnostic result. Two were flagged as uncertain because the input DNA was too low to give reliable results. The presence or absence of the paternal mutant allele was correctly determined with more than 96.6% (28/29) accuracy. In comparison, the simultaneous assessment of total plasma DNA samples, without size-fractionation, resulted in almost 50% of the cases of paternally inherited allele being incorrectly evaluated.

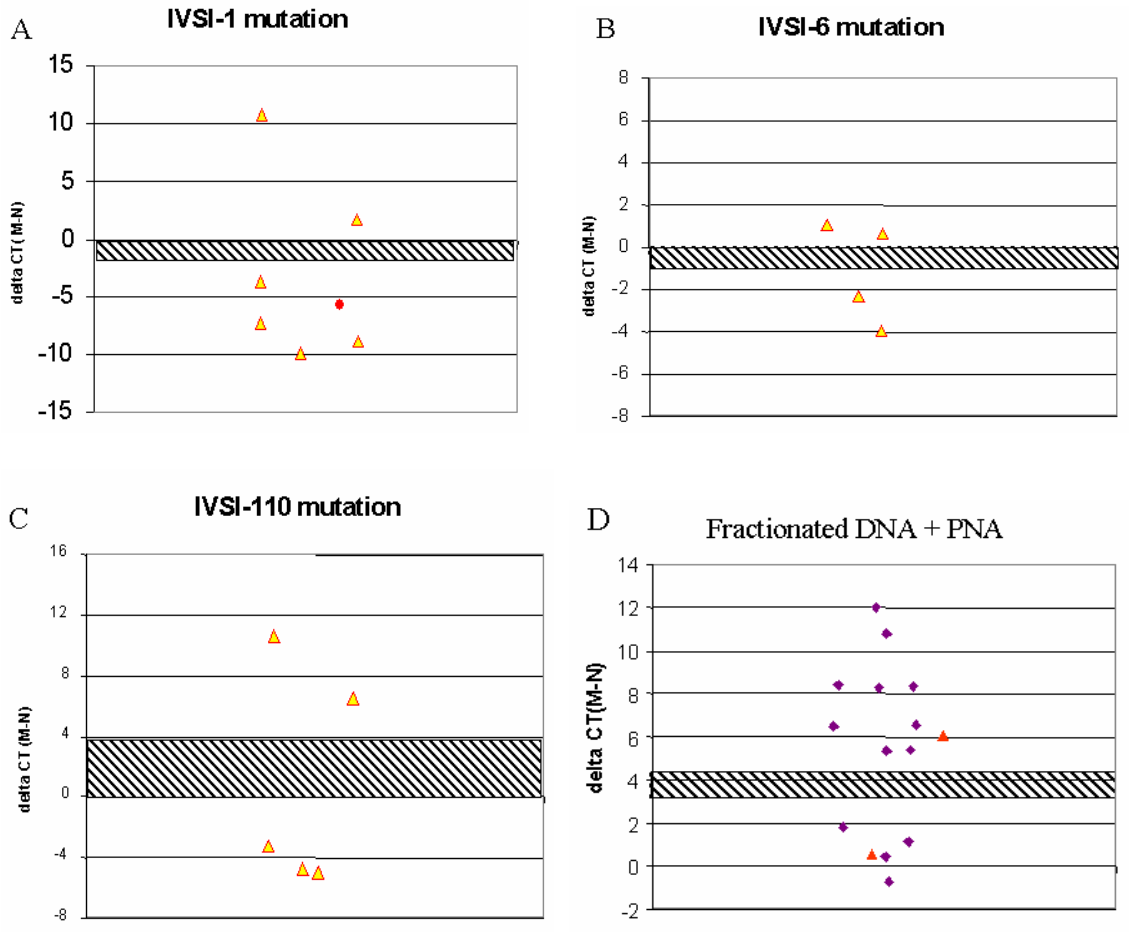

Figure 8. Scatter blot of the real-time PCR analysis for the IVSI-1, IVSI-6, IVSI-110 and Codon 39 mutations. The samples were clearly separated into two groups: the upper group was wild type allele, and the lower group was mutant allele.

The advantage of being able to detect paternally inherited mutant allele in maternal circulation is that their absence can be used to exclude pregnancies at risk for compound heterozygous, such as β thalassemia, thereby avoiding an invasive prenatal diagnostic procedure. The approach that we developed for the detection of low amount single gene mutations in wild-type background is relatively simple and could be used for routine clinical laboratories.

The studies also indicated that the size-fractionation of circulating DNA is very useful for non-invasive prenatal determination of fetal point mutations, as well as fetal aneuploidies. The enriched circulating fetal DNA might, in the near future, be useful for non-invasive prenatal determination of fetal genetic traits in clinic.

Urinary DNA is not a marker for prenatal diagnosis, but a marker for renal transplantation (papers 4, 5)

Studies have suggested that circulating fetal DNA is cleared very rapidly from maternal plasma [60]. Recently, two reports of particular interest have shown that fetal DNA could be detected in maternal urine [64, 65]. To the contrary, we were not able to reproduce these results (paper 4).

In the first of the reports, Botezatu and colleagues examined the urine samples from women immediately before the termination of first-trimester pregnancies (7-10wks). The second report, Al-Yatama et al examined urine samples from pregnant women between 7 and 40 wks gestation. In our report, we chose the samples close to term, in which the amount of cell free fetal DNA in maternal plasma is at a maximum [51], as well as 2 samples affected by pre-eclampsia-associated HELLP (hemolysis, elevated liver

enzymes, and low platelets) syndrome, in which condition the cell-free DNA in maternal plasma is significantly elevated [8, 70]. In the two cases, the cell-free fetal DNA in maternal plasma was as approximately 20-30 folds greater than normal controls (Table 2). If the circulating fetal DNA was cleared by kidney, it is expected that its presence in urine would be increased.

Table 2: Levels of total and fetal cell-free DNA in maternal plasma and urine

Subject	Total cell-free DNA in maternal plasma	Cell-free fetal DNA in maternal plasma	Total cell-free DNA in maternal urine	Cell-free fetal DNA in maternal urine
C1	15,867.2	215.8	12,561.6	0.0
C2	27,046.3	224.7	19,443.2	0.0
C3	7986.6	0.0	9898.2	0.0
P1	358,463.1	6683.1	11,214.2	0.0
P2	9,528,103.1	4088.7	11,256.1	0.0

C = pregnant women with normal outcome; P = pregnancy affected by HELLP syndrome.

Values are indicated as genome equivalents/mL urine

C3 gave birth to a girl; hence, no fetal DNA was detectable in maternal plasma

Botezatu et al used the Guanidine/Promega Wizard Resin methods for extracting urine DNA, instead of commercial DNA extraction kits. They emphasized that commercial DNA extraction kits can lead to the loss of the low-molecular weight DNA during the DNA isolation step. To address this issue, we compared the efficacy of DNA extraction method that we used, namely High pure PCR templates Purification kit (Roche), to that of the other two methods used by Botezatu et al and by Al-Yatama et al, respectively, by quantifying the levels of total DNA with real time PCR assay specific for the GAPDH gene. The results showed that the total levels of urinary cell-free DNA were significantly

greater using the method we had chosen. Furthermore, we isolated plasma DNA with Roche column for 1% agarose gel electrophoresis and subsequent southern blot analysis. We could observe the small DNA fragments about 180bp either on the agarose gel or on the blot hybridized with Alu sequences (Figure 3). Thus it is unlikely that our failure to detect any fetal DNA in maternal urine resulted from the incorrectness of the plasma DNA isolation.

One further possibility for the discrepancy between our data and that of Botezatu et al is that they detected the highly repetitive Y-chromosomal target-DYZ1, with repeats up to 5000 times per male cell in maternal urine by nested PCR assay. Using such highly repetitive sequences is very prone to contamination. Furthermore, if fetal DNA in maternal urine can only be detected by such an approach, then it is of limited value, as most genes of interest clinically exist as single-copy genes. Of interest is that Al-Yatame et al detected a single-copy gene on Y-chromosome by nested PCR assay. However, the sensitivity for detection of fetal DNA in maternal urine was only 38%. Such a low sensitivity, compared to > 95% of examining plasma fetal DNA, is not good enough for clinical diagnosis.

The exact mechanisms of plasma fetal DNA clearance have remained incompletely understood. Botezatu and colleagues's studies supposed the hypothesis that kidney play a role in the clearance of plasma fetal DNA. However, our data, especially those from the analysis of samples affected by HELLP syndrome strongly denied the hypothesis made by Botezatu et al. As we know, the kidneys of pregnant women affected by HELLP syndrome are damaged, as determined by the presence of elevated levels of urinary albumin. Despite increase of renal permeability and of cell-free fetal DNA in maternal

plasma, the cell-free fetal DNA was not able to be detected in maternal urine, more interestedly, the total urinary DNA from those samples was not elevated compared to the controls (Table2). Lau et al showed that in pre-eclampsia, cell-free fetal DNA had an increase in half-life of almost 2 hours compared with approximately 15 minutes in normal control pregnancies [89]. Our data and those of Lau et al directly argue against the potential role of the kidney in the clearance of cell-free DNA from maternal plasma. The reason for this is that under the conditions of increased renal permeability, cell-free DNA displays a decreased rate of clearance from the maternal circulation.

That the inability to detect fetal DNA in maternal urine indicated that the fetal DNA in maternal circulation is removed by other mechanisms such as by the liver. Because the liver is affected under the conditions of pre-eclampsia and especially HELLP syndrome, it is expected that the rate of removal be reduced, thereby leading to greater accumulation of these molecules in the peripheral circulation as described by Lau and colleagues. Alternatively, there are other mechanisms that maybe involve in the clearance of plasma fetal DNA. (more discussion see part : clearance of circulating fetal DNA)

After renal transplantation, quantitative analysis of urinary donor-derived DNA has indicated that it may serve as a new marker to monitor kidney engraftment. However, the quantitative analysis of donor-derived DNA in the recipient's urine by real-time PCR assay relies on the analysis of the Y chromosome specific gene in the cases in which female recipient had received male kidney. We examined the donor-derived DNA in recipient's urine by analysis of donor-derived microsatellite markers (STR) in those cases in which the donor and the recipient were either of the same sex or the donor was female

and the recipient male. Our results showed that donor-specific STR alleles could be detected in all 5 cases (Figure 8).

A B C

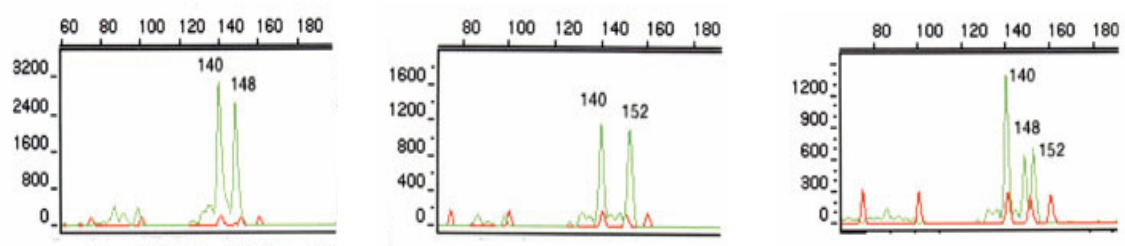

Figure 8:Capillary electropherograms of D21S1432 microsatellite amplicons as detected with use of the ABI 310 automated sequencer. A).donor genotype; B) recipient genotype; C) recipient urine.

We next examined the genetic polymorphisms in the glutathione S-transferase M1 (GSTM1) gene and angiotensin-converting enzyme (ACE) [90]. We were able to obtain an informative case in which the GSTM1 gene was present in the donor and was absent from the recipient. Our quantitative analysis of this sample indicated that the donor-derived DNA in the recipient's urine was very high immediately after transplantation, and decreased dramatically by day 7 (The patient with successful transplantation). The concentration of total cell-free urinary DNA by analysis of the GAPHD gene presented a similar curve pattern. The result also indicated that almost the entire cell-free DNA in the recipient urine was donor-derived (Figure9).

Our and others data indicated that measuring urinary DNA is a non-invasive diagnosis for monitoring graft rejection. Microsatellite assay is a good alternative detection. However, the caveat of this analysis is labor consuming and cannot be used for precise quantification. The polymorphic GSTM1 gene and ACE gene are new alternative

markers for real time PCR assay. The limited usefulness of the two genes is only ~50% of the population does not posses the locus and the informative pairs of donor-recipient for this locus would have the donor possessing the GSTM1 gene and the recipient possessing the null allele. In the future, the new markers for PCR-based assays will need to be developed to guarantee effective analysis for all donor-recipient constellations.

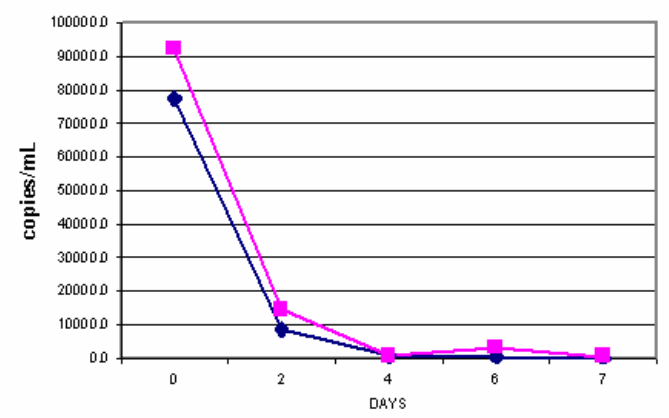

Fig 9. Changes in the concentrations of GSTM1 gene and GAPDH gene after transplantation.

Determination of paternal RhD zygosity using real-time PCR assay (paper 6)

For the pregnancies of sensitive RhD-negative women, it is important to know the paternal RhD genotype because the knowledge of the paternal RhD zygosity situation is essential for counseling a couple about the risk of HDN (hemolytic disease of the newborn) in future pregnancies. 56% of Rh-positive Caucasian individuals are heterozygous at D locus [91]. The father, heterozygous at RhD locus, has a 50% chance of producing a RhD-positive fetus, whereas homozygous produces only a RhD-positive fetus.

Recent development of the real-time PCR technique has shown that it is sufficiently sensitive to detect differences in gene dosage even as low as 3:2. To test the possibility that the real-time PCR could be used to determine the paternal RhD genotype, we

examined 39 samples obtained from males who had been serologically typed to be RhD. We designed two multiplex real-time quantitative PCR assays to simultaneously detect the RhD gene in relation to the SRY sequence and the GAPDH sequence, respectively. In theory, the ratio of RhD/SRY from a homozygous father is 2:1 and the ratio of RhD/GAPDH is 2:2. On the other hand, the ratio of RhD/ SRY from a heterozygous father is 1:1 and the ratio of RhD/ GAPDH is 1:2. Thus, The RhD zygosity was determined by calculating the RhD gene relative dosage. Our study showed two assays, which were in complete concordance (Figure 10). In the sample cohort of 39 samples we determined that 26 (66%) were heterozygous for the RhD gene and 13(34%) were homozygous.

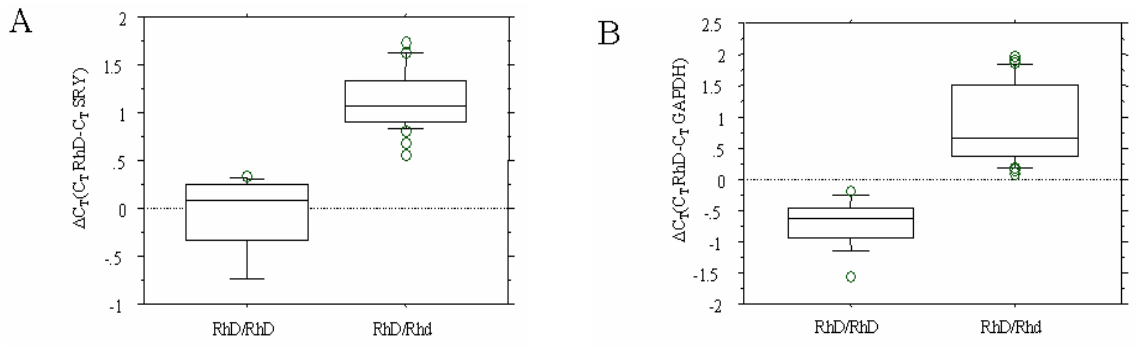

Figure 10. Box-plot showing the discrimination of RhD zygosity by real time PCR. A). The RhD gene has been examined in comparison with the SRY gene. B). The RhD gene has been examined in comparison with the GAPDH gene.

The prediction of paternal RhD genotype is very useful in conjunction with the maternal plasma fetal DNA-based test for fetal RhD status, for better prevention and management of hemolytic disease of the newborn.

Although our data do indicate that real-time PCR assay can potentially be used for the determination of RhD zygosity, we caution against the premature clinical use of this technology, since the efficacy has not yet been determined in large scale studies, nor is it yet known how they may be influenced by the RhD polymorphisms prevalent in many ethic populations.

Conclusions:

Maternal plasma DNA displays apoptotic characteristics, and circulating fetal DNA exists in maternal plasma as small fragments of less than 300bp. The latter feature provides a method to enrich circulating fetal DNA from maternal circulation. Such a selection permits easier to detect paternally inherited DNA polymorphisms and fetal single gene mutations from maternal plasma.

Cell-free fetal DNA is not readily detected in maternal urine, even under conditions known to increase kidney permeability. The result showed that circulating fetal DNA is not cleared by kidney. Thus, urine DNA is not a marker for prenatal diagnosis. However, the quantitative analysis of urinary DNA indicated that it can server as a marker for monitoring and prognosticating the graft rejection of renal transplantation.

Real-time PCR can be used for discrimination of paternal RhD zygosity, which is an alternative non-invasive risk-free manner to predict fetal RhD genotype.

Future directions:

A long-term goal of all obstetricians and gynecologists is the development of simple, rapid, accurate, non-invasive tests for prenatal diagnosis. There is no doubt that fetal genetic material in maternal circulation is the first target for this purpose.

Although there are many exciting aspects that have been well covered in this research field, especially diagnostic applications, some issues about circulating DNA remain to be elucidated. For example, the tissue sources of the circulating DNA, their mechanisms of production and metabolism, and the functional role of the fetal DNA in the maternal circulation.

To date, the quantification of the fetal DNA in maternal circulation depends mainly on gender dependent marker, namely Y chromosome–specific sequences. That is limited to only male fetuses. Therefore, gender independent markers need to be developed. Furthermore, only paternally inherited alleles could be detected in maternal circulation. It is technically difficult to identify the fetal origin of maternally inherited alleles because of maternal DNA background. For example, the detection of paternally inherited β-globin gene mutations in maternal circulation can only exclude heterozygous β thalassemia major. Techniques need to be developed to determine the fetal genotype in those instances where both partners are carriers for the same disease allele. Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOP) mass spectrometry would be a good solution for those cases [92].

Our discovery that circulating fetal DNA exists in maternal plasma as small fragments would speed the non-invasive prenatal diagnosis by the size-selection of circulating fetal DNA. However, more tests and a large number of samples are needed to be done to

confirm its use in clinical applications. The enrichment methods of circulating fetal DNA should be updated to look for more efficient and more rapid procedures.

More recently, increased awareness has been paid to the existence of fetal RNA in the plasma of pregnant women. Those studies indicated that fetal-derived mRNA might be used as gender independent markers that provide a suitable screening tool for pregnancy-associated disorders. However, a significant amount of work remains in selecting the specific fetal-derived markers as well as the selection of appropriate samples handling and detection methods due to the instability of the target molecules and variable efficiencies of the reverse transcription step. With the rapid advancements being made in molecular techniques, plasma RNA has the potential to transform molecular diagnosis in the near future.

Publication list:

Peer reviewed publications:

1. **Y. Li**, B. Zimmerman, C. Rusterholz, A.Kang, W.Holzgreve and S.Hahn. **Size separation of circulatory DNA in maternal plasma permits ready detection of fetal DNA polymorphisms.** Clinical Chemistry 2004 50: 1002-1011

2. **Y. Li**, W. Holzgreve, L. Christiaens, JJP. Gille and S. Hahn. **Improved prenatal detection of a fetal point mutation for achondroplasia by the use of size-fractionated circulatory DNA in maternal plasma – case report.**
Prenatal diagnosis 2004 24:896-898

3. **Y. Li**, E. Di Naro, A. Vitucci, B. Zimmermann, W. Holzgreve and S. Hahn. D**etection of paternally inherited fetal point mutations for β- thalassemia by the use of size-fractionated cell-free DNA in maternal plasma**. JAMA 2005 293:843-849

4**. Y. Li**, X.Y. Zhong, A. Kang, C. Troger, W. Holzgreve and S. Hahn. **Inability to detect cell free fetal DNA in the urine of normal pregnant women nor in those affected by preeclampsia associated HELLP syndrome.** The Journal of the Society for Gynecologic Investigation. 2003, 10: 503-508

5. **Y. Li**, D. Hahn, X.Y. Zhong, P.D. Thomson, W. Holzgreve, and S. Hahn. **Detection of Donor-specific DNA Polymorphisms in the Urine of Renal Transplant Recipients.** Clinical Chemistry 2003, 49: 655-658

6. **Y. Li,** B. Zimmerman, X. Y Zhong, A. Gupta, W. Holzgreve and S. Hahn. **Determination of Rhesus D Zygosity Using Real-time Quantitative PCR**. Swiss Medical Weekly 2003, 133:442-445

7. I. Hoesli, M. Danek, D. Lin, **Y. Li**, S. Hahn and W. Holzgreve. **Circulating Erythroblasts in Maternal Blood Are Not Elevated Before Onset of Preterm Labor.** Obstet and Gyneco. 2002, 100: 992-996

Book contributions:

1. X.Y. Zhong, **Y. Li**, W. Holzgreve and S. Hahn. **Primer Extension Preamplification (PEP) of single cells: Efficiency and Bias.** PCR Technology: Current Innovations, Second Edition. Editors: Thomas Weissensteiner, Hugh G.Griffin and Annette Griffin. Chapter 35, p343-349.

2. S. Hahn, X.Y. Zhong, S. Hristokova, B. Zimmermann, **Y. Li**. **Prenatal diagnosis using fetal cells and cell free fetal DNA in maternal blood: current status in Basel.** Early Prenatal Diagnosis, Fetal Cells and DNA in the Mother -present state and perspectives. Editors: Milan Macek Sr, Diana W.Binachi and Howard Cuckle. P 183-193.

Part III: Publications

Paper 1

Size separation of circulatory DNA in maternal plasma permits ready detection of fetal DNA polymorphisms

Y. Li, B. Zimmerman, C. Rusterholz, A. Kang, W. Holzgreve and S. Hahn

University Women's Hospital/Department of Research, University of Basel, Basel, Switzerland

Published in: Clinical Chemistry 2004, 50: 1002-1011

Paper 2

Improved prenatal detection of a fetal point mutation for Achondroplasia by the use of size-fractionated circulatory DNA in maternal plasma – case report

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Published in: Prenatal Diagnosis 2004, 24: 896-898

Paper 3

Detection of paternally inherited fetal point mutations for β- thalassemia by the use of size-fractionated cell-free DNA in maternal plasma

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Published in : The journal of the American Medical Association 2005, 293:843-849

Paper 4

Inability to detect cell free fetal DNA in the urine of normal pregnant women nor in those affected by preeclampsia associated HELLP syndrome

Y. Li, X.Y. Zhong, A. Kang, C. Troger, W. Holzgreve and S. Hahn

University Women's Hospital/Department of Research, University of Basel, Basel, Switzerland

Published in: The Journal of the Society for Gynecologic Investigation 2003, 10: 503-508

Paper 5

Detection of Donor-specific DNA Polymorphisms in the Urine of Renal Transplant Recipients

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Published in: Clinical Chemistry 2003, 49: 655-658

Paper 6

Determination of Rhesus D Zygosity Using Real-time Quantitative PCR

Y. Li, B. Zimmerman, X. Y Zhong, A. Gupta, W. Holzgreve, and S. Hahn

University Women's Hospital/Department of Research, University of Basel, Basel, Switzerland

Published in: Swiss Medical Weekly 2003, 133: 442-445

Paper 7

Circulating Erythroblasts in Maternal Blood Are Not Elevated Before Onset of Preterm Labor

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Published in: Obstetrics and Gynecology 2002, 100: 992-996

Circulating Erythroblasts in Maternal Blood Are Not Elevated Before Onset of Preterm Labor

Irene Hoesli, MD, Milan Danek, MD, Dexin Lin, Ying Li, Sinuhe Hahn, PhD, and Wolfgang Holzgreve, MD

OBJECTIVE: Preterm labor has recently been reported to be associated with an increased release of cell free fetal deoxyribonucleic acid (DNA) into the maternal circulation. We have previously observed increases in both fetal cell traffic and cell free fetal DNA in preeclamptic pregnancies. In this study, we investigated whether fetal cell traffic is also disturbed in pregnancies with preterm labor.

METHODS: In a case-control study, we examined 47 pregnancies complicated by preterm contractions that occurred between 20 and 34 weeks' gestation and an equal number of matched controls. Erythroblasts were enriched for by magnetic cell sorting and enumerated. These values were then correlated with subsequent pregnancy outcome.

RESULTS: In the study group 16 patients delivered prematurely (subgroup A). The other 31 (subgroup B) delivered at term, as did all those in the control group. No significant difference was noted in erythroblast numbers between either one of the subgroups and the controls.

CONCLUSION: Contrary to the reported increased levels of free fetal DNA in maternal serum, erythroblasts in maternal blood are not elevated significantly in pregnancies with threatened premature labor or in those that deliver preterm. (Obstet Gynecol 2002;100:992–6. © 2002 by The American College of Obstetricians and Gynecologists.)

Research into the use of fetal cells (specifically, erythroblasts) enriched from the maternal circulation as a noninvasive method for prenatal diagnosis has yielded some interesting new insights into pathologic conditions of pregnancy.[1,2] Our group made the novel observation that significant elevations in fetal cell traffic into the maternal periphery occur in pregnancies affected by preeclampsia.[3] In a large-scale prospective study,[4] we showed that this disturbance occurs as early as 20 weeks' gestation in pregnancies at risk for preeclampsia. Similar observations have been made in independent studies.[5] In addition, increases in fetal-maternal cell traffic have been noted in a pregnancy with polyhydramnios[6] and in pregnancies with certain fetal aneuploidies. It is currently unclear if fetal cell traffic is elevated in pregnancies with growth-retarded fetuses, because of conflicting reports in the literature.[4,7,8]

A recent observation that has received much attention in this field is that of cell free fetal deoxyribonucleic acid (DNA) in maternal plasma or serum.[9] As the analysis of this material is relatively facile by polymerase chain reaction (PCR), it has been shown that it can be readily used for the analysis of certain fetal genetic traits, such as fetal sex and rhesus D status in pregnancies with a rhesus constellation.[1,10] The development of real-time PCR methods has permitted the accurate quantitation of this acellular fetal genetic material.[1] By the use of this technology, we and others have shown that cell free fetal DNA levels are elevated in a manner analogous to fetal cells in pregnancies affected by preeclampsia[11,12] and hydramnios[6] and in pregnancy with trisomy 21 fetuses.[13,14]

Of particular interest is a recent report made by Leung et al,[15] who found that pregnancies at risk for preterm labor were associated with elevated levels of cell free fetal DNA. In this report it was proposed that these elevations in cell free fetal DNA concentrations might be able to distinguish between true and false preterm labor. Because prematurity is one of the major unresolved problems in perinatal medicine,[16] the ability to distinguish between true and false labor would be of considerable obstetric benefit. To evaluate this phenomenon more closely, we have investigated whether fetal cell traffic is altered in a manner similar to that of the reported release of cell free DNA in pregnancies with threatened prematurity.[15] Previous studies from our group using both PCR and fluorescent in situ hybridization (FISH)[3,6,17] have indicated that a significant proportion of the erythroblasts in maternal blood are of fetal origin. Therefore, in this study we made no attempt to distinguish between the two groups, but have solely used erythroblasts identified by morphology as a marker of fetal-maternal cell traffic.

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The authors thank Dr. Maria Sophocles for editorial assistance.

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MATERIALS AND METHODS

We conducted a case-control study with approval from our institutional review committee. Pregnant women with singleton pregnancies between 20 and 34 weeks' gestation, who were admitted to our university's department of obstetrics and gynecology because of preterm contractions, were asked to participate in the study. The gestational age was confirmed by first-trimester ultrasound in all cases. Preterm contractions were defined as four or more contractions every 20 minutes according to the Canadian preterm labor investigators' group.[18] We excluded pregnancies with known fetal malformations as well as those who received in utero–administered glucocorticoid application for lung maturation. An equal number of control patients matched for gestational age were included at the time of blood sampling on a one to one basis. Control patients were recruited from our ambulatory care service. They all belonged to a low-risk group and all delivered at term (more than 37 + 0 weeks' gestation). In all cases we obtained written informed consent before blood sampling. In all patients, cultures for bacterial vaginosis, *Ureaplasma urealyticum*, and group B streptococcus were performed.

For the enrichment of fetal erythroblasts, 20 mL of heparinized venous blood was collected. All samples were analyzed using our well-established protocol, whose performance we have previously validated.[3,4,6,19] Analytic protocol included a single 1077 Ficol density gradient (Sigma, St. Louis, MO) and separation with magnetic cell sorting (Milteny Biotec, Bergisch Gladbach, Germany) using anti–CD 71 conjugated with magnetic microbeads (Milteny Biotec). The positively enriched cell fraction was transferred on to glass slides by cytocentrifugation (Shandon, Frankfurt, Germany), and the number of erythroblasts was enumerated after May Grünwald staining (Sigma) using an Axioscope light microscope (Carl Zeiss, Jena, Germany). All blood samples were processed immediately or stored at room temperature up to a maximum delay of 24 hours. Analysis of erythroblast numbers was carried out without the knowledge of the outcome of the pregnancy.

To determine the size of the study, a power analysis was performed based on the results published by Leung et al,[15] which indicated that we needed to examine 34 patients and an equal number of controls for a power of 80% and a significance of .05. The variance of the number of fetal cells in maternal blood was taken from our previous findings.[20] The data were analyzed using the SPSS statistics software package for Windows (SPSS Inc., Chicago, IL).

Table 1. Pregnancy Characteristics

	Study group (n = 47)	Control group (n = 47)	P
Age (y; mean ± SD)	29.91 ± 4.63	29.11 ± 6.55	.491*
GA at time of blood sampling (mean ± SD)	27.9 ± 3.9	27.7 ± 3.9	.853*
GA at delivery (mean ± SD)	36.2 ± 5.0	40.3 ± 1.1	<.001*
Preterm labor [n (%)]	47 (100)	0	<.001†
Preterm delivery [n (%)]	16 (34)	0	<.001†
Delivery at term [n (%)]	31 (66)	47 (100)	
Vaginal bleeding [n (%)]‡	6 (12.8)	0	.026†
Preterm rupture of membranes [n (%)]‡	3 (6.4)	0	.242†
IUGR (<5th percentile) [n (%)]‡	4 (8.5)	1 (2.13)	.361†
Infections (GBS, BV, *Ureaplasma urealyticum* [n (%)]‡	20 (42.55)	4 (8.5)	.003†

SD = standard deviation; GA = gestational age; IUGR = intrauterine growth restriction; GBS = group B streptococcus; BV = bacterial vaginosis.
* Unpaired t test.
† Fisher exact test.
‡ Overlapping possible.

RESULTS

The maternal characteristics and gestational ages at delivery are summarized in Table 1. Of the study group, 16 pregnant women delivered prematurely (subgroup A), whereas 31 delivered at term (subgroup B). Table 2 summarizes the data of all 16 patients who delivered preterm, including the various and overlapping risks for prematurity and the number of erythroblasts. All the pregnant women in the control cohort delivered healthy, normal babies at term. In the study group we recorded six cases with vaginal bleeding, three instances of premature rupture of membranes, and four deliveries of growth-retarded fetuses. Twenty instances of infections (positive culture for bacterial vaginosis, *Ureaplasma urealyticum*, and/or group B streptococcus) occurred in the study group and four similar instances in the control group. No patients developed preeclampsia. Four patients in the study group had polyhydramnios, and two of them delivered preterm. Erythroblasts were elevated in maternal blood of nine patients who delivered preterm and in one patient who delivered at term. No patients in the control group developed polyhydramnios.

Our study on erythroblast numbers indicated that no significant difference was discernible between the study group and control group, regardless of whether premature contractions were associated with preterm delivery (Table 3 and Figure 1).

Table 2. Specific Data on the 16 Preterm Patients

Patient No.	GA at time of blood collection	GA at delivery	NRBCs	Vaginal bleeding (3)	PROM (1)	IUGR (2)	Vaginal infection (7)
1	20	21	12	−	−	−	+
2	20	24	0	−	−	−	+
3	25	36	3	−	−	−	+
4	27	29	0	−	−	+	−
5	25	26	0	+	−	−	+
6	26	33	0	+	−	−	+
7	27	35	10	−	−	−	+
8	28	34	34	−	−	−	−
9	30	30	23	−	−	−	−
10	30	34	0	−	−	−	+
11	32	34	0	−	−	−	−
12	32	37	0	+	−	−	−
13	32	36	6	−	−	−	−
14	34	34	6	−	−	+	−
15	34	37	12	−	−	−	−
16	34	35	3	−	+	−	−

NRBC = nucleated red blood cell; PROM = premature rupture of membranes. Other abbreviations as in Table 1.

DISCUSSION

Previous studies have shown that certain pregnancy-related pathologies such as preeclampsia are associated with an increased traffic of fetal cells into the maternal periphery.[3,4] Recent studies using real-time PCR have also indicated that the release of cell free DNA is affected in a similar manner.[21]

Because increments in cell free fetal DNA have recently been reported to precede the onset of true preterm labor,[15] we have examined whether the traffic of fetal cells is affected in a similar manner. In our study we examined a cohort of 47 pregnant women hospitalized at our institution with premature contractions, of whom 16 delivered their newborns prematurely and 31 at term. A control cohort of 47 gestationally matched controls was examined at the same time. Our enumeration of enriched erythroblasts from these samples indicated that there was no significant difference between any of the groups. Because we have previously shown that almost half the erythroblasts in the maternal circulation are of fetal origin, both by FISH[3] and single-cell PCR,[17] the total number of enriched erythroblasts can be used as a reliable marker for fetal cell traffic.

From our results two conclusions can be drawn:

1. Preterm contractions are not associated with an increased traffic of fetal cells into the maternal periphery.
2. Fetal cell traffic is not elevated in those pregnancies with subsequent premature delivery.

These data imply that the placenta provides a relatively impermeable barrier because gross physiologic pressures, such as those that occur during contractions, do not lead to an increased influx of fetal cells into the maternal periphery. They also indicate that the placental

Table 3. Numbers of Enriched Erythroblasts in the Study Group With Preterm Delivery (Subgroup A), the Study Group With Term Delivery (Subgroup B), and With the Matched Control Group

	Study group			
	Subgroup A (n = 16)	Subgroup B (n = 31)	Control group (n = 47)	P
Median no. of erythroblasts (range)	2* (0–24)	1† (0–74)	2*† (0–29)	.6*‡ .8†‡
Blood sampling to delivery interval (mean ± SD) (d)	25.13 (± 2.06)*	57.11 (± 34.76)†	86.46 (± 24.0)*†	.001*‡ .09†‡
GA at delivery (mean ± SD)	30.88 ± 5.07*	39.64 ± 1.32†	40.45 ± 1.21*†	.001*‡ .7†‡

Abbreviations as in Table 1.

P values for the two sections of the study group are shown relative to the control cohort.

*‡ Unpaired t test: subgroup A vs control group.

†‡ Unpaired t test: subgroup B vs control group.

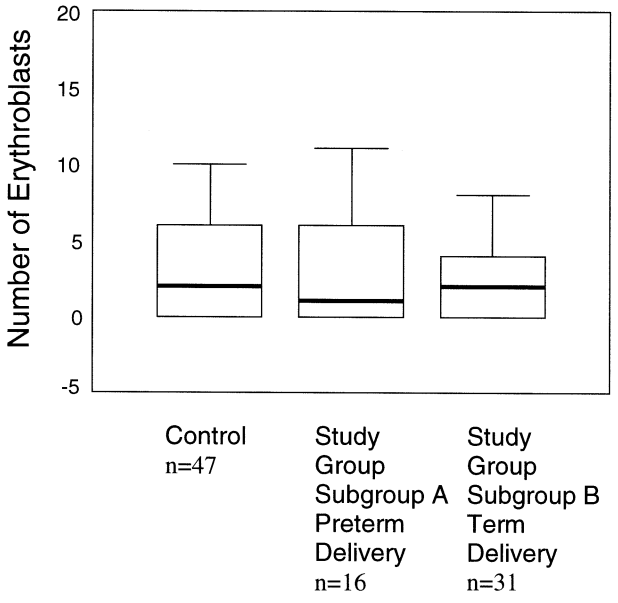

Figure 1. Box plot illustrating the numbers of enriched erythroblasts in pregnancies with true prematurity (subgroup A) and those with threatened prematurity (subgroup B) in comparison to a control cohort. Values shown are median *(heavy rule)*, interquartile range *(box limits)*, and extreme values *(error bars)*.

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changes leading to preterm delivery are not such that they lead to an increase in fetal-maternal cell traffic.

Consequently, a notable feature of our study is that our data concerning fetal cell traffic do not parallel those of Leung and colleagues[15] regarding the release of cell free fetal DNA in pregnancies with preterm labor. Currently the relationship between these two parameters is unclear because the main source of cell free fetal DNA appears to be the placenta, whereas fetal cell traffic occurs when a few rare fetal hemopoietic cells actually traverse the placenta. Indeed, studies from our laboratory have indicated that there is no significant correlation between the levels of cell free fetal DNA and fetal cell numbers in normal or preeclamptic pregnancies.[22] This suggests that these two phenomena may occur independently of each other. Consequently, it is possible that certain conditions, such as preeclampsia, are associated with a placental lesion leading to both the increased release of cell free fetal DNA and an influx of fetal cells into the maternal periphery. On the other hand, in preterm labor it appears that only the release of cell free fetal DNA but not fetal cell traffic is affected. It will be of interest to examine cell free fetal DNA levels in the described study group and to compare these to fetal cell levels, as this will indicate the relationship between these two parameters. A further consequence of our findings is that alterations in fetal cell traffic do not have predictive value in determining pregnancies at risk for premature delivery in contrast to pregnancies at risk for preeclampsia.[4]

Increased levels of fetal DNA in the maternal periphery without increased fetal-maternal cell traffic, however, could mean that some degree of increased cellular apoptosis or necrosis in the placenta may precede premature labor.

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Determination of RHD zygosity using real-time quantitative PCR

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Summary

At present RHD incompatibility is still an obstetric problem despite prophylactic treatment. A very welcome recent technical advance has now made it possible to determine the foetal RHD status in a non-invasive risk-free manner using cell free foetal DNA in maternal plasma. In some cases, however, where there is a high risk that the foetus may be affected by HDN (haemolytic disease of the newborn), it may be of interest to determine whether the father is hetero- or homozygous for the RHD gene, since in the former instance there is only a 50% chance that the pregnancy is affected. It has recently been shown that quantitative PCR assays, in particular real-time Taqman PCR, can be used to determine the RHD gene dosage, and also to determine foetal aneuploidies. We demonstrate that the same real-time Taqman PCR assay we had previously developed for non-invasive analysis of the foetal RHD gene and the foetal Y chromosome from maternal plasma can be used to determine the paternal RHD genotype.

Key words: RHD; paternal genotype; prenatal diagnosis; real-time PCR

The determination of RHD zygosity has until recently been a tedious procedure and is usually inferred from the serotype, family history and/or complex PCR-based RFLP assays [1–3]. To address this issue, two recent publications have shown that quantitative PCR assays can be used to accurately genotype the predominant Caucasian RHD locus. In the first instance, Chiu and colleagues determined RHD zygosity using a real-time Taqman PCR assay in which the dosage of the RHD gene was compared with a control locus, namely the albumin gene [4]. In the second approach, described by Pertl and colleagues, a quantitative fluorescent PCR assay compared the dosage of the RHD gene to that of the related RhCE gene locus [5]. A further development of the real-time PCR approach by our group has shown that this technology is sufficiently sensitive to detect even smaller differences in gene dosage, namely those which occur in foetal aneuploidies, in which instance only a 50% increase in gene copy number occurs and not 100% as is the case for the RHD gene [6].

Since it is only of interest to determine the RHD genotype of the male partner (the mother by definition being RHd), we were curious as to whether a real-time Taqman PCR assay we had previously developed for another purpose, non-invasive risk-free determination of foetal RHD status and sex from maternal plasma [7], could be used for the determination of RHD zygosity.

The Taqman® real-time PCR assay centres upon the detection of a fluorescent signal generated from the cleavage of a target sequence specific probe by the Taq polymerase during each cycle of the PCR reaction [8]. As this signal is directly proportional to the PCR product being amplified, it permits very precise quantitation of the amount of initial input template. This is ascertained from the so-called threshold cycle, also termed the C_T value, the point where the exponential phase of the amplification curve crosses a defined threshold line. As this C_T value is a reflection of the number of PCR cycles required to reach this threshold, the lower the C_T value is, the higher is the concentration of input target template.

By using a real-time PCR assay in which two genetic loci are amplified simultaneously in a multiplex reaction, it is possible to determine the relative ratio of these two loci by subtracting their respective C_T-values, e.g.:

$\Delta C_T = C_T$ (target A) – C_T (target B) = C_T (RHD) – C_T (SRY)

Since 1 cycle entails a doubling of the PCR product, the ratio of RHD: SRY = $2^{-(\Delta CT)}$

Hence, if both the RHD and SRY genes are present with the same gene dosage, e.g. 1 copy (heterozygous RHD/RHd), the difference in

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threshold cycle number (ΔC_T) will be 0 cycles, whereas if 2 copies of the RHD gene are present (homozygous RHD/RHD) the difference in threshold cycle number (ΔC_T) will be 1 cycle. It should be noted that these are theoretical values and that slight differences are bound to occur due to minor variations in the PCR assay, especially if one reaction proceeds with slightly greater efficiency than the other.

These minor deviations are bound to occur even if considerable care has been taken to optimise the paired PCR reactions in such a manner that no significant difference is discernible between their efficiencies, as measured by the slope of the PCR assays [4, 6].

Precautions which need to be taken in order to obtain a correct result include the use of multiplex PCR reactions whereby both target gene sequences are analysed simultaneously in the same reaction vessel, as well as the inclusion of genotypically defined samples in each analysis [4, 6, 9].

By using such precautions it has previously been shown by Chiu and colleagues [4] that real-time PCR can be used to determine the paternal RHD genotype. More recently we have shown that real-time PCR can also be used to detect more subtle increments in gene dosage (only 50%), such as those occurring in foetal trisomies [6].

To test the possibility that the real-time PCR assay we had previously developed for analysis of the foetal RHD and SRY genes in maternal plasma [7] could be used to determine the paternal RHD genotype, we examined 39 DNA samples obtained from males who had been serologically typed to be RHD (Swiss Red Cross Blood Bank, Basel, Switzerland). To confirm the accuracy of our assay we also determined RHD zygosity in these samples using a modification of the real-time PCR described by Chiu and colleagues [4]. In our investigation the dosage of the RHD gene was compared to another control locus, which in our case was the GAPDH (glyceraldehyde 3-phosphate dehydrogenase) gene and not the albumin gene. The reason for this modification was that we have previously described the use of the Taqman real-time PCR assay for this GAPDH control locus in a number of studies, using either cell free DNA [7, 10] or genomic DNA [6]. In our analysis, all samples were run in triplicate.

The results of our analysis indicated that the samples in our investigation clustered into two specific groups, one having a median of almost 0 and the other of approximately 1 (see Figure 1A).

To ascertain the genotype of these two groups we also tested our samples using a modification of the assay described by Chiu and colleagues [4], whereby we compared the dosage of the RhD gene with that of the GAPDH gene [6]. Here we would expect an opposite pattern, as the GAPDH gene is normally present in 2 copies, i.e. the RHD/RHD genotype should yield a ΔC_T value of 0, whereas the RHD/RHd genotype should differ by 1 cycle from the C_T value for the GAPDH reference. In this analysis we again found that the samples

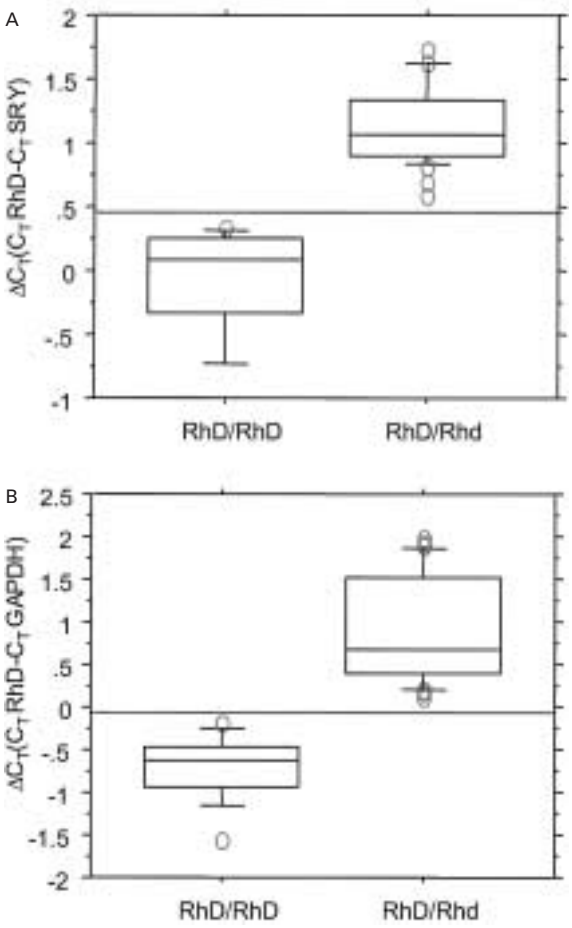

Figure 1

Box-plot showing the discernment of RHD zygosity by real-time PCR. In Figure 1A, the RHD gene has been examined in comparison with the SRY gene. In Figure 1B, the RHD gene has been examined in comparison with the GAPDH gene. The lines inside the boxes indicate the median value, whereas the upper and lower limits of the boxes represent the 75th and 25th percentiles respectively. The upper and lower horizontal bars indicate the 90th and 10th percentiles respectively. Outliers are indicated by open circles. The X axis indicates the RHD genotype determined, whereas the Y axis indicates the pertinent ΔC_T values. No overlap was observed between the two genotypic groups. The cut-off values are indicated by stippled lines.

clearly clustered into two groups, one with a median ΔC_T value of approximately –0.5 and the other a median ΔC_T value of approximately 0.5 (see figure 1B). It is of interest that a 100% concordance was observed between the 2 groups of samples in both assays.

Interpretation of the results, however, is not as straightforward as it would seem, due to a significant deviation from the expected ΔC_T values. In this manner, even though the expected ΔC_T values in the first SRY:RHD assay would be –1 for the RHD/RHD genotype and 0 for the RhD/Rhd genotype, it is apparent that the PCR reaction for the SRY gene has proceeded with slightly better efficiency than that for the RHD gene. Consequently the expected RHD:SRY ΔC_T value in the case of the RHD/RHD genotype has shifted up from the theoretical value of –1 to almost 0, whilst that for the RHD/RHd genotype has similarly also been shifted up by 1 cycle from the theoretical value of 0 to almost 1.

In an analogous manner the GAPDH PCR reaction has proceeded slightly more efficiently than that for the RHD gene, resulting in a shift in the expected RHD:GAPDH ΔC_T value. In this case the ΔC_T value for the homozygous RHD/RHD genotype has shifted down by almost half a cycle from the theoretical value of 0 to almost –0.5. Equally, that for the heterozygous RHD/RHd genotype has been shifted down by 0.5 cycles from the theoretical value of 1 to almost 0.5.

We have previously indicated that to counter these unwanted shifts it is possible to use ΔC_T values, whereby the C_T value of the sample being analysed is compared to a mean C_T value comprised of the analysis of a large number of samples of known genotype [6, 9]. Furthermore, these drifts in ΔC_T values stress the importance of including samples of known genotype in each analysis, to counter inter-run variations [4, 6, 9]. It is also imperative to analyse the samples in a multiplex manner whereby both target genes are analysed simultaneously in the same reaction vessel, and not to attempt this type of analysis by comparing the assessed gene dosage against a standard curve [4, 6, 9].

An important feature that is evident from our analysis is that the two groups can be segregated with 100% accuracy by the use of particular cut-off values. With regard to the RHD/SRY assay, we determined that a cut-off ΔC_T (C_T RHD – C_T SRY) value of 0.5 could be used to distinguish between the heterozygous (RHD/RHd) and homozygous (RHD/RHD) genotypes, in that the values for the RHD/RHd genotype had ΔC_T values which clustered around 1, while RHD/RHD genotype had ΔC_T values which clustered around 0 (Figure 1A). No overlap between the two groups was found to occur.

Similarly, for the RHD/GAPDH assay, a ΔC_T (C_T RHD – C_T GAPDH) cut-off value of 0.0 could be used to distinguish the homozygous RHD/RHD genotype form the heterozygous RHD/RHd one. In this test the heterozygous (RHD/RHd) genotypes have ΔC_T values above 0.0, while the homozygous (RHD/RHD) genotype had ΔC_T values below 0.0 (Figure 1B). Once again, no overlap between the two groups was found.

As explained previously, the reason for the difference in the ΔC_T cut-off values between these two assays is that for the RHD/GAPDH assay the RHD gene is compared with both alleles of the GAPDH gene on chromosome 12, whereas for the RHD/SRY assay, the dosage of the RHD gene is compared to the single SRY allele on the Y chromosome.

The validity of our analysis is underscored by our subsequent examination of 3 samples known to be from RHD heterozygous males, in which case we were able to determine the correct genotype in a blinded manner (data not shown).

In the sample cohort of 39 samples we determined that 26 (66%) were heterozygous for the RHD gene (RHD/RHD) and 13 (34%) were homozygous (RHD/RHD). Once again there was complete concordance between the 2 assays. Although the expected frequency of RHD heterozygosity would be 56% [1], it is probable that our results are slightly skewed by the small number of cases examined.

Although our data obtained by the use of two independent real-time PCR assays do serve to indicate that this technology can potentially be used for the determination of RHD zygosity, we have also shown that the employment assays and their subsequent analysis require considerable experience if a correct diagnostic outcome is to be achieved. Furthermore, the data also emphasise the importance of running genotypically well defined control samples in parallel with the sample being analysed, as the theoretically expected ΔC_T values cannot be used. Our study also indicates the usefulness of running two independent analyses in parallel as a potential safeguard against erroneous results, a feature we have also observed previously when attempting to discern foetal trisomies by the use of real-time PCR [6, 9].

Despite these promising results we caution against the premature clinical use of these assays, since their efficacy has not yet been determined in large scale studies, nor is it yet known how they may be influenced by the RHD polymorphisms [1–3] prevalent in many ethnic populations.

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Inability to Detect Cell Free Fetal DNA in the Urine of Normal Pregnant Women nor in Those Affected by Preeclampsia Associated HELLP Syndrome

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OBJECTIVE: *Recent reports have indicated that cell-free fetal DNA can be detected in the urine of pregnant women. We attempted to reproduce those data.*

METHODS: *Urine samples were collected from 18 normal pregnant women (11 with a male fetus). Urinary DNA was examined by Y-chromosome-specific nested polymerase chain reaction (PCR) or real-time PCR. Samples were also examined from two pregnancies complicated by HELLP (hemolysis, elevated liver enzymes, and low platelets) syndrome, which had very high levels of cell-free fetal DNA in the maternal plasma. To validate our data, a quantitative comparison of different DNA extraction procedures used in the previous reports was performed.*

RESULTS: *In no instance were we able to detect any fetal DNA in maternal urine, although copious quantities of cell-free fetal DNA were present in the maternal plasma of those pregnancies affected by HELLP syndrome. Our quantitative analysis of the various extraction procedures used indicated that the commercial column elution method we used was comparable, if not superior, to the noncommercial methods used in previous reports.*

CONCLUSION: *Our data strongly suggest that cell-free fetal DNA is not readily detectable in maternal urine, even under conditions known to increase kidney permeability. (J Soc Gynecol Investig 2003;10: 503–8)* *Copyright © 2003 by the Society for Gynecologic Investigation.*

KEY WORDS: Fetal DNA, maternal urine, PCR, prenatal diagnosis, preeclampsia, HELLP syndrome.

Currently two main approaches have emerged for noninvasive risk-free detection of fetal genetic traits, namely, the enrichment and isolation of fetal cells from the maternal circulation or the analysis of extracellular fetal DNA in maternal plasma or serum.[1,2] Recent reports have indicated that cell-free fetal DNA may be detected by polymerase chain reaction (PCR) in maternal urine, thereby raising hopes of a noninvasive method for prenatal diagnosis.[3,4]

In the first of these reports, Botezatu and colleagues[3] attempted to address an important issue regarding the very short half-life of cell-free DNA in the peripheral circulation.[5] They hypothesized that cell-free DNA may be cleared by the kidney. In their studies they examined urine samples obtained from women immediately before the termination of first-trimester pregnancies, in whom they were able to detect Y-chromosome-specific sequences in eight of ten cases with a male fetus. No false-positive results were observed in any of the nine control cases, who were pregnant with female fetuses. Their studies further indicated that the DNA present in the urine had a size of less than 200 bp.

The second report, by Al-Yatama and colleagues,[4] examined urine samples from 80 pregnant women. In their study using a nested PCR assay they were able to detect Y-chromosome-specific sequences in 38% of the urine samples from women carrying a male fetus. One false-positive result was recorded.

Encouraged by those reports, we attempted to confirm those data. In our study, we examined urine samples from normal healthy pregnancies, as well as two cases affected by preeclampsia-associated HELLP (hemolysis, elevated liver enzymes, and low platelets) syndrome. The reason for examining this specific pregnancy-related disorder is that we and others have previously observed that the levels of circulatory fetal DNA are significantly elevated in such conditions.[6–8] Furthermore, renal function is known to be affected in such affected

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Preliminary data from this study were presented at the Second Conference on Circulation Nucleic Acids in Serum and Plasma (CNAPS), Hong Kong, February 20–21, 2001, and have been published in the conference proceedings (reference 18).

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pregnancies, evidenced by the enhanced presence of protein in the urine.[9] Hence, it is to be expected that, if cell-free DNA is cleared by the kidney, its presence in urine may be enhanced under conditions of preeclampsia or the therewith associated HELLP syndrome.

MATERIALS AND METHODS

Sample Collection

This study was approved by the Cantonal Institutional Review Board of Basel, Switzerland. Informed consent was requested in all instances. We chose to examine pregnancies close to term, as it has been shown that the amount of cell-free fetal DNA in maternal plasma is then at a maximum.[5] Urine samples were carefully collected from 20 pregnant women; 18 of them delivered normal healthy babies and had an unremarkable pregnancy history, the other two pregnant women had manifest HELLP syndrome. This was defined by blood pressure over 160/110 mm Hg on at least two occasions, proteinuria of at least 5 g on a 24-hour urine collection, hemolysis (total bilirubin $>$ 1.2 or lactate dehydrogenase $>$ 600 IU/L), elevated liver enzymes (aspartate aminotransferase $>$ 70 IU/L) and low platelets ($<$100,000 platelets/dL), unremitting headache, nausea, visual impairment, and right upper quadrant pain. Both fetuses were delivered prematurely (28 weeks and 32 + 6 weeks) and had growth retardation, with fetal weights at delivery of 840 g and 1250 g.

To examine the efficacy of the different DNA extraction procedures, samples (n = 12) were collected from healthy nonpregnant volunteers.

Sample Processing

The urine samples were collected in standard Sarstedt Monovette tubes (Sarstedt, Sevelen, Switzerland) used for the collection of blood samples. These tubes contained 1.6 mg potassium ethylenediamine tetraacetic acid (KEDTA)/mL total volume, as the presence of this chelating agent may aid in the stabilization of cell-free urinary DNA by inactivating any nucleases present. The same tubes were used for the collection of maternal blood samples, thereby reducing the margin of error which could be caused by inappropriate sample collection. Urine samples were centrifuged at 1200 $\times$ g for 10 minutes. The supernatant was recentrifuged at 3000 $\times$ g for 10 minutes to ensure that it was cell free. The cell-free urine was aliquoted and stored at $-$20C.

DNA was extracted using High Pure PCR Templates Purification Kit (Roche, Basel, Switzerland) according to the manufacturer's protocol. An 800-μL cell-free urine sample was used for DNA extraction. The DNA was eluted with 50 μL of elution buffer. The DNA was then either analyzed by a highly sensitive nested PCR assay or real-time PCR. We have used this approach for the successful analysis of cell-free fetal DNA in maternal plasma and transplant-derived cell-free DNA in the urine of kidney transplant recipients.

To compare the three different extraction protocols, we split each urine sample in three parts. For the method used by Botezatu and colleagues,[3] cell-free DNA was extracted from 2.5 mL of the cell-free urine sample using 3.75 mL of 6 M guanidine isothiocyanate (Invitrogen Life-Technologies/GIBCO-BRL, Basel, Switzerland), 400 μL of a commercial DNA binding resin, and coupled column technology (Wizard Minipreps DNA Purification Resin; Promega, Madison, WI) as described by those authors.[3] The DNA was eluted with 50 μL of sterile distilled water and then analyzed by real-time PCR.[7] We also examined the method used by Al-Yatama and colleagues,[4] where again the 2.5-mL cell-free portion of the paired urine sample was used. In this method, the DNA from the 2.5-mL cell-free urine aliquot was extracted using 1.5 mL of a solution containing 6M guanidine isothiocyanate (Invitrogen), 13 mM ethylenediamenetetra-acetic acid (EDTA) (Sigma-Aldrich, St. Louis, MO), 0.5% sodium N-lauryl-sarcosine (Sigma-Aldrich), 10 μg glycogen (Sigma-Aldrich), and 26 mM Tris-HCl (pH 8.0) (Sigma-Aldrich). This mixture was incubated at 60C for 10 minutes, after which the DNA was precipitated by addition of 4 mL isopropanol (Sigma-Aldrich) and incubated at $-$20C for 15 minutes. After high-speed centrifugation (12,000 $\times$ g for 15 minutes), the pellet was carefully washed with 70% ethanol and resuspended in 50 μL TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). Finally, 50 μL 2% Chelex-100 (Sigma-Aldrich) was added to remove inhibitors.

Nested and Real-Time PCR

A multiplex PCR was performed to amplify the β-globin and SRY genes simultaneously. This assay has been described in detail previously, where we have used it both for the analysis of single cells and well as cell-free fetal DNA in the maternal circulation.[10,12,13] Briefly, the 50-μL PCR amplification mix contained 5 μL of template DNA, 25 pM of each external primer pair, 1.5 mM magnesium chloride, 300 nM dNTPs, and 2 IU *Taq* polymerase (Promega). Amplification was done using a hot start at 95C for 5 minutes and 30 subsequent cycles of 95C for 30 seconds, 55C for 30 seconds, and 72C for 1 minute. For the nested PCR, a 1-μL aliquot of the external PCR product was transferred to a second 20-μL PCR reaction mix containing 10 pM of the nested internal primer pairs, 1.5 mM magnesium chloride, 300 nM dNTPs, and 2 IU *Taq* polymerase (Promega). The amplification was carried out as described above. The PCR products were analyzed on a 2% agarose gel.

Real-time PCR specific for the SRY and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) genes was carried out using a Perkin Elmer Applied Biosystems 7700 Sequence Detector (Taqman; Perkin Elmer Applied Biosystems, Boston, MA) and conditions previously established in our laboratory for the analysis of cell-free fetal DNA in maternal plasma.[7] In brief, the 25-μL amplification reaction mixture consisted of 2 μL of template DNA, 300 nM of each primer, 150 nM of dual-labeled Taqman probe, 12.5 μL TaqMan Universal PCR Master Mix, 3.5 mM magnesium chloride, 100 mM dNTPs, 0.025 U Ampli*Taq* Gold, and 0.01 U Amp Erase (Perkin-Elmer). The following conditions were used for the PCR

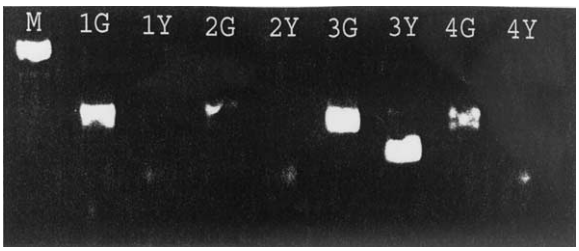

Figure 1. Analysis of total cell-free DNA and male fetal DNA in maternal urine by multiplex nested PCR. Total cell-free DNA was detected by a PCR assay for the ubiquitous β-globin gene (G). Male fetal DNA was detected by an assay specific for the SRY gene on the Y chromosome (Y). M = 25-bp DNA ladder molecular weight marker (Invitrogen). G = β-globin PCR product (240 bp); Y = SRY gene PCR product (133 bp). Samples 1 and 2 = amplification of urine samples from pregnant women carrying a male fetus. No Y-chromosome-specific DNA was detectable. Sample 3 = positive control from male genomic DNA, in which both globin- and SRY-specific signals were detected. Sample 4 = negative control from female genomic DNA, in which only the globin-specific amplicon was detected.

amplification: an initial incubation at 50C for 2 minutes and at 95C for 10 minutes, followed by 40 cycles of 60C for 1 minute and 95C for 15 seconds. Each sample was analyzed in triplicate. The concentration of the DNA template was determined from a standard curve, which was included in each real-time PCR analysis.

RESULTS

In our initial set of experiments we obtained 15 urine samples from women with normal pregnancies. Eight of these women were pregnant with singleton male fetuses. Extracellular DNA was extracted from these samples using commercial column technology, which we previously found to be effective for isolating cell-free fetal DNA from maternal plasma.[7,10,11] In this study we made extensive use of commercial column technology marketed by Roche; however, we previously obtained analogous results with technology marketed by Qiagen.[10,11]

For the analysis of these urine samples we used a highly sensitive nested PCR assay specific for the SRY gene on the Y chromosome, which we had previously shown to be suitable for the analysis of single fetal cells and cell-free fetal DNA in maternal plasma.[10,12,13] Despite the sensitivity of this assay, we were not able to detect any Y-chromosome-specific sequences in the maternal urine samples from those pregnancies bearing male fetuses (Figure 1). No false-positive results were recorded in the pregnancies with female fetuses. DNA was present in all of these samples, which was evident from the results obtained using a PCR assay specific for the ubiquitous β-globin gene (Figure 1).

Our data were therefore in disagreement with the reports of Botezatu et al[3] and Al-Yatama et al.[4] One possibility for this discrepancy could be the use of different DNA isolation procedures. In this regard, the procedure used by Botezatu and colleagues involved a denaturing step with guanidinium isothiocyanate, followed by adsorption of the DNA on commercial column technology (Wizard column; Promega).[3] Al-Yatama and colleagues also used a guanidinium isothiocyanate step, followed by conventional precipitation of the DNA using isopropanol.[4]

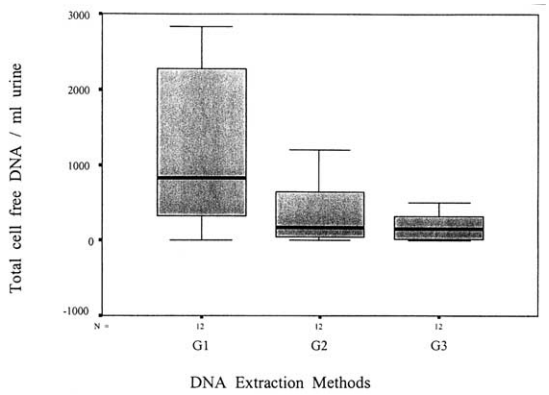

Figure 2. Box plot illustrating the levels of total cell-free DNA obtained from the same urine samples using three different extraction methods. The concentration of total cell-free DNA was quantified using real-time PCR for the GAPDH gene. The median and range values are indicated in Table 1. The lowest, second lowest, middle, second highest, and highest box points represent the 10th, 25th, median, 75th, and 90th percentiles, respectively. Extreme outliers have been excluded from the graphical representation to aid clarity. Although the median DNA concentration obtained by the Roche column technology (G1) appears to be the highest, no significant difference was noted when compared with the two other methods (G2 and G3, Botezatu et al[3] and Al-Yatama et al[4]).

For this reason, we investigated whether these two protocols were more effective than the method we had chosen, and could thereby account for the discrepancy in the results we had obtained. Consequently, we compared the efficacy of the three methods on the same urine samples (n = 12), by quantifying the levels of total free DNA as determined by real-time PCR specific for the ubiquitous GAPDH gene.[7] Surprisingly, these experiments indicated that the total levels of cell-free DNA obtained were significantly greater using the method we had chosen (Figure 2 and Table 1). It is, therefore, unlikely that our failure to detect any fetal DNA in maternal urine resulted from a technical deficit.

We and others have previously reported that circulatory fetal DNA concentrations are elevated in maternal plasma in pregnancies affected by preeclampsia[6,7] and especially HELLP syndrome.[7,8] Therefore, we also examined urine samples from two such patients. As controls, we examined urine and maternal plasma samples from three normal healthy pregnant

Table 1. Comparison of the Three DNA Extraction Methods Tested on the Same 12 Urine Samples Using Real-Time PCR for the GAPDH Gene

Method	Median	Range
Roche column technology[7]	832.5	0–16, 238.4
Botezatu et al[3]	177.3	0–16, 057.2
Al-Yatama et al[4]	154.2	0–21, 369.6

Values are genome equivalents/mL of urine.

Table 2. Levels of Total and Fetal Cell-Free DNA in Maternal Plasma and Urine

Subject	Total cell-free DNA in maternal plasma*	Cell-free fetal DNA in maternal plasma*	Total cell-free DNA in maternal urine	Cell-free fetal DNA in maternal urine
C1	15, 867.2	215.8	12, 561.6	0.0
C2	27, 046.3	224.7	19, 443.2	0.0
C3†	7986.6	0.0	9898.2	0.0
P1	359, 463.1	6683.1	11, 214.2	0.0
P2	9, 528, 103.1	4088.7	11, 256.1	0.0

C = pregnant women with normal outcome; P = pregnancy affected by HELLP syndrome.
Values are indicated as genome equivalents/mL urine.
* Total cell free DNA was measured using a real-time PCR assay for the GAPDH gene, and cell free fetal DNA was detected using a assay specific for the Y chromosome.
† C3 gave birth to a baby girl; hence, no male fetal DNA was detectable in maternal plasma.

women. Two of them delivered a healthy boy, the other a girl. We examined both the level of cell-free fetal DNA in the maternal plasma and that in maternal urine. This study found that the concentration of cell-free fetal DNA in the maternal plasma was indeed highly elevated in pregnancies affected by the preeclampsia-associated HELLP syndrome (4088 copies and 6683 copies/mL plasma, respectively), compared with slightly over 200 copies/mL of maternal plasma detected in the control pregnancies (Table 2). No male fetal DNA, however, was detected in the maternal urine of any of these samples tested.

DISCUSSION

Recent reports have indicated that cell-free fetal DNA may be detectable in the urine of pregnant women, thereby opening up the possibility for a noninvasive method of prenatal analysis of fetal genetic traits.[3,4] In our experiments we were not able to reproduce these results, although we took considerable precautions to ensure that we were not mislead by a technical deficit. These precautions included the use of two different PCR approaches, a highly sensitive nested PCR assay that we have successfully used for the analysis of single cells,[12,13] as well as a robust real-time PCR assay, which we have used for highly reproducible analysis of cell-free fetal DNA in maternal plasma.[8,11,13]

We have also made a quantitative comparison of the various DNA extraction protocols used, to ensure that we had extracted a comparable amount of cell-free DNA by our procedure. This study found that the method we had chosen was comparable, if not superior, to the other methods used. Although it can be argued that the Roche column we used is not as efficient for the absorption of small DNA molecules as the Promega Wizard column, we nevertheless feel confident that our results are correct, as we have used these columns on several hundred occasions for the analysis of cell-free fetal DNA in maternal plasma, most of which is in the form of small nucleosome-associated fragments.[14] By using a real-time PCR assay we were also able to determine that our amplification of DNA templates obtained by all three DNA extraction methods tested proceeded with equal efficiency, as we did not observe any significant alterations in the respective amplification profiles (data not shown). It is, therefore, unlikely that toxic guanidinium isothiocyanate compounds hindered the PCR analysis.

Furthermore, in a separate series of studies, we and others have shown that the Roche column DNA extraction method and PCR assays described above can be used successfully to analyze cell-free kidney transplant-derived DNA in the urine of kidney transplant recipients.[17–19]

One further possibility for the discrepancy between our data and that of Botezatu and colleagues[3] is that they used the DYZ1 highly repetitive sequence (3000–5000 reiterated copies). Our reason for not using this approach is that PCR assays for such highly repetitive sequences are very prone to contamination. Furthermore, if fetal DNA in maternal urine can only be detected by such an approach, then it is of limited value, as most genes of interest clinically exist as single copy genes. Of interest is that Al-Yatama and colleagues[4] performed a nested PCR assay for a single-copy gene previously described by Lo and colleagues.[15] Apart from the low sensitivity this group attained in their analysis of cell-free fetal DNA in maternal urine samples, the specificity of their assay, however, has to be questioned because, for their analysis of circulatory fetal DNA in maternal plasma, they obtained a specificity of only 88%, which is significantly lower than that which we and others have achieved using real-time PCR (sensitivity $>$ 95%; specificity 100%).[2]

Our data, however, not only challenge the premise that cell-free fetal DNA can be detected in maternal urine, but they also question the hypothesis made by Botezatu and colleagues that the short half-life of this material in the circulation can in part be accounted for by its clearance by the kidney.[3] This aspect is most evident from our data regarding the samples analyzed from pregnancies affected by HELLP syndrome, in which discernible damage to the kidneys was evident, as determined by the presence of elevated levels of urinary albumin. Yet, despite this evidence of increased renal permeability and the presence of copious amounts of circulatory fetal DNA in the maternal plasma (approximately 20- to 30-fold greater than in the controls), we were in both instances not able to detect any cell-free fetal DNA in these maternal urine samples.

A further interesting aspect of our data is that once again we observed that the total levels of cell-free DNA, which are representative of the levels of cell-free maternal DNA are dramatically elevated in pregnancies affected by HELLP syndrome compared with the control group (again approximately 30-fold), thereby confirming earlier observations.[7,8] In these two patients, however, no increase in the total levels of cell-

free DNA were noted in their urine. Indeed, the cell-free urinary DNA levels were of the same order as those recorded in the control group.

Hence, these combined data regarding cell-free fetal and maternal plasma DNA suggest that neither of these cell-free DNA species is removed from the maternal circulation by the kidney. These data, however, do not address the origin of urinary cell-free DNA. This pertinent question has been explored most extensively by the use of kidney transplant recipients.[17–19] In those studies, it has been observed that most, if not all, of the cell-free DNA in the urine is derived from the donor kidney.[18,19] A further pertinent observation we made during these studies on kidney-transplant recipients was that the levels of transplant-derived cell-free DNA in recipient plasma did not correlate with the corresponding levels in recipient urine.[18] Because the cell-free DNA in the plasma does not appear to be transported by the kidney into the urine, this result implies that there are two different sources for these discrete cell-free DNA species: circulatory cell-free DNA in the plasma is derived from cells in direct contact with the blood, whereas urinary cell-free DNA is derived from cells in contact with the urinary system. This hypothesis is supported by a recent report indicating that almost all of the cell-free DNA in the circulation is derived from the hemopoietic system itself[20] and that the solid organs make only a small contribution to the total amount of circulatory cell-free DNA.[21]

Our data, furthermore, complement a recent report made by Lo and colleagues,[15] in which they found that clearance of cell-free fetal DNA from the maternal circulation was significantly reduced in pregnancies affected by preeclampsia. Lau and colleagues[16] showed that in preeclampsia, circulatory fetal DNA had an increase in half-life of almost 2 hours compared with approximately 15 minutes in normal control pregnancies. Therefore, our data and those of Lo et al[15] directly argue against the potential role of the kidney in the clearance of cell-free fetal DNA from the maternal circulation. The reason for this is that under conditions of increased renal permeability, evident from the entry of large protein molecules into the urine, cell-free DNA displays a decreased rate of clearance from the maternal circulation. From our data this is evident by the increased accumulation of both cell-free fetal and maternal DNA species in the plasma of the two patients with HELLP syndrome. If the premise of Botezatu and colleagues were correct, one would expect to see increased clearance of cell-free DNA from the maternal circulation by the urinary system under conditions of preeclampsia and HELLP syndrome, and as such one would expect a reduction in the levels of these two species in the maternal circulation. Because neither of these events appears to occur, ie, increased clearance from the periphery or increased presence of fetal and maternal cell-free DNA in maternal urine under conditions of increased renal permeability, it is highly unlikely that the kidney plays a role in the removal of cell-free DNA from the peripheral circulation.

The pertinent question that now remains to be addressed is how cell-free DNA is cleared from the circulation. Without delving too deeply into speculation, it is likely that the cell-free DNA is removed by detoxification organs, such as the liver. Because the liver is affected under conditions of preeclampsia and especially HELLP syndrome, it is to be expected that the rate of removal is reduced, thereby leading to greater accumulation of these molecules in the peripheral circulation as described by Lau and colleagues.[16] Alternatively, it is possible that circulatory cell-free DNA is absorbed and removed by the endothelium or even by circulating blood cells, because these cells are in constant contact with this material. The endothelium as well as the maternal immune system are also affected in preeclampsia, so one would again expect that these alterations could be associated with a decreased rate of clearance. It is clear, however, that further studies will be necessary to clarify this important aspect of circulatory cell-free DNA physiology.

In summary, our current investigations strongly suggest that cell-free fetal DNA is not present in maternal urine and rather that the observations made in the two previous reports may have been the result of spurious contaminants detected by the use of highly repetitive sequences or nested PCR protocols without adequate precautions.

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stable, showing a relative decrease in cholesteryl ester FAs and increase in phospholipid FAs. In both cholesteryl esters and phospholipids, very-long-chain n-6 FAs and n-3 FAs were the most reliable and stable. Eicosapentaenoic acid (C20:5n-3) and docosahexaenoic acid (C22:6n-3), both n-3FAs of marine origin, were highly reliable in all fat fractions (Table 1). Although the proportion of n-3 PUFAs was lower in cholesteryl esters than in erythrocyte membrane phospholipids (2.8% vs 9.4%), the variability was larger in cholesteryl esters than in erythrocyte membrane phospholipids (SD, 1.7% vs 1.4%).

Mailing blood samples offers a cost-effective approach and enables the study of large numbers of samples *(13)*. In practice, blood specimens can be sampled by phlebotomists at individuals' homes without the need for strict preanalytic procedures (i.e., direct centrifugation, separation, dispensing, and freezing). This approach ensures lower numbers of missing values, high comparability of groups, and thereby, a high internal validity of the study. We also tested whether direct centrifugation of SST tubes provided extra advantages, which was not the case.

Although the systematic error for some of these analytes (glucose, lipids, and C-reactive protein) was statistically significant, the degree of error was small (~3%), which is in accordance with studies that found a high stability during storage *(2–8)*. EDTA-plasma cholesteryl esters, serum cholesteryl esters, and phospholipids in EDTA-erythrocyte membranes yielded similar reliability coefficients. Major FAs and their composites were found to be especially reliable, but saturated FAs and minor FAs that constitute <1% of total FAs were less so. The CVs in the present study (~4–5%) were small when balanced first against the intra- and interassay measurement errors (~2–5%) and second against the within-person variability of FAs over time (~9%) *(14, 15)*. Essential n-6 and n-3 PUFAs were especially reliable, both in EDTA plasma and serum and, to a somewhat lesser extent, in erythrocyte-membrane phospholipids. These FAs are not synthesized endogenously, but their circulating concentrations depend on the amounts in foods and reflect dietary intake well *(16, 17)*.

We conclude that after a delay of 1 or 2 days in blood processing, glucose, lipids, C-reactive protein, and individual FAs adequately rank individuals according to baseline values. These analytes are generally stable after next-working-day mail delivery at room temperature; this procedure may therefore be suitable for many epidemiologic investigations. For the FA composition, use of EDTA plasma is the most practical and reliable, whereas for glucose, lipids, and C-reactive protein, plasma and serum are equivalent. Mailing blood samples offers a cost-effective approach for risk factor assessment with acceptable stability and reliability.

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Detection of Donor-specific DNA Polymorphisms in the Urine of Renal Transplant Recipients, *Ying Li,[1] Deirdré Hahn,[2] Xiao Yan Zhong,[1] Peter D. Thomson,[2] Wolfgang Holzgreve,[1] and Sinuhe Hahn[1*]* ([1] University Women's Hospital/Department of Research, University of Basel, CH 4031 Basel, Switzerland; [2] Division of Paediatric Nephrology, University of the Witwatersrand and Johannesburg Hospital, Johannesburg, South Africa; * address correspondence to this author at: Laboratory for Prenatal Medicine, University Women's Hospital/Department of Research, Schanzenstrasse 46, CH 4031 Basel, Switzerland; fax 41-61-325-9399, e-mail shahn@unbs.ch)

Recently, a novel form of chimerism, termed urinary DNA chimerism, has been described in kidney transplant recipients in that cell-free DNA from the donor kidney was detected in the recipient's urine *(1)*. Quantitative analysis of this urinary donor-derived DNA has indicated that it may serve as a new marker to monitor kidney

transplant engraftment because increased concentrations were present under conditions of graft rejection, which decreased to basal values after immunosuppressive treatment *(2)*. A caveat of these studies was that they relied on sex-disparate donor–recipient conditions: because the PCR assays used were specific for the Y chromosome, cell-free DNA from the donor kidney could be detected only in the urine of female recipients who had received male kidneys *(1, 2)*.

We examined whether other kidney donor-derived DNA sequences could be detected in the urine of transplant recipients, using PCR assays specific for highly polymorphic short tandem repeat (STR) loci, also termed microsatellite markers. Previous examinations using such polymorphic genetic loci have shown that they can be used for differentiating female fetal cells from maternal ones *(3, 4)* or for the gender-independent detection of cell-free fetal DNA in maternal plasma *(5, 6)*. For this purpose, we tested for the presence of donor-specific STR loci in the urine of cases in which the donor and recipient were either of the same sex or the donor was female and the recipient was male.

For our study, which was approved by our respective ethics review boards, five cases involving living-donor (four related and one unrelated) transplants were enrolled.

Blood samples from both the recipient and donor were obtained before the transplantation, and spontaneous urine samples were obtained from the previously anuric recipients post transplantation. Because there is some tentative evidence that DNA in urine can be stabilized by the presence of the chelating agent EDTA *(7)*, the urine samples were collected and shipped in standard Monovette tubes (Sarstedt) used for the collection of blood samples (containing 1.6 mg of potassium EDTA/mL of total volume).

Whole-blood DNA and cell-free urinary DNA were extracted with use of the High Pure PCR Template reagent set (Roche), according to the manufacturer's instructions.

The donor–recipient pairs were first genotyped using 100 ng of total genomic DNA to monitor microsatellite markers on chromosome 21 in a fluorescent PCR assay established previously in our laboratory *(3)*:

D21S11: forward, 5′-TAT GTG AGT CAA TTC CCC AAG TGA-3′; reverse, 5′-GTT GTA TTA GTC AAT GTT CTC CAG-3′
D21S1432: forward, 5′-CTT AGA GGG ACA GAA CTA ATA GGC-3′; reverse, 5′-AGC CTA TTG TGG GTT TGT GA-3′
D21S1435: forward, 5′-CCC TCT CAA TTG TTT GTC TAC C-3′; reverse, 5′-GCA AGA GAT TTC AGT GCC AT-3′
D21S1440: forward, 5′-GAG TTT GAA AAT AAA GTG TTC TGC-3′; reverse, 5′-CCC CAC CCC TTT TAG TTT TA-3′

The D21S11 and D21S1435 forward primers were 5′-labeled with the fluorescent dyes carboxyfluorescein (FAM) and 2,7-dimethyloxy-4,5-dichloro-6-carboxyfluorescein (HEX), whereas the D21S1432 and D21S1440 reverse primers were 5′-labeled with tetrachlorofluorescein (TET) and HEX, respectively. All primers were obtained form Microsynth Incorporated. This step allowed us to determine which of the STR loci on chromosome 21 were informative, in that a particular STR allele present in the donor genome was absent from that of the recipient. We should then be able to determine whether we could detect this donor-specific informative STR allele in the urine of the transplant recipient.

Because the concentration of cell-free DNA in urine was previously found to be very low *(2)*, this material was examined by use of a seminested PCR assay we have used previously for the analysis of single cells *(3)*. In this assay the following external seminested primers were used:

D21S11 (forward): 5′-GGG ACT TTT CTC AGT CTC CAT A-3′
D21S1432 (forward): 5′-TTC TAA AAG AAA TCA AAA TGA TGC-3′
D21S1435 (forward): 5′-TTG ACA TTC TTC TGT AAG GAA GAG-3′
D21S1440 (reverse): 5′-ATG TGT GAT TGC CAG CCT CTG-3′

Table 1. Microsatellite analysis of donor–recipient pairs and recipient urine.

		Allele size, bp		
Case	STR locus	Donor	Recipient	Urine
1	D21S11	216	216	216
		220		220
			242	242
	D21S1432	140	140	140
		148		148
			152	152
2	D21S11	216		216
		220	220	220
			248	
	D21S1435	172	172	172
			176	176
		184		184
3	D21S11	220		220
		228	228	228
			238	
	D21S1435	172		172
		176	176	176
			180	
4	D21S11	238		238
		242	242	242
	D21S1432	140	140	140
		148		148
5	D21S1435	168		168
			172	172
		176	176	176
	D21S1440	154		154
			164	164
		172	172	172

In brief, PCR amplification was performed in a total volume of 30 μL containing 100 ng of template DNA, 200 nM deoxynucleotide triphosphates, 10 pM each of the primers, 3.5 mM $MgCl_2$, and 1.5 U of AmpliTaq Gold (Applied Biosystems Inc.). After denaturation at 95 °C for 10 min, PCR was performed for 25 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s, with a final extension step at 72 °C for 7 min. For the urine samples, 1 μL of the PCR amplicon was used as template for a subsequent seminested PCR amplification. This nested PCR was performed as above except that the annealing temperature was increased to 58 °C. After amplification, the PCR products were analyzed by capillary electrophoresis on a *ABI* 310 gene analyzer (Applied Biosystems) equipped with GeneScan software (Applied Biosystems). Fluorescently labeled GeneScan 500 molecular weight markers were included in each run.

Our analysis of these STR loci showed that informative allelic differences could be obtained in each of the cases studied (Table 1). These were then used to study the corresponding urine samples. Subsequent analysis showed that donor-specific STR alleles could be detected in each case examined (Table 1 and Fig. 1). In general, the recipient urine samples contained both recipient- and donor-derived STR sequences (e.g., cases 1, 2, and 5) in that informative donor and recipient alleles could be detected in these samples. In one recipient (case 3), donor-derived sequences appeared to dominate in that the informative recipient allele was lacking. In case 4, the recipient was homozygous for both of the STR markers tested. In the urine of this patient, however, the unique donor-derived STR allele as well as the allele common to both donor and recipient were detectable for both the STR markers examined.

Because our investigation used a nested PCR assay in which a post-linear amplification phase amplicon was reamplified, no statement concerning the relative quantities of the donor and recipient cell-free DNA species is possible. For this reason, we examined two genetic polymorphisms in the glutathione *S*-transferase M1 (*GSTM1*) and angiotensin-converting enzyme (*ACE*) genes, recently described for the quantitative analysis of fetomaternal cell traffic and transfer of cell-free DNA *(8)*. Unfortunately, in our study, we were able to obtain an informative constellation only in a solitary instance for only one of these loci, i.e., the *GSTM1* gene, in which instance the gene was absent from the recipient. Of interest is that this case involved the transplantation of a kidney from an unrelated donor. Our analysis of this sample indicated that the recipient's urine contained >77 000 copies of cell-free donor-derived DNA/mL immediately post transplantation, which decreased to slightly more than 100 copies/mL of urine by day 7. The concentration of total cell-free DNA was initially determined to be >92 000 copies/mL of recipient urine, which decreased to 560 copies/mL of urine by day 7, based on a real-time PCR assay for the *GAPDH* gene *(9)*. This analysis indicated that almost all of the cell-free DNA in the recipient urine was donor-derived, a feature that is in good accord with previous reports *(1, 2)*.

The limited usefulness of the polymorphic *ACE* and *GSTM1* loci in our study could be a reflection of the rather small study size (only five cases). Nevertheless, it does indicate that assays for other markers will need be developed in the future to guarantee effective analysis of all donor–recipient constellations. Because we were readily able to detect informative donor-derived STR alleles in all of the samples tested, our results do suggest that it should

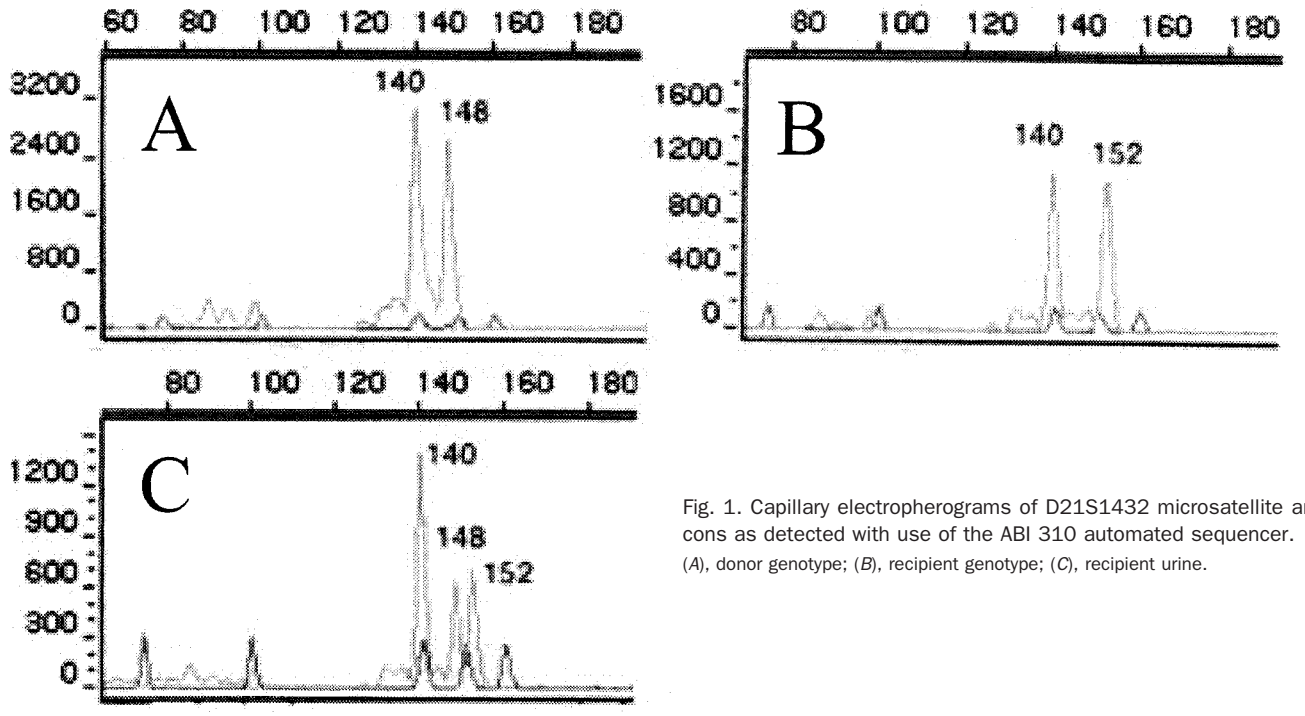

Fig. 1. Capillary electropherograms of D21S1432 microsatellite amplicons as detected with use of the ABI 310 automated sequencer.
(*A*), donor genotype; (*B*), recipient genotype; (*C*), recipient urine.

be possible to detect other polymorphic markers more amenable to quantification by real-time PCR.

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Measurement of Cortisol in Small Quantities of Saliva,

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The determination of cortisol in saliva has become popular for human research on stress reactions *(1–5)*. Depending on the sensitivity and reliability of the assays used, the required sample volume varies between 0.025 and 2 mL of saliva *(6–8)*. Infants and toddlers, however, often produce only small amounts of saliva and are usually sampled by swabbing the mouth with cotton dental rolls *(5)* or commercial cotton swabs (Salivette; Sarstedt Inc.) *(9)*, or by pipettes or alternative devices that aspirate saliva from the floor of the mouth *(10–13)*. Cotton rolls must either be centrifuged to obtain saliva *(9)* or be placed in the barrel of a syringe (needleless), from which the saliva is expressed into a vial by compression of the plunger *(5)*. With these procedures, saliva remaining in the swabs is thus lost for analysis. When we tested seven different types of cotton rolls, we found that, depending on the individual type, 135–450 μL of saliva could not be centrifuged from the rolls.

Oral stimulants (such as presweetened Kool-Aid crystals) can increase saliva production, but they affect the concentration of cortisol *(14)*. Finally, in the case of Salivettes, the material covering the cotton swab is hard and makes sampling unpleasant.

In this report, we present a new method that uses soft cotton swabs without hard covering material and solvent extraction of cortisol from saliva in the cotton.

Saliva was collected from volunteers in the laboratory and from infants and toddlers participating in studies on cortisol and behavior. Volunteers and the parents of the infants gave informed consent. These studies had been approved by the Medical Ethical Committee of the University Medical Center Utrecht. After collection, either direct or with use of cotton rolls, the samples were stored in closed containers at −20 °C for periods of up to several weeks. We placed 4-cm cotton rolls with a diameter of 8 mm (article no. 900-2005; Henri Schein) individually in disposable 5-mL syringes (PE + PP; Becton Dickinson), closed the syringes with a small plastic cap, and weighed them. For the saliva collection, the cotton roll was taken out of the syringe and the child's mouth was swabbed by introducing one end of the cotton roll into the buccal cavity. The experimenter moved the roll in the child's mouth, trying to induce sucking. To obtain as much saliva as possible, after 1–2 min, the experimenter took the roll out of the child's mouth, turned it around, and introduced the dry end into the child's mouth. After an additional 1–2 min, the cotton roll was put back in the syringe. The syringe was stored in the dark at −18 to −20 °C and later transported to the laboratory where it was once again weighed. The increase in weight was caused by the amount of saliva on the cotton, 1 mg being equivalent to 1 μL of saliva.

When the volume of saliva was 50–250 μL, cortisol was extracted from the cotton by opening the syringe at both sides and rinsing the cotton roll in the syringe with 1 mL of 960 mL/L ethanol, followed by centrifugation of the syringe at 1500*g* for 5 min. The resulting liquid was evaporated, and when the volume of saliva was <0.1 mL or the volume was equivalent to the volume of saliva collected, the residue was dissolved in 100 μL of 0.01 mol/L phosphate-buffered saline (pH 7.0) containing 2 g/L bovine serum albumin. After the solution had stood for at least 15 min with repeated mixing with a vortex-mixer, 25 μL was used for the measurement of cortisol by RIA *(15)*.

Direct measurements of cortisol in saliva required 25 μL of saliva. The detection limit of the direct assay was 0.5 nmol/L; the within-assay imprecision (CV) was 4% at 10 nmol/L (n = 10), and the between-assay CV was 9% at 4 nmol/L (n = 69) and 5% at 10 nmol/L (n = 69).

Over an experimental range of 2–20 nmol/L cortisol, concentrations measured after extraction were highly comparable to those measured directly. At the lowest volumes, the imprecision of the measurements increased, but the concentrations with and without extraction did not differ significantly: with solvent extraction they were 117% (22%), 98% (12%), and 107% (11%) of the values measured directly for volumes of 50, 100, and 200 μL, respectively (n = 24 at each volume).

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Size Separation of Circulatory DNA in Maternal Plasma Permits Ready Detection of Fetal DNA Polymorphisms

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Background: **Analysis of fetal DNA in maternal plasma has recently been introduced as a new method for noninvasive prenatal diagnosis, particularly for the analysis of fetal genetic traits, which are absent from the maternal genome, e.g., RHD or Y-chromosome-specific sequences. To date, the analysis of other fetal genetic traits has been more problematic because of the overwhelming presence of maternal DNA sequences in the circulation. We examined whether different biochemical properties can be discerned between fetal and maternal circulatory DNA.**

Methods: **Plasma DNA was examined by agarose gel electrophoresis. The fractions of fetal and maternal DNA in size-fractionated fragments were assayed by real-time PCR. The determination of paternally and maternally inherited fetal genetic traits was examined by use of highly polymorphic chromosome-21-specific microsatellite markers.**

Results: **Size fractionation of circulatory DNA indicated that the major portion of cell-free fetal DNA had an approximate molecular size of <0.3 kb, whereas maternally derived sequences were, on average, considerably larger than 1 kb. Analysis of size-fractionated DNA (≤0.3 kb) from maternal plasma samples facilitated the ready detection of paternally and maternally inherited microsatellite markers.**

Conclusions: **Circulatory fetal DNA can be enriched by size selection of fragment sizes less than ~0.3kb. Such selection permits easier analysis of both paternally and maternally inherited DNA polymorphisms.**

Since its discovery in 1997 by Lo et al. *(1)*, circulatory fetal DNA in maternal plasma or serum has rapidly emerged as the prime focus for the development of risk-free methods for prenatal diagnosis of fetal genetic traits *(2, 3)*. Indeed, because of the relative abundance of this fetal genetic material, which is present in concentrations several orders of magnitude higher than those of trafficking fetal cells *(4)*, the determination of fetal genetic loci that are totally absent from the maternal genome is relatively easy. Consequently, the analysis of circulatory fetal DNA in maternal plasma is already being offered clinically by several centers to determine fetal RHD status in pregnancies with a Rhesus constellation or fetal sex by the detection of Y-chromosome-specific sequences in pregnancies at risk for an X-linked disorder (e.g., hemophilia, fragile X syndrome) *(3)*.

Quantitative analysis of this new-found fetal analyte by real-time PCR strategies has also indicated that the concentrations of circulatory fetal DNA are increased in a variety of pregnancy-related pathologies, including preeclampsia *(5, 6)*, preterm labor *(7, 8)*, and hyperemesis gravidarum *(8)*, and in pregnancies with fetal aneuploidies, most notably trisomy 21 *(9, 10)*. These studies have suggested that fetal DNA concentrations may serve as a new screening marker for such pregnancy-related anomalies *(11)*.

A caveat of current investigations is that the overwhelming amount of circulatory DNA in the maternal circulation is of maternal origin (>90%) *(4)*, which has rendered the differentiation of more subtle genetic differences between mother and child considerably more difficult *(2, 3)*. This is generally true for Mendelian genetic disorders involving point mutations *(12)* or those instances where both parents are carriers for the same

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disease allele, as well as for the examination of DNA polymorphisms *(13, 14)* that could be used for the determination of fetal ploidy *(15)*. Consequently, few reports exist regarding the successful use of circulatory fetal DNA for such applications *(12, 16–18)*.

To date, few studies have addressed the biochemical properties of circulatory fetal DNA *(19, 20)*. In the most recent of these, Chan et al. *(20)* used differently sized PCR amplicons to discern the respective size distributions of circulatory fetal and maternal DNA species; their results indicated that fetal DNA molecules are generally smaller than comparable maternal ones. Because this approach permitted a precise delineation of only rather small DNA species (<800 bp), we have examined this aspect, using a combination of agarose gel electrophoresis, Southern blotting, and real-time PCR, because this would permit an analysis of large DNA molecules (>10 to 20 kb). Our studies have shown that circulatory DNA has apoptotic characteristics, displaying a typical ladder obtained by nucleosomal cleavage. We also observed that circulatory fetal DNA is generally of a smaller size than maternally derived cell-free DNA fragments, in good agreement with recent observations *(20)*. By exploiting this observation, we have shown that even a simple strategy, such as size separation using conventional agarose gel electrophoresis and subsequent PCR analysis *(21)*, can lead to the selective enrichment of circulatory fetal DNA sequences. These in turn can be used for the determination of DNA polymorphisms that are masked by maternal sequences in the native plasma sample.

Materials and Methods

SOUTHERN BLOT ANALYSIS

Plasma sample collection. After receiving approval by the Cantonal Institutional Review Board of Basel, Switzerland, we obtained 18 mL of peripheral blood from pregnant women who had given written informed consent. EDTA (Movovette tubes; Sarstedt) was used as anticoagulant. The blood samples were first cleared by centrifugation at 1600*g* for 10 min, after which the plasma was subjected to a second centrifugation step at 16 000*g* for 10 min. Plasma was immediately used for DNA extraction in each analysis.

Southern hybridization. Circulatory DNA was extracted from ~7–10 mL of maternal plasma (gestational age, 11–17 weeks) and 18 mL of cord blood plasma by a conventional phenol–chloroform procedure, with a slight modification in that the plasma sample was first treated with a chaotropic guanidinium isothiocyanate solution to denature any nucleases (Qiagen) *(22)*. As a control, we used plasma from nonpregnant women.

The extracted DNA was separated on a 1.0% agarose gel. A 100-bp ladder and *Hin*dIII-digested Lambda phage DNA were used to estimate molecular size (New England Biolabs). The DNA was transferred to nylon membranes (Roche) with 20× standard saline citrate by a standard capillary transfer method *(22)*.

Transferred DNA was detected with the Roche® DIG labeling and detection system, according to the manufacturer's instructions (Roche) *(23)*. The highly repetitive Alu sequence was used as a hybridization probe and was directly digoxigenin-labeled by a PCR process using the PCR DIG Probe Synthesis Kit (Roche). The primer for the Alu sequence was as follows: 5′-ATC TCG GCT CAC TGC AA-3′. Prehybridization was carried out at 42 °C in DIG Easy Hyb solution (Roche). Hybridization was performed at 42 °C overnight, after which the membrane was washed at high stringency and incubated with the chemiluminescent alkaline phosphatase substrate (CSPD) according to the manufacturer's instructions. The resulting blot was exposed on x-ray film.

DETERMINATION OF SIZE DISTRIBUTION OF CIRCULATORY FETAL-DERIVED DNA IN MATERNAL PLASMA

Preparation of circulatory DNA. Plasma samples were prepared as described above. Peripheral blood was collected from pregnant women carrying a singleton male fetus. Six samples were obtained early in pregnancy (median gestational age, 13 + 2 weeks), and eight samples were collected in the third trimester (median gestational age, 34 + 4 weeks). Blood from three nonpregnant women and three healthy males was used as controls. Routinely, 5–7 mL of plasma was used for DNA extraction, which was performed with a combination of the Roche High Pure Template DNA Purification Kit (Roche) and a custom-made vacuum pump for the isolation of circulatory plasma DNA. In brief, as described in the manufacturer's instructions, the plasma sample was incubated with binding buffer and proteinase K at 70 °C for 10 min, after which the required volume of isopropanol was added and the sample was passed through the Roche column under reduced pressure. The column was then washed with inhibitor removal buffer and twice with wash buffer, respectively, as recommended by the manufacturer. The column-bound circulatory DNA was eluted in 40 μL of elution buffer.

Gel electrophoresis and isolation of circulatory DNA fragments. The total circulatory DNA was subjected to agarose gel electrophoresis on a 1.0% agarose gel (Invitrogen) containing 0.5 mg/L ethidium bromide (Sigma). Size markers were a 100-bp ladder and *Hin*dIII-digested Lambda phage DNA (New England Biolabs). Electrophoresis was carried out at 80 V for 1 h. Each lane of the gel containing circulatory DNA was then cut with a sterile scalpel blade into six discrete sections, with the molecular weight markers used as a guide. The sizes of the fragments in the sections, based on the molecular weight markers, were 0.09–0.3, 0.3–0.5, 0.5–1.0, 1.0–1.5, 1.5–23, and >23 kb. Because the location of the 23-kb marker is imprecise in 1% gels, we used it only as a rough guide to estimate the

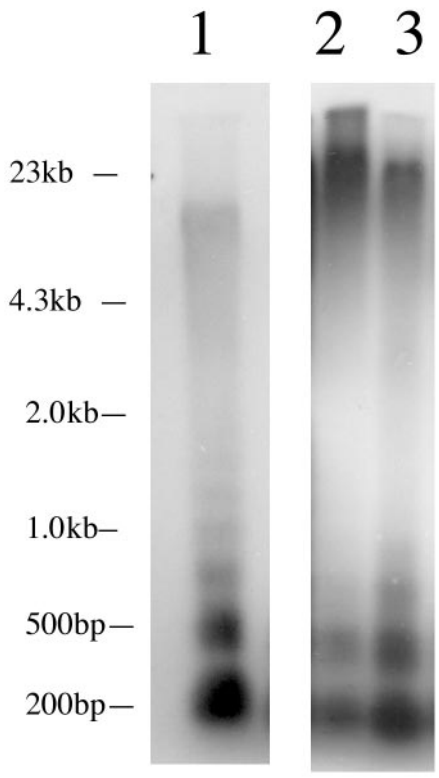

Fig. 1. Southern blot analysis of plasma circulatory DNA, using a highly repetitive Alu probe.

Lane 1, plasma from cord blood; *lane 2*, maternal plasma (13 weeks of gestation); *lane 3*, plasma from nonpregnant woman.

size of DNA fragments larger than 10 kb. The circulatory DNA was extracted from the agarose sections with a QIAEX®II Gel Extraction Kit (Qiagen) and eluted in 40 μL of sterile 10 mmol/L Tris-HCl (pH 8.0). Procedures to prevent contamination were used during these experiments, including ultraviolet irradiation of the gel tray and tank, use of fresh buffers with each electrophoretic run, use of plasma samples from women carrying female fetuses (n = 2), and examination of blank gel slices (n = 14) in parallel with each analysis. In no instance were any false-positive results recorded.

Determination of the proportion of circulatory fetal and total DNA. The relative proportions of fetal and total circulatory DNA eluted from the individual agarose gel sections were determined by use of a well-established TaqMan® real-time PCR assay for the *SRY* gene on the Y chromosome and the ubiquitous glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene *(6)*. The only modifications were that a new-generation Perkin-Elmer Applied Biosystems 7000 Sequence Detector was used and that minor groove binding probes were used instead of the previous 6-carboxytetramethylrhodamine-conjugated probes (Applied Biosystems). The PCR reactions were carried out in a final reaction volume of 25 μL, which consisted of 6 μL of eluted DNA, 300 nM each primer, 150 nM each probe, and 2× TaqMan Universal PCR Master Mix (Applied Biosystems). PCR was carried out with an initial incubation at 50 °C for 2 min to activate uracil-N-glycosylase, followed by incubation at 95 °C for 10 min and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. This analysis permitted determination of the total amount of DNA present in each fraction, by use of the *GAPDH*-specific assay, and the fraction that was fetal, by use of the *SRY*-specific assay. The relative proportions of each were expressed as percentages.

DETECTION OF HIGHLY POLYMORPHIC MICROSATELLITE MARKERS

Sample preparation. We collected 18 mL of maternal blood from three third-trimester pregnancies and collected cord blood subsequent to delivery. After plasma separation by high-speed centrifugation, the buffy coat was collected, washed with phosphate-buffered saline, and used for the preparation of maternal genomic DNA. Fetal genomic DNA was prepared similarly from the cord blood sample. To verify that this same approach can be used to examine clinically relevant samples, we examined four samples taken early in pregnancy (median gestational age, 13 + 5 weeks). In this instance the fetal genotype was determined from archived amniocyte or chorionic villus cultures. One of these samples was obtained from a trisomy 21 fetus.

Fluorescent PCR analysis of highly polymorphic microsatellite markers. We used the same standardized fluorescent PCR assay for highly polymorphic short tandem repeat (STR)

Table 1. Size distribution of total and fetal circulatory DNA in third-trimester maternal plasma samples.[a]

	Median (range)		
Size of DNA fraction, kb	Size distribution of total DNA,[b] %	Size distribution of fetal DNA,[c] %	Proportion of fetal DNA per fraction,[d] %
<0.3	22.4 (15.7–26.7)	70.0 (51.0–82.3)	68.7 (22.2–87.1)
0.3–0.5	28.4 (15.7–35.2)	24.3 (13.8–31.6)	15.4 (6.4–31.4)
0.5–1.0	23.0 (15.0–26.8)	3.8 (0.0–17.4)	2.6 (0.0–7.8)
1.0–1.5	7.5 (2.2–11.4)	0.0 (0.0–8.7)	0.0
1.5–23	21.1 (10.3–35.7)	0.0	0.0

[a] Six samples were analyzed in this study. Median gestational age was 34 + 4 weeks.

[b] Size distribution of total circulatory DNA was determined by a real-time PCR assay for the *GAPDH* gene. The values are indicative of the percentage of total DNA in each fraction examined.

[c] Size distribution of circulatory fetal DNA as determined by a real-time PCR assay for the *SRY* gene. These values are indicative of the percentage of fetal DNA with regard to the total amount of fetal DNA in each fraction examined.

[d] The proportion of fetal DNA indicates the percentage of fetal DNA in each of the examined fractions with regard to the total amount of circulatory DNA in that fraction.

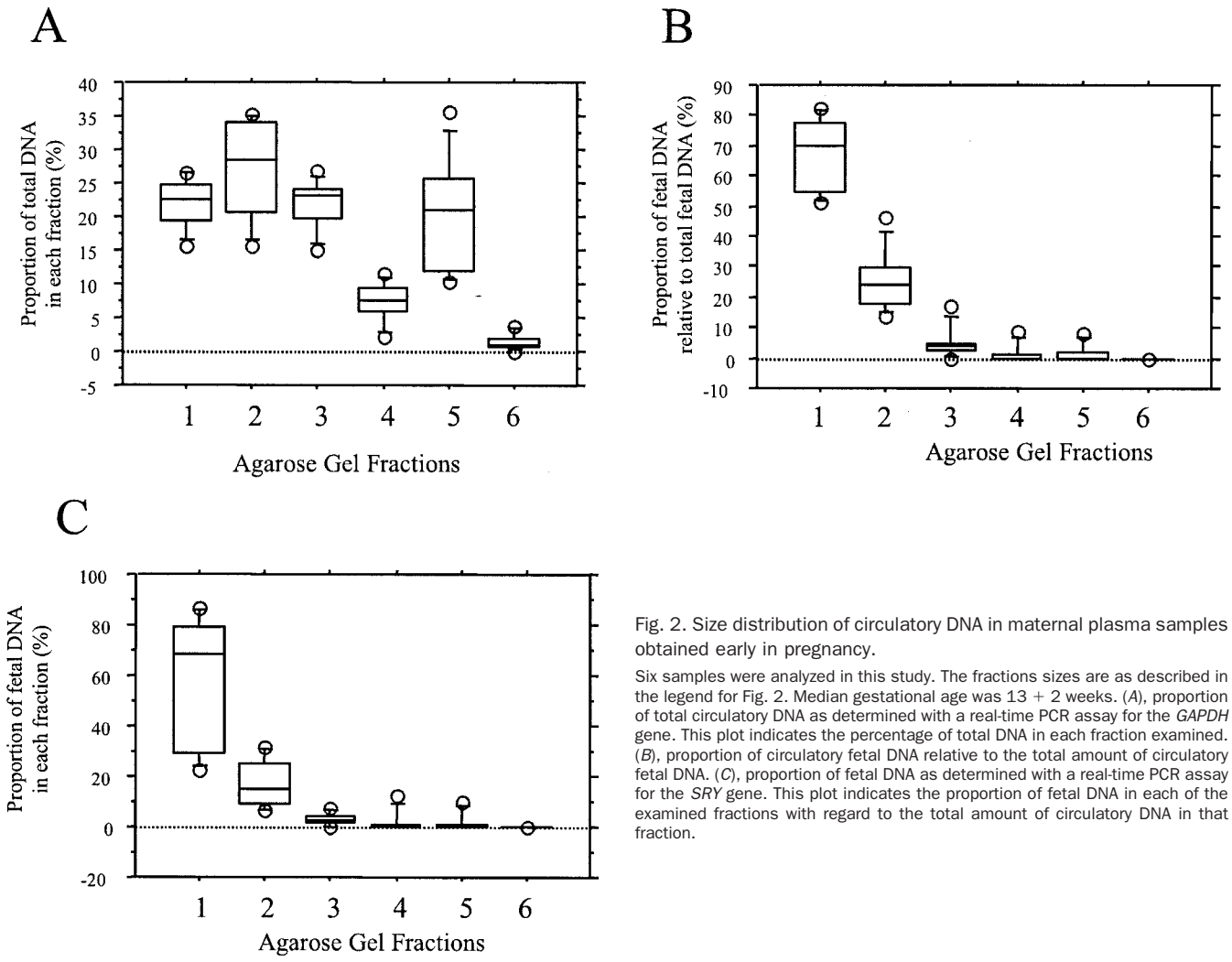

Fig. 2. Size distribution of circulatory DNA in maternal plasma samples obtained early in pregnancy.

Six samples were analyzed in this study. The fractions sizes are as described in the legend for Fig. 2. Median gestational age was 13 + 2 weeks. (*A*), proportion of total circulatory DNA as determined with a real-time PCR assay for the *GAPDH* gene. This plot indicates the percentage of total DNA in each fraction examined. (*B*), proportion of circulatory fetal DNA relative to the total amount of circulatory fetal DNA. (*C*), proportion of fetal DNA as determined with a real-time PCR assay for the *SRY* gene. This plot indicates the proportion of fetal DNA in each of the examined fractions with regard to the total amount of circulatory DNA in that fraction.

markers on chromosome 21 that we used previously for our analysis of urinary DNA *(24)*. The fetal and maternal genomic DNA sources were used to determine which STR markers on chromosome 21 were informative. These informative STR markers were then used for the detection of paternally and maternally inherited fetal alleles in total maternal plasma circulatory DNA as well as in size-separated circulatory DNA fractions. The low-molecular-weight circulatory DNA fraction was prepared as described above.

Because the concentration of circulatory DNA after size fractionation was very low, we used a seminested PCR assay as described previously *(24)*. The PCR products were analyzed by capillary electrophoresis on an ABI 310 gene analyzer (Applied Biosystems). For samples taken early in pregnancy, in which the concentration of circulatory fetal DNA has been shown to be lower than at term, our investigation showed that the PCR procedure had to be modified to obtain optimum results. For this reason,

Table 2. Size distribution of total and fetal circulatory DNA in maternal plasma samples obtained early in pregnancy.[a]

	Median (range)		
Size of DNA fraction, kb	**Size distribution of total DNA, %**	**Size distribution of fetal DNA, %**	**Proportion of fetal DNA per fraction, %**
<0.3	26.9 (12.7–41.3)	85.5 (67.8–100.0)	28.4 (11.6–56.6)
0.3–0.5	29.1 (26.1–54.4)	11.7 (0.0–15.5)	4.0 (0.0–13.5)
0.5–1.0	28.5 (14.5–25.9)	1.2 (0.0–16.8)	0.4 (0.0–5.2)
1.0–1.5	8.2 (4.5–12.1)	0.0	0.0
1.5–23	8.6 (7.6–23.7)	0.0	0.0

[a] Eight samples were analyzed in this study. Median gestational age was 13 + 2 weeks. Size distributions of total circulatory DNA, circulatory fetal DNA, and the proportion of fetal DNA were determined as described in the footnotes for Table 1.

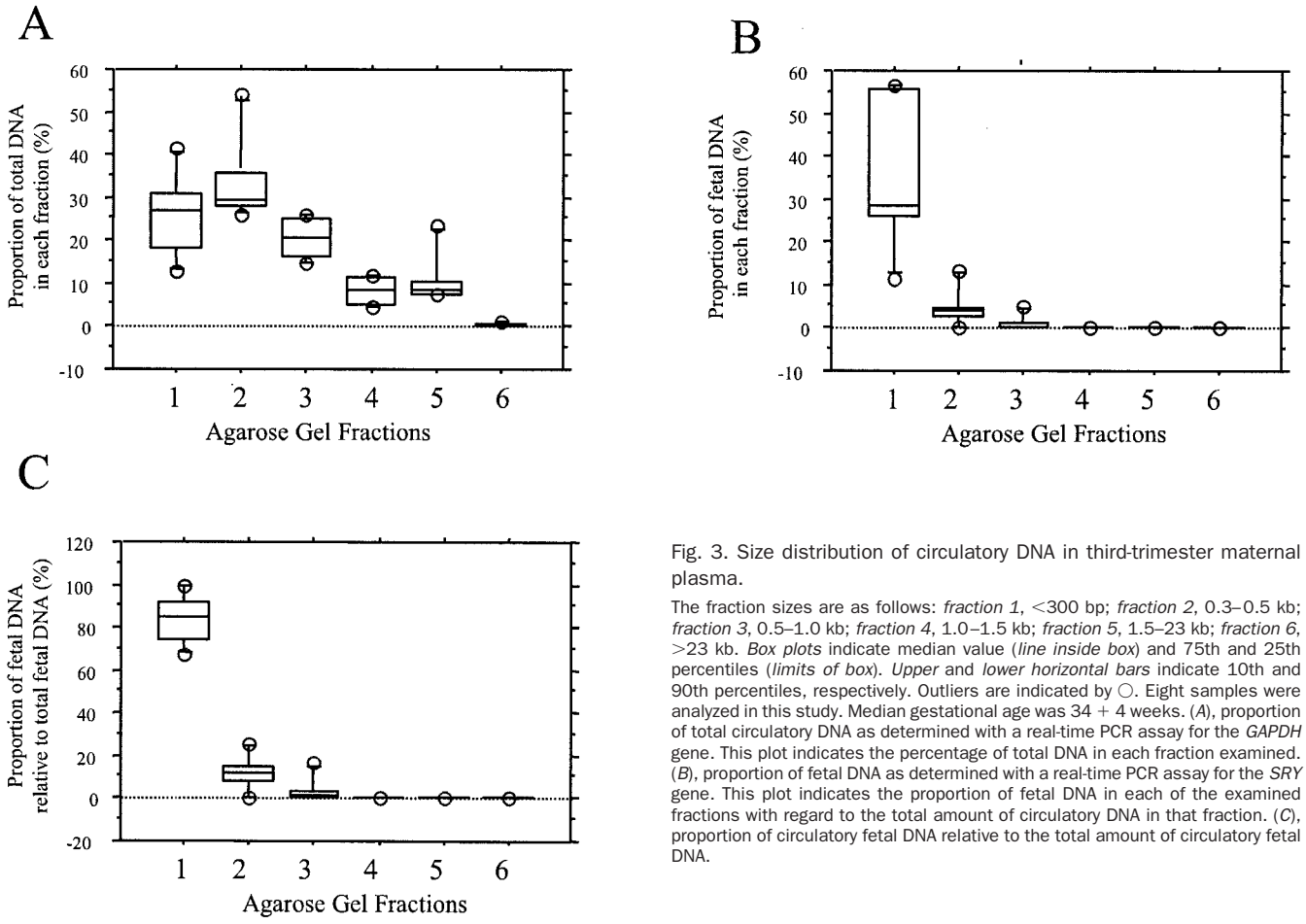

Fig. 3. Size distribution of circulatory DNA in third-trimester maternal plasma.

The fraction sizes are as follows: *fraction 1*, <300 bp; *fraction 2*, 0.3–0.5 kb; *fraction 3*, 0.5–1.0 kb; *fraction 4*, 1.0–1.5 kb; *fraction 5*, 1.5–23 kb; *fraction 6*, >23 kb. *Box plots* indicate median value (*line inside box*) and 75th and 25th percentiles (*limits of box*). *Upper* and *lower horizontal bars* indicate 10th and 90th percentiles, respectively. Outliers are indicated by ○. Eight samples were analyzed in this study. Median gestational age was 34 + 4 weeks. (*A*), proportion of total circulatory DNA as determined with a real-time PCR assay for the *GAPDH* gene. This plot indicates the percentage of total DNA in each fraction examined. (*B*), proportion of fetal DNA as determined with a real-time PCR assay for the *SRY* gene. This plot indicates the proportion of fetal DNA in each of the examined fractions with regard to the total amount of circulatory DNA in that fraction. (*C*), proportion of circulatory fetal DNA relative to the total amount of circulatory fetal DNA.

we used a "touch-down" PCR method instead of our conventional seminested PCR method described above. In this procedure, a total of 50 cycles were run, using PCR reactions containing 3 μL of 10× buffer, 3.5 mM $MgCl_2$, 160 μM deoxynucleotide triphosphates, 0.1 μM each of forward and reverse primers *(24)* (one primer of each set was fluorescent labeled), 1U of AmpliTaq Gold polymerase, and 3 μL of DNA in a final volume of 30 μL. After incubation at 95 °C for 10 min, 10 cycles (95 °C for 15 s, 65 °C for 15 s, 72 °C for 30 s) of thermal cycling were carried out in which the annealing temperature was decreased by 1 °C/cycle. This was then followed by 40 cycles of 95 °C for 15 s, 56 °C for 15 s, and 72 °C for 30 s. The PCR was terminated after an extension phase at 72 °C for 7 min. As described above, the PCR products were analyzed by capillary electrophoresis on an ABI 310 gene analyzer *(24)*.

Results

Circulatory DNA has been proposed to exhibit apoptotic hallmarks such as oligonucleosomal laddering and nucleosome association *(25, 26)*. In our initial investigation, we attempted to determine whether circulatory DNA in pregnant women also displayed such characteristics. For this examination we used Southern blot analysis of total circulatory plasma DNA that had been subjected to agarose gel electrophoresis. The analysis of such blots with the ubiquitous highly repetitive Alu sequence indicated that oligonucleosomal fragments could indeed be detected and were present in all three of the plasma sources examined: maternal blood, nonpregnant female control blood, and cord blood (Fig. 1). This examination also indicated that a substantial proportion of the circulatory DNA had a molecular size >10 or even >23 kb (Fig. 1). The presence of such high-molecular-weight DNA species cannot be attributed to the plasma sample being contaminated by maternal cells because we took extreme care to obtain cell-free plasma samples. It is of interest that these high-molecular-weight DNA molecules are quite similar to the very large ones we observed in terminally differentiated erythroblasts before enucleation (S. Hristoskova et al., manuscript in preparation).

Unfortunately, we were not able to determine the characteristics of circulatory fetal DNA in the samples obtained from pregnant women because the concentrations of fetal DNA were too low to be detectable even when we used a very high copy probe (DYS14) specific for the Y chromosome.

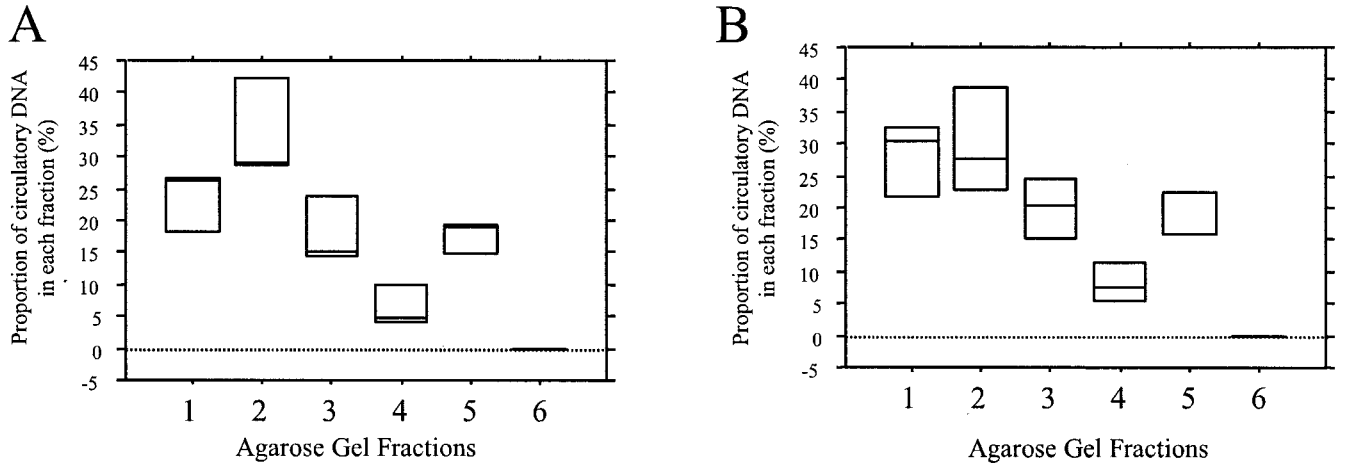

Fig. 4. Size distribution of circulatory DNA in plasma samples from healthy nonpregnant women or healthy males.

Three samples per group were analyzed in this study. The fractions sizes are as described in the legend for Fig. 2. (*A*), proportion of total circulatory DNA in each fraction examined in samples obtained from nonpregnant women. (*B*), proportion of total circulatory DNA in each fraction examined in samples obtained from healthy male volunteers.

Because we were, however, able to detect circulatory apoptotic DNA fragments in our Southern blot analysis, we used an alternative strategy to determine whether circulatory fetal DNA displayed a similar pattern. For this analysis, we used an approach that had previously been used successfully for the characterization of rare linear extrachromosomal DNA species *(21)*. In this procedure, the circulatory DNA was first subjected to agarose gel electrophoresis, after which individual gel fragments containing the size-fractionated DNA were examined by PCR. For our examination, after electrophoresis the agarose gel was cut into six discrete sections containing fragments with approximate sizes of <0.3, 0.3–0.5, 0.5–1.0, 1.0–1.5, 1.5–23.0, and >23 kb. Once the circulatory DNA was extracted from these gel fragments, the proportions of fetal and maternal DNA in these fractions were then determined by well-established real-time PCR assays for the *SRY* locus on the Y chromosome and the ubiquitous *GAPDH* gene *(6)*. To ensure that we were not being misled by any PCR artifacts, we included several procedures to prevent contamination in our study, including use of plasma samples from women pregnant with female fetuses (n = 2) and the parallel examination of blank gel slices in each analysis (n = 14). No false-positive results were recorded in any of these instances.

Our examination of plasma samples from third-trimester pregnancies with a male fetus indicated that the vast proportion of circulatory fetal DNA, as detected by the *SRY*-specific PCR assay, had an approximate molecular size <300 bp (Table 1 and Fig. 2), with very little or no

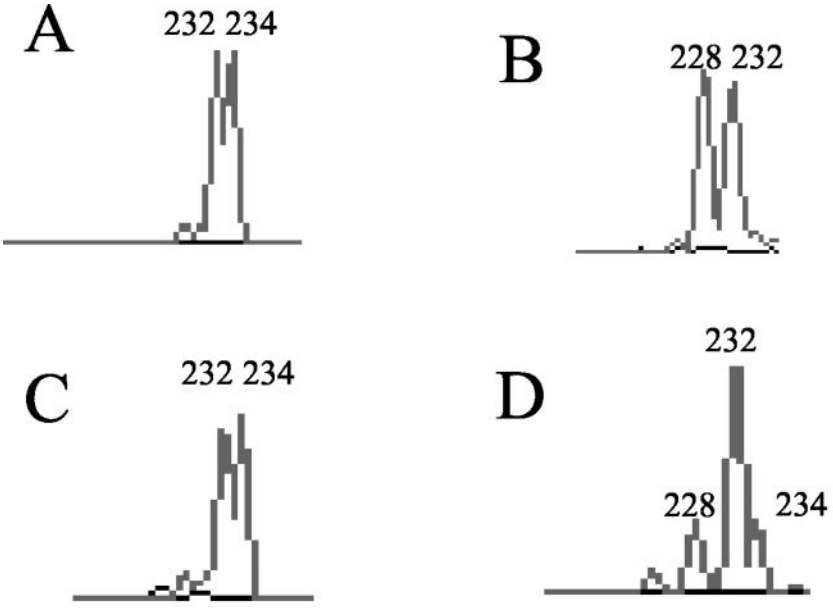

Fig. 5. Detection of both paternally and maternally inherited highly polymorphic microsatellite markers in size-fractionated circulatory DNA.

In this analysis a maternal plasma taken near term was used. Shown are capillary electropherograms of D21S11 alleles. (*A*), maternal genomic DNA. Two maternal alleles with sizes of 232 and 234 bp were detected. (*B*), fetal genomic DNA. The paternally inherited allele with a size of 228 bp and the maternally inherited allele with a size of 232 bp were detected. (*C*), total circulatory DNA. The predominant alleles detected were those of the mother (D21S11 alleles with sizes of 232 and 234 bp). (*D*), size-fractionated circulatory DNA with a size <300 bp. Three D21S11 alleles with sizes of 228, 232, and 234 bp were detected.

fetal DNA having a molecular size >1 kb. On the other hand, maternally derived sequences, as determined by the *GAPDH*-specific PCR assay, were estimated to be larger than 0.5–1.0 kb and included molecular-weight species larger than 10–20 kb. A similar pattern for both fetal and maternal circulatory DNA was also observed in plasma samples obtained early in the second trimester of pregnancy (Table 2 and Fig. 3).

With regard to the size distribution of total circulatory DNA, we determined that the pattern we had observed in pregnant women was very similar to that observed in samples taken from nonpregnant women as well as healthy male volunteers (Fig. 4). In none of these analyses were we able to detect large amounts of DNA with a molecular size greater than that indicated by the 23-kb molecular weight marker, in contrast to what we observed in our Southern blot analysis (Fig. 1). The reason for this anomaly may be that these large fragments are not easily eluted from the agarose gel under the conditions we are using, unlike in the Southern blotting, where the DNA is first treated with alkali to generate the small fragments required for efficient capillary transfer.

Our data also indicated that selective enrichment of circulatory fetal DNA sequences may be possible by examination of DNA fragments with a size less than ~300 bp. Our next step, therefore, was to determine whether such size-dependent separation would facilitate the determination of more subtle fetal genetic traits.

For this purpose we examined whether both paternally and maternally inherited DNA polymorphisms could be discerned from such size-fractionated circulatory DNA. For our analysis we used highly polymorphic STR sequences on chromosome 21, which have previously been demonstrated to be suitable for the reliable distinction of mother and child *(24)*. To test the feasibility of this approach, we first examined samples taken close to term because they would contain the maximum concentrations of circulatory fetal DNA and because it was possible to obtain the fetal genotype, a prerequisite for such studies, from a cord blood sample after birth. In this manner, we could select easily discernible polymorphic markers between mother (Fig. 5A) and fetus (Fig. 5B).

In this analysis, the benefits of selectively enriching for circulatory fetal DNA species became readily apparent in that the paternally inherited STR allele (with a size of 228 bp) was barely detectable in the total plasma extracted DNA (Fig. 5C) but was clearly present in the DNA fraction with a size <300 bp (Fig. 5D). Furthermore, the method could not differentiate the maternally inherited STR allele (with a size of 232 bp) from the predominantly maternal pattern obtained from the analysis of total plasma extracted DNA (Fig. 5C). This fetal allele, however, was detected in the analysis of the DNA fraction with a size <300 bp (Fig. 5D) because the peak for that STR allele had a much larger area than either the paternally inherited fetal allele (228 bp) or the solitary maternal allele (234 bp). This indicates that both fetal and maternal loci are contributing to the presence of this particular PCR product. Similar results were obtained in the analysis of the DNA fraction with a size of 300–500 bp, although the results in this instance were less evident than those obtained with the smaller DNA fraction (<300 bp; data not shown). The reproducibility of this approach was verified in the analysis of two additional samples, which were analyzed at a several different polymorphic loci; we obtained similar results for these samples (Table 3).

Table 3. Detection of paternally and maternally inherited fetal highly polymorphic microsatellite markers in size-fractionated circulatory DNA obtained from maternal plasma samples taken close to term.[a]

Case no. (D21 locus)	Method of sample preparation[b]	Maternal alleles detected	Fetal alleles detected
1 (D21S11)	Maternal genomic DNA	232/234	
	Fetal genomic DNA		228/232
	Total plasma DNA	232/234	Not detectable
	Plasma DNA <300 bp	232/234	228/232
1 (D21S1435)	Maternal genomic DNA	172/180	
	Fetal genomic DNA		172/176
	Total plasma DNA	172/180	176[c]
	Plasma DNA <300 bp	172/180	172/176
2 (D21S1270)	Maternal genomic DNA	184/188	
	Fetal genomic DNA		180/184
	Total plasma DNA	184/188	Not detectable
	Plasma DNA <300 bp	184/188	180/184
2 (D21S1432)	Maternal genomic DNA	138/152	
	Fetal genomic DNA		134/138
	Total plasma DNA	138/152	134[c]
	Plasma DNA <300 bp	138/152	134/138
3 (D21S1435)	Maternal genomic DNA	168/176	
	Fetal genomic DNA		168/172
	Total plasma DNA	168/176	172[c]
	Plasma DNA <300 bp	168/176	168/172

[a] Three samples were analyzed in this study.

[b] Genomic DNA was prepared directly from maternal or fetal lymphocytes. "Total plasma DNA" indicates analysis of circulatory DNA extracted from an nonfractionated DNA samples, whereas "Plasma DNA <300 bp" indicates analysis of a discrete fraction of circulatory DNA that had been size-fractionated by gel electrophoresis.

[c] Minimal detection of paternally inherited allele.

To determine whether this approach could also be applied to clinically relevant samples, we examined four samples taken early in the second trimester of pregnancy. In these analyses the fetal genotype was determined from archived amniocyte or chorionic villus cultures. One of these samples was from a fetus affected by Down syndrome (trisomy 21). This was evident from our microsatellite analysis for the D21S1432 marker, in which three equivalent peaks with sizes of 133, 137, and 141 bp, respectively, were detected (Fig. 6B), implying that the fetus had inherited a copy of each of the two maternal chromosomes 21 in addition to the paternally inherited chromosome 21. In our analysis of the total circulatory cell-free DNA in the maternal plasma sample, only the

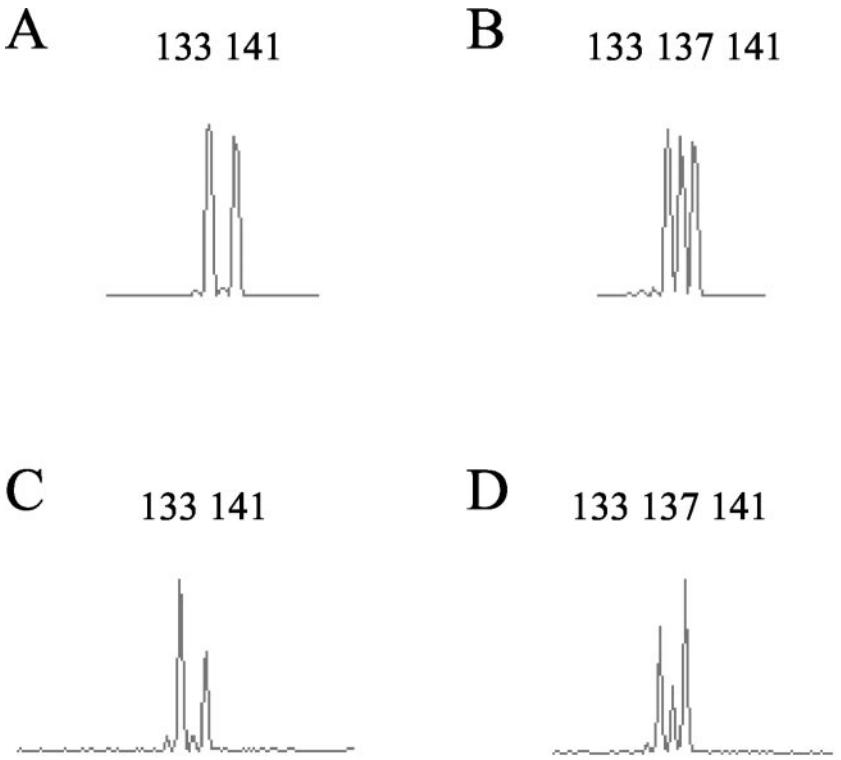

Fig. 6. Analysis of highly polymorphic microsatellite markers in the plasma of a mother carrying a fetus with trisomy 21.

In this analysis we used an early second-trimester maternal plasma sample. Shown are capillary electropherograms of D21S1432 alleles. (*A*), maternal genomic DNA. Two maternal alleles with sizes of 133 and 141 bp were detected. (*B*), fetal genomic DNA. The paternally inherited allele (137 bp) and both of the inherited maternal alleles (133 and 141 bp) were detected. (*C*), total circulatory DNA. The predominant alleles detected were those of the mother (D21S1432 alleles; 133 and 141 bp). (*D*), size-fractionated circulatory DNA with a size <300 bp. The paternal D21S1432 allele, with a size of 137 bp, was readily detected.

two maternal markers were readily detected (133 and 141 bp; Fig. 6C), whereas our analysis of size-fractionated DNA with an approximate size of <300 bp facilitated the ready detection of the paternally inherited 137-bp marker (Fig. 6D). We observed a similar feature when we examined this sample for a different microsatellite marker, D21S1270 (Table 4). In this instance, it was not possible to determine whether the maternally inherited polymorphisms could be detected because the fetus had the same pattern as the mother for both loci. In the three other cases, which had a normal karyotype, we were in all three cases readily able to detect the paternally inherited polymorphic locus in the size-fractionated DNA sample (Table 4). However, in only one instance could we discern the presence of the maternally inherited locus (Table 4, case 3). In case 2, this was not possible because the mother was homozygous for the locus interrogated, whereas in the other cases [case 2 (D21S1435) and case 4 (D21S1440)], the maternal locus that had not been inherited by the fetus appeared to have been preferentially amplified (Table 4).

Discussion

Our investigation supports the current hypothesis that circulatory DNA has apoptotic attributes *(25, 26)* in that we could readily discern oligonucleosomally cleaved fragments by Southern blot analysis. Our analysis also indicated that a substantial proportion of the circulatory DNA has a very large molecular size (>20 kb). Independent investigations in our laboratory indicated that these large circulatory DNA species may be derived from the erythropoietic system, in that DNA isolated from terminally differentiating erythroblasts exhibited similar characteristics (S. Hristoskova et al., manuscript in preparation). It is currently unclear whether these large DNA molecules are subsequently cleaved into smaller oligonucleosomal fragments in the maternal plasma or whether the smaller fragments we detected are derived from another source.

Using an approach that had previously been used to examine rare linear extrachromosomal DNA species *(21)*, in which DNA size-fractionated by gel electrophoresis was subsequently extracted and analyzed by PCR, we made the surprising finding that a large discrepancy existed in the size of circulatory fetal and maternal DNA species. In this regard our study indicated that fetal DNA molecules predominantly have an approximate size ≤300 bp, whereas most maternally derived DNA molecules are considerably larger than this. The fact that no large circulatory fetal DNA species were detected (i.e., >20 kb) implies that the mechanism contributing to the formation of the large maternally derived DNA species is not involved in the liberation of circulatory fetal DNA. The explanation for this difference could be that circulatory fetal DNA appears to be exclusively derived from the placenta *(27)*, whereas the vast proportion of normal maternal circulatory DNA is of hemopoietic origin *(28)*.

With regard to the size distribution of circulatory maternal and fetal DNA species, our results are remarkably similar to those reported recently by Chan et al. *(20)*, who also observed that fetal cell DNA molecules were

Table 4. Detection of paternally and maternally inherited fetal highly polymorphic microsatellite markers in size-fractionated circulatory DNA obtained from maternal plasma samples obtained early in the second trimester.[a]

Case no. (D21 locus)	Method of sample preparation[b]	Maternal alleles detected	Fetal alleles detected
1 (D21S1270)	Maternal genomic DNA	182/191	
	Fetal genomic DNA		182/187/191
	Total plasma DNA	182/191	Not detectable
	Plasma DNA <300 bp	182/191	187
1 (D21S1432)	Maternal genomic DNA	133/141	
	Fetal genomic DNA		133/137/141
	Total plasma DNA	133/141	Not detectable
	Plasma DNA <300 bp	133/141	137
2 (D21S1440)	Maternal genomic DNA	154	
	Fetal genomic DNA		154/157
	Total plasma DNA	154	Not detectable
	Plasma DNA <300 bp	154	157
2 (D21S1435)	Maternal genomic DNA	141/172	
	Fetal genomic DNA		172/176
	Total plasma DNA	141/172	Not detectable
	Plasma DNA <300 bp	141/172	176
3 (D21S1440)	Maternal genomic DNA	157/160	
	Fetal genomic DNA		154/157
	Total plasma DNA	157/160	Not detectable
	Plasma DNA <300 bp	157/160	154/157
4 (D21S1440)	Maternal genomic DNA	154/157	
	Fetal genomic DNA		157/160
	Total plasma DNA	154/157	Not detectable
	Plasma DNA <300 bp	154/157	160

[a] Four samples were analyzed in this study.

[b] Genomic DNA was prepared directly from maternal or fetal lymphocytes. "Total plasma DNA" indicates analysis of circulatory DNA extracted from an nonfractionated DNA samples, whereas "Plasma DNA <300 bp" indicates analysis of a discrete fraction of circulatory DNA that had been size-fractionated by gel electrophoresis.

generally smaller than those of maternal origin. For their study, they made use of differently sized PCR amplicons ranging in size from 105 to 798 bp to determine the size of predominantly maternally derived DNA molecules, and amplicons of 107–524 bp to examine circulatory fetal DNA molecules. Although their approach permitted a much more precise delineation of the size distribution of circulatory DNA molecules within this given range, they were not able to demonstrate the existence of very large (>10 to 20 kb) maternal circulatory DNA species, as our analysis could.

Another important point that the study of Chan et al. *(20)* did not address, and which has been the major focus of our study, is that this observation permits the development of a strategy facilitating the selective enrichment of circulatory fetal DNA sequences. The exploitation of this approach in turn permits the determination of highly polymorphic fetal genetic traits not discernible from the analysis of total extracted plasma circulatory DNA. In this manner we were able to detect the presence of both paternally and maternally inherited STR markers in size-separated circulatory DNA fractions, which was not possible when the same analysis was attempted on unfractionated samples *(13, 14)*. Our study does, however, indicate that the method we have chosen for this proof-of-concept study is too imprecise and inefficient to be used for potential clinical applications. This became clear in our analysis of paternally and maternally inherited polymorphic markers; we could not determine the precise proportion that the fetal markers contribute to the analyzed pattern. This feature is very important because numerous clinical studies have clearly shown that the analysis of such highly polymorphic STR markers can be very useful for the determination of fetal ploidy *(15)*. Hence, if our approach using size fractionation were optimal, it should be feasible to determine fetal chromosomal anomalies directly from maternal plasma. Because in our study we were not able to determine fetal ploidy from the size-fractionated DNA sample for the one case with a trisomy 21 fetus (Fig. 6 and Table 4), we believe that this application will have to await developments that permit better separation of maternal and fetal DNA species. It is, however, possible that even in its current form, our approach could be used for the noninvasive determination of paternity.

Another important aspect of our observation is that it may in future aid in the examination of Mendelian disorders, particularly those involving point mutations, because these analyses should no longer be hindered by the large excess of maternal DNA sequences in the circulation *(2, 3)*. Indeed, the use of quantitative assays may make it possible to determine the fetal genotype in those instances where both partners are carriers for the same disease allele.

In summary, our findings showed that circulatory fetal DNA molecules are generally of a smaller size than comparable maternally derived sequences and that selective enrichment of fetal DNA sequences can be achieved by size-dependent separation. This latter feature permits the detection of fetal genetic traits not detectable in total plasma circulatory DNA. For clinical applications, more efficacious separation modes will need to be developed that facilitate better differentiation and recovery of fetal and maternal circulatory DNA species.

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PRELIMINARY COMMUNICATION

Detection of Paternally Inherited Fetal Point Mutations for β-Thalassemia Using Size-Fractionated Cell-Free DNA in Maternal Plasma

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Context Currently, fetal point mutations cannot be reliably analyzed from circulatory fetal DNA in maternal plasma, due to the predominance of maternal DNA sequences. However, analysis of circulatory fetal DNA sequences in maternal plasma have been shown to selectively enrich for fetal DNA molecules on the basis of a smaller molecular size than maternal DNA.

Objective To examine the prenatal analysis of 4 common β-thalassemia point mutations: *IVSI*-1, *IVSI*-6, *IVSI*-110, and codon 39.

Design, Setting, and Patients A total of 32 maternal blood samples were collected at 10 to 12 weeks of gestation (mean, 10.7 weeks) between February 15, 2003, and February 25, 2004, in Bari, Italy, from women with risk for β-thalassemia in their newborns immediately prior to chorionic villous sampling. Samples in which the father and mother did not carry the same mutation were examined. Circulatory DNA was size-fractionated by gel electrophoresis and polymerase chain reaction (PCR) amplified with a peptide-nucleic-acid clamp, which suppresses amplification of the normal maternal allele. Presence of the paternal mutant allele was detected by allele-specific real-time PCR.

Main Outcome Measure Detection of paternally inherited β-globin gene point mutations.

Results Presence or absence of the paternal mutant allele was correctly determined in 6 (86%) of 7 cases with the *IVSI*-1 mutation, 4 (100%) of 4 with the *IVSI*-6 mutation, 5 (100%) of 5 with the *IVSI*-110 mutation, and 13 (81%) of 16 with the codon 39 mutation. One false-positive test result was scored for the *IVSI*-1 mutation. Two cases with the codon 39 mutation were classified as uncertain and 1 case was excluded due to lack of a diagnostic test result at the time of analysis. These results yielded an overall sensitivity of 100% and specificity of 93.8%, with classified cases removed.

Conclusion Our recently described technique of the size-fractionation of circulatory DNA in maternal plasma may be potentially useful for the noninvasive prenatal determination of fetal point mutations.

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MONOGENIC DISORDERS frequently involve point mutations. This single nucleotide exchange makes the analysis of point mutations more complex as stringent assays need to be established that permit a clear distinction between normal and mutant alleles. The prenatal diagnosis of this multitude of hereditary genetic disorders currently relies on invasive procedures,[1] such as amniocentesis or chorionic villous sampling, which are associated with a small but significant risk of fetal loss.[2,3] To avoid this procedure-related risk, several strategies have been considered for noninvasive assessment of fetal genetic traits, including the isolation of rare fetal cells from the maternal circulation and the analysis of circulatory fetal DNA in maternal plasma.[1,4-6]

Although proof-of-principle studies have indicated that the analysis of isolated fetal cells by single-cell polymerase chain reaction (PCR) can be used for the noninvasive prenatal diagnosis of hemoglobinopathies,[7,8] this strategy is too complex, labor intensive, and not sufficiently efficient for routine clinical settings. The analysis of fetal genetic traits by the analysis of cell-free fetal DNA in maternal plasma has proven to be remarkably reliable for the assessment of fetal loci absent from the maternal genome, such as Y-chromosome–specific sequences or the RhD gene in pregnant women who are Rh-negative, especially in European medical centers.[1,4]

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This approach, however, is unsuitable for the analysis of fetal loci that do not differ largely from the maternal alleles, due to the vast predominance of cell-free maternal DNA in the maternal samples.[9] As such, the analysis of fetal point mutations has been restricted to single-case articles.[10,11]

It has recently been shown that circulatory fetal DNA sequences are generally smaller (<300 base pairs [bp]) than comparable circulatory maternal DNA species (>500 bp).[12,13] By exploiting this observation, we have previously shown that this phenomenon can be used to selectively enrich for fetal DNA molecules, which permitted the detection of otherwise masked highly polymorphic fetal microsatellite markers.[12] We examined whether this approach will permit the detection of fetal point mutations. The advantage of such a development is that it would permit the detection of paternal mutations, which could be used to determine which pregnancies are at risk for a compound heterozygous genetic disorder. We have focused on one of the most common monogenic disorders, β-thalassemia, and have examined 4 point mutations, which occur with high frequency in the Mediterranean population.[14,15]

METHODS

Sample Collection and Processing

Following ethical approval from both participating institutions' review boards and written informed consent from all participants, blood samples were obtained from 32 pregnant women with risk for β-thalassemia in their newborns between February 15, 2003, and February 25, 2004, in Bari, Italy. No one refused to participate and all women were self-declared white (southern Italian origin). Approximately 18-mL maternal blood samples were collected into two 9-mL EDTA blood collection tubes (Sarstedt, Sevelen, Switzerland) at 10 to 12 weeks of gestation (mean, 10.7 weeks; median, 11.2 weeks) before chorionic villous sampling. Initially, 21 samples were sent as whole blood by overnight commercial express courier service. Because of concern that this 24-hour delay before processing of the maternal plasma sample might be detrimental, the remaining 11 samples were processed directly on-site in Bari, Italy, and the plasma was shipped frozen to Basel, Switzerland.

All samples were sent coded and examined to Basel in a blinded manner. None of the samples examined have been used in any prior investigations. Plasma was prepared from the maternal blood samples by high-speed centrifugation as described previously and stored at –70°C before analysis.[12] In addition, the frozen plasma samples shipped from Bari were again subjected to high-speed centrifugation (16 000*g* for 10 minutes) before analysis.[16] We focused exclusively on samples in which the father was a carrier for 1 of the 4 following β-globin gene mutations (*IVSI*-1, *IVSI*-6, *IVSI*-110, and codon 39) and the mother had been genotyped to carry another β-globin gene mutation.

The chorionic villus sampling sample was obtained by transabdominal puncture with a 23-gauge needle under ultrasonic guidance. The samples were processed and analyzed at the diagnostic laboratory at the University of Bari, using an allele refractory mutation system and PCR procedure, followed by combined reverse dot blot analysis.[7,8]

Circulatory DNA Extraction and Size-Fractionation

Circulatory DNA was extracted from 5- to 10-mL maternal plasma using commercial column technology (Roche High Pure Template DNA Purification Kit; Roche, Basel, Switzerland) in combination with a vacuum pump.[12] After extraction, the DNA was separated by agarose gel (1%) electrophoresis (Invitrogen, Basel, Switzerland), and the gel fraction containing circulatory DNA with a size of approximately 300 bp was carefully excised. The DNA was extracted from this gel slice by using an extraction kit (QIAEX II Gel Extraction Kit; Qiagen, Basel, Switzerland) and eluted into a final volume of 40-μL sterile 10-mM *tris*-hydrochloric acid, pH 8.0 (Roche).[12] Strict anticontamination procedures were used throughout the procedure, including the analysis of on average 2 blank gel slices per samples examined, which were all negative.

PCR Amplification Using a Peptide-Nucleic-Acid Clamp

Peptide-nucleic-acids (PNAs) bind with very high affinity to specific DNA sequences (eg, to a wild-type or mutant allele), which may differ by as little as a single-base change.[17] These molecules can be used when examining DNA samples that contain a mixture of wild-type and mutant alleles to suppress the specific amplification of either allele.[17] In this manner, the mutant or wild-type allele can be selected specifically from a mixture of both alleles. We used a PNA sequence specific for the maternal normal allele to suppress amplification of the wild-type maternal allele, thereby enriching for the presence of paternally inherited mutant sequences. The PCR/PNA clamping reactions were performed in a total volume of 30 μL, consisting of 8-μL size-separated circulatory DNA, 1× buffer with 3.5-mM magnesium, 0.2-mM dNTPs (nucleotides), 0.13-μM of each primer (all the primers used in this study were synthesized by Microsynth, Basel, Switzerland, and high performance liquid chromatography [HPLC] purified), and 0.6-U TaqGold DNA polymerase (Applied Biosystems, Rotkreuz, Switzerland), using the following PNA probe concentrations (Applied Biosystems): 0.67-μM for the *IVSI*-1 mutation, 0.5-μM for *IVSI*-6 mutation, 1-μM for *IVSI*-110 mutation, and 1-μM for codon 39 mutation. The detailed primer sequences and PCR/PNA clamping reactions are shown in TABLE 1. The clamping reaction was performed in a thermal cycler (Mastercycler, Eppendorf, Hamburg, Germany).

Allele-Specific Real-Time PCR

Following the PCR/PNA clamping step, the presence of the mutant paternal allele was detected by a real-time allele-specific PCR reaction, which was performed on a sequence detector (Perkin Elmer Applied Biosystems 7000 Se-

quence Detector, Applied Biosystems). A total of 1 µL of the PCR clamping product was amplified in duplicate in a final reaction volume of 25 µL containing 160 nM of each primer, and a mixed solution (1X SYBR Green Master Mix, Applied Biosystems). The specificity of each of the allele-specific assays for the 4 β-globin gene mutations was optimized by evaluating a series of conditions concerning buffers composition (magnesium ions), temperature and length of PCR amplification cycles, as well as use of different oligonucleotide primers.[18]

These experiments were performed on artificial mixtures of mutant DNA diluted into wild-type DNA. The final conditions are listed in Table 1. For the real-time PCR analysis, the mixed solution (1X SYBR Green Master Mix) was used to monitor the PCR reaction. The quantitative process used by real-time PCR makes use of a defined threshold value, which is determined by the crossing of a defined threshold by the accumulated PCR product,[19] which is termed the *threshold value* or C_T. This value can be used for the accurate determination of the exact amount on specific input template DNA, by comparison with a standard curve. To measure the quantitative differences between 2 genetic loci (eg, wild-type and mutant), the difference between the respective C_T values (ΔC_T) can be used.[20] We used this ΔC_T system to determine the ratio of wild-type to mutant, whereby the extent of the amplification of the normal wild-type allele (CT_N) was subtracted from that of the mutant allele (CT_M).

By the use of this $\Delta CT_{(M-N)}$ approach, we observed a clear discrimination of normal wild-type DNA samples from those samples heterozygous for the mutant allele, even with experimental conditions in which the mutant allele constituted less than 10% of the total DNA examined. This analysis also permitted us to assign arbitrary $\Delta CT_{(M-N)}$ cut-off areas for the 4 allele-specific PCR assays; the normal allele yielding higher and the mutant allele yielding lower values (FIGURE).

Statistical Analysis

The χ^2 test was used to evaluate whether a significant difference existed between the results obtained by the analysis of size-fractionated circulating DNA and by the analysis of total-circulatory DNA. The analysis was performed by using Stata version 8.0 (StataCorp LP, Lausanne, Switzerland). $P<.05$ was considered statistically significant.

RESULTS

The laboratory components of the study were performed from October 1, 2003, through May 30, 2004, in Basel, Switzerland. Four distinct point mutations of the β-globin gene—*IVSI*-1 (n=7), *IVSI*-6 (n=4), *IVSI*-110 (n=5), and codon 39 (n=16)—were examined. For each of these mutations, an allele-specific real-time PCR assay was developed. In the case of the codon 39 mutation, the development of an allele-specific assay was more complex due to the number of repetitive sequences in the vicinity of the mutation, which initially hindered the specificity of the PCR amplification.

Because we were concerned that circulatory fetal DNA sequences may still be outnumbered by maternal DNA sequences, even after selective enrichment on the basis of size, an additional PCR step was used before the allele-specific PCR assay to ensure that the presence of mutant fetal alleles could be detected in a mixture of mu-

Table 1. Oligonucleotide Sequences and PCR Conditions

	PNA Clamping		Allele-Specific Real-Time PCR	
Mutation	Primers and PNA Probes	Conditions of PNA Clamping	Primers	Conditions of Allele-Specific Real-Time PCR
IVSI-1	F-primer: GTG AAC GTG GAT GAA GTT GGT R-primer: TCT CCT TAA ACC TGT CTT GTA ACC TTC TAT PNA probe: OO-GAT ACC AAC CTG CCC	Denatured at 95°C for 10 min, followed by 25 cycles of 95°C for 15 s, 70°C for 1 min, 60°C for 15 s, 72°C for 30 s, final extension at 72°C for 5 min	F-primer: GTG AAC GTG GAT GAA GTT GGT *IVSI*-1/N: TTA AAC CTG TCT TGT AAC CTT GAT ACG AAC *IVSI*-1/M: TTA AAC CTG TCT TGT AAC CTT GAT ACG AAT	Incubated at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 63.5°C for 15 s, 72°C for 30 s
IVSI-6	F-primer: GTG AAC GTG GAT GAA GTT GGT R-primer: CTT AAA CCT GTC TTG TAA CCT TGA PNA probe: OO-GAT ACC AAC CTG CCC	Denatured at 95°C for 10 min, followed by 25 cycles of 95°C for 15 s, 70°C for 1 min, 60°C for 15 s, 72°C for 30 s, final extension at 72°C for 5 min	F-primer: GTG AAC GTG GAT GAA GTT GGT *IVSI*-6/N: CT TAA ACC TGT CTT GTA ACC TTC ATA *IVSI*-6/M: CT TAA ACC TGT CTT GTA ACC TTC ATG	Incubated at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 62.5°C for 15 s, 72°C for 30 s
IVSI-110	F-primer: ACT CTT GGG TTT CTG ATA GGC ACT R-primer: CAG CCT AAG GGT GGG AAA ATA G PNA probe: OO-TAG ACC AAT AGG C	Denatured at 95°C for 10 min, followed by 25 cycles of 95°C for 15 s, 71°C for 1 min, 62°C for 15 s, 72°C for 30 s, final extension at 72°C for 5 min	F-primer: ACT CTT GGG TTT CTG ATA GGC ACT *IVSI*-110/N: CAG CCT AAG GGT GGG AAA ATA CAC C *IVSI*-110/M: CAG CCT AAG GGT GGG AAA ATA CAC T	Incubated at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 59.5°C for 15 s, 72°C for 30 s
Codon 39	F-primer: CTC TGC CTA TTG GTC TAT TTT CCC R-primer: ATC CCC AAA GGA CTC AAA GAA CC PNA probe: OO-ACC TCT GGG TCC A	Denatured at 95°C for 10 min, followed by 25 cycles of 95°C for 15 s, 72°C for 1 min, 63°C for 15 s, 72°C for 30 s, final extension at 72°C for 5 min	F-primer: CTC TGC CTA TTG GTC TAT TTT CCC Codon 39/N: ATC CCC AAA GGA CTC AAA GAA CCT GTG Codon 39/M: ATC CCC AAA GGA CTC AAA GAA CCT GTA	Incubated at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 61.5°C for 15 s, 72°C for 30 s

Abbreviations: M, mutant allele; N, wild-type allele; OO, ethylene glycol linker; PCR, polymerase chain reaction; PNA, peptide-nucleic-acid.

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Improved prenatal detection of a fetal point mutation for achondroplasia by the use of size-fractionated circulatory DNA in maternal plasma—case report

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Introduction The efficacious analysis of fetal loci involving point mutations from circulatory fetal DNA in maternal plasma is hindered by the preponderance of maternal DNA. It has recently been shown that the size difference between fetal and maternal DNA species can be used for the selective enrichment of circulatory fetal DNA in maternal plasma. We have now tested this approach for the detection of a fetal point mutation in the fibroblast growth factor receptor 3 (FGFR3) gene that causes achondroplasia.

Methods Circulatory DNA was extracted from maternal plasma and size-fractionated by agarose gel electrophoresis. The fraction with a size less than 300 bp was examined by a touchdown PCR assay specific for the FGFR3 gene, and the mutation was identified by SfcI restriction analysis.

Result Our analysis indicated that although the fetal mutation was discernible in the analysis of total plasma DNA, the result using size-fractionated DNA was much more evident.

Conclusion The enrichment of circulatory fetal DNA in maternal plasma by size-fractionation facilitates the detection of subtle feto-maternal genetic differences, such as those involving point mutations. This approach can easily be extended for the non-invasive prenatal determination of other fetal loci. (190) Copyright © 2004 John Wiley & Sons, Ltd.

KEY WORDS: prenatal diagnosis; point mutation; fetal DNA; maternal plasma

INTRODUCTION

The discovery of circulatory fetal DNA in maternal plasma has opened a new avenue for non-invasive prenatal diagnosis and has readily been seized upon for the risk-free assessment of fetal loci absent from the maternal genome (Lo *et al*., 1997). As such, it is already used clinically for the determination of fetal Rhesus D status in Rhd mothers with a positive partner or for fetal sex determination in pregnancies at risk for X-linked disorders (Faas *et al*., 1998; Lo *et al*., 1998; Zhong *et al*., 2000; Costa *et al*., 2002; Rijnders *et al*., 2004).

Since only a small percentage of the total circulatory DNA in maternal plasma is of fetal origin (approximately 3–5%), the analysis of more subtle feto-maternal genetic differences, such as Mendelian disorders involving point mutations is more complex, as they are masked by the preponderance of maternal sequences (Hahn and Holzgreve, 2002).

It has recently been shown that the major proportion of circulatory fetal DNA fragments is of a smaller size than corresponding maternal ones (Chan *et al*., 2004; Li *et al*., 2004). It has also been shown that by selecting fragments with a size of less than 300 bp a selective enrichment of fetal DNA can be achieved and that this will facilitate the detection of otherwise masked fetal polymorphic loci (Li *et al*., 2004).

We have now examined whether this approach can be used for the detection of fetal loci involving single point mutations, using achondroplasia as a model case. Achondroplasia (ACH) is the most common genetic form of dwarfism, and is caused by dominant mutations in the fibroblast growth factor receptor 3 (FGFR3) gene. More than 90% of ACH patients have the same mutation in the transmembrane domain of FGFR 3, a G-A transition at nucleotide 1138 (G380R mutation) (Shiang *et al*., 1994).

In our study, circulatory fetal DNA sequences were enriched by size-separation and examined by a combination of touchdown polymerase chain reaction (PCR) and restriction enzyme analysis. Our analysis indicates that this approach permits a more precise detection of the fetal G380R mutant allele than conventional analysis of total plasma circulatory DNA.

METHODS AND MATERIALS

Patient

Our patient is a healthy parous woman, with a gestational age of $34\frac{3}{7}$ weeks. Her husband and her previous

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child have ACH. Amniocentesis had been performed at 16 weeks because of maternal age (38 years). The parents requested simultaneous analysis for achondroplasia so as to prepare for the eventual birth of an affected child and also because in the previous pregnancy mid-trimester ultrasonography had not identified the fetal condition. The second fetus had a normal karyotype, but was also affected by ACH.

Sample processing

Eighteen millilitre peripheral blood, using EDTA as an anti-coagulant, was obtained from the mother (34 + 3 weeks) and simultaneously from the father, who is affected with ACH caused by the G380R mutation. This study was approved by our Institutional Review Boards, and informed consent was obtained prior to venipuncture. Following transport by overnight express courier, the plasma was separated by an initial centrifugation at 1600 g for 10 min, and a further one at 16 000 g for 10 min. The plasma and the buffy coat were then stored at −70 °C.

DNA isolation and size fraction

Circulatory DNA was extracted from 7-mL maternal plasma using the Roche ® High Pure Template DNA Purification Kit (Roche, Switzerland), in combination with a custom-made vacuum pump (Li *et al*., 2004). Maternal and paternal DNA were extracted from the respective buffy coats.

The extracted DNA was fractionated by agarose gel electrophoresis (1% agarose gel containing 0.5 ug/mL ethidium bromide (Sigma, USA)). Using 100-bp ladder DNA (New England Biolabs, USA) as a guide, a gel slice containing DNA fragments with an approximate size of 100–300-bp fragments was excised using a sterile scalpel blade. DNA was eluted from the agarose section using QIAEX® II Gel Extraction Kit (Qiagene, Switzerland) (Li *et al*., 2004).

Touchdown PCR and restriction digestion

The small amount of template DNA necessitated the use of a high number of amplification cycles (n = 57) for the amplification of the target DNA. As conventional PCR assay would under these conditions have led to erroneous amplification, we used a touchdown PCR protocol, as this leads to a greater specificity by reducing the amount of mispriming associated with high cycle numbers (Don *et al*., 1991). This is achieved by using a temperature gradient in the first few cycles (in our instance, seven cycles), until the optimal primer-annealing conditions are attained. This is then followed by a conventional PCR amplification for the remaining number of cycles.

For our touchdown PCR amplification, a total of 57 cycles were performed, using a PCR reaction mix containing 5 uL of 10× buffer; 3.5 mM MgCl2; 160 μM dNTPs; 0.1 μM each of forward and reverse primers (Microsynth, Switzerland), 0.75U of AmpliTaq Gold polymerase (Perkin Elmer, USA) and 5 uL of DNA in a total volume of 50 uL. After incubation at 95 °C for 10 min, a temperature gradient was carried out for the seven cycles (95 °C for 15 s, 65 °C for 15 s, 72 °C for 30 s), whereby the annealing temperature was decreased 1 °C every cycle. This was then followed by a further 50 cycles of 95 °C for 15 s, 58 °C for 15 s, and 72 °C for 30 s. The PCR was terminated with an extension phase at 72 °C for 7 min. The PCR product was purified using a MinElut kit (Qiagene).

The purified PCR product (15 uL) was digested with SfcI (New England BioLabs, USA) at 37 °C overnight. Digested fragments were separated by polyacrylamide gel (6%) and the products were visualised by staining with SYBRGreen (Molecular Probes, The Netherlands).

RESULTS

The FGFR 3 mutation at 1138 (G-A) creates a unique SfcI restriction site, which permits the 164-bp amplicon to be digested to yield two fragments having sizes of 109 bp and 55 bp respectively (Shiang *et al*., 1994). Our analysis indicates that the mother has two normal copies of the FGFR3 gene, as the 164-bp amplicon was not digested by the SfcI restriction enzyme (Figure 1, lane 2). The father, on the other hand, is heterozygous for this mutation, as determined by the presence of a normal FGFR 3 allele and a mutant ACH allele, in that both the normal 164-bp amplicon and the digested mutant 109-bp fragment is detected (Figure 1, lane 4). In our analysis the 55-bp fragment was not discernible, as it could not be adequately resolved. In our examination of circulatory fetal DNA, we determine that while the 109-bp ACH mutant fragment could be detected in the analysis of total circulatory DNA (Figure 1, lane 6), this was far more obvious in the analysis performed on size-fractionated DNA (Figure 1, lane 8).

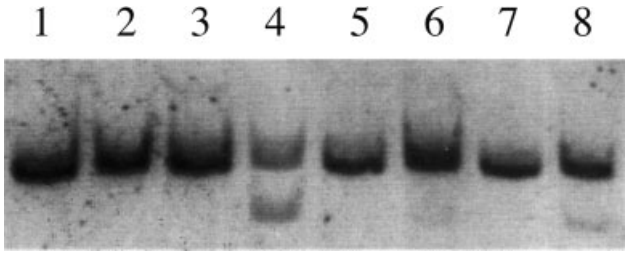

Figure 1—PCR amplification and restriction analysis of PCR products for the achondroplasia mutation of the FGFR3 gene. Lane 1: Maternal DNA (undigested). Detection of 166 bp fragments. Lane 2: Maternal DNA (digested with SfcI). Lane 3: Paternal DNA (undigested). Lane 4: Paternal DNA (digested with SfcI). Detection of 166 bp and 109-bp fragments. Lane 5: Total circulatory DNA in maternal plasma (undigested). Lane 6: Total circulatory DNA in maternal plasma (digested with SfcI). Lane 7: Size-fractionated circulatory DNA in maternal plasma (undigested). Lane 8: Size-fractionated circulatory DNA in maternal plasma (digested with SfcI)

DISCUSSION

The vast excess of circulatory maternal DNA sequences hinders the reliable analysis of fetal loci from circulatory fetal DNA in maternal plasma, especially when examining point mutations or small genomic differences. Recent findings have shown that circulatory fetal DNA molecules in maternal plasma predominantly have a size of less than 300–500 bp, whereas the majority of circulatory maternal DNA molecules are larger than this (Chan *et al*., 2004; Li *et al*., 2004). This feature can be exploited to facilitate a selective enrichment of circulatory fetal DNA sequences, thereby permitting the detection of otherwise masked fetal genetic traits (Li *et al*., 2004).

To test this approach, we examined a pregnancy at risk for ACH. Our data indicate that the presence of the mutant fetal allele could be more readily detected in the circulatory DNA fraction, which had been subjected to size-separation when compared to the analysis of total circulatory DNA. This is the second case report on non-invasive prenatal diagnosis of fetal achondroplasia by the use of circulatory fetal DNA in maternal plasma (Satio *et al*., 2000). Our present study indicates that the enrichment of fetal DNA by size-fractionation is a significant step forward towards the more optimal detection of fetal sequences, differing only discretely from the maternal background.

In this context, the use of mass spectrometry for the examination of paternally inherited fetal genetic loci in maternal plasma has very recently been reported (Ding *et al*., 2004). In this study, point mutations for β-thalassaemia as well as associated–single nucleotide polymorphisms (SNPs) could be reliably detected from maternal plasma samples. The immediate advantage of this approach is that it can be used on total plasma cell-free DNA and does not require any additional processing, such as size-fractionation. Furthermore, it is amenable to high-throughput automated analysis. The disadvantage of this new approach is that it requires very sophisticated and expensive equipment, not readily available to the vast majority of diagnostic or research laboratories.

Our approach on the other hand, while somewhat more labour intensive and unsophisticated, does not require any specialised equipment and as such can be readily implemented in any standard routine diagnostic or research laboratory. Hence, under these circumstances, the size-fractionation approach may be particularly useful for the prenatal analysis of other Mendelian disorders, which cannot be performed reliably on total circulatory DNA in maternal plasma.

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Oral presentation:

Detection of paternally inherited fetal point mutations in maternal plasma by the use of size-fractionated circulating DNA.

Toronto, Canada. October 26-31, 2004. The 54th American society of human genetics.

Poster presentation:

1. **Size separation of circulatory DNA in maternal plasma permits detection of fetal DNA polymorphisms**.

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2. **Detection of Donor-specific DNA Polymorphic Markers in the Urine of Renal Transplant Recipient by Fluorescent PCR and Real-time PCR.**

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