

— REVIEW PAPER —

Fetal cells in maternal circulation – potentials for prenatal control

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Multiple studies have already showed that intact fetal cells and fetal nucleic acids circulate freely in maternal blood, in contrast to the traditional knowledge that placenta is an impermeable barrier, which prevents any communication between maternal and fetal blood. Fetal cells in maternal blood are very rare and a major issue is to determine the real number of fetal cells present in maternal circulation. Numerous reports do not agree about the numbers of fetal cells and DNA, not only in various gestational ages but also in different pregnancies. Until now, five types of fetal cells have been isolated and detected from maternal blood: trophoblasts, lymphocytes, granulocytes, nucleated erythrocytes and progenitor cells. These cell types could potentially be used for prenatal diagnosis. However, there are some difficulties concerning the use of these cells for accurate prenatal control.

Key words: prenatal control, fetal cells, free DNA.

INTRODUCTION

Prenatal control for fetal abnormalities has become a standard procedure of modern obstetrics. Obstetricians, offering prenatal control or screening, take a heavy responsibility. It is a fact that there is always the risk for diagnostic error, which could result either in loss of a normal pregnancy or in an avoidable birth of a handicapped child. Before the 1960s, prenatal detection and diagnosis of genetic disorders and birth defects did not exist. The only available method was practically the family history. Penrose first described the association between maternal age and Down syndrome in 1933. It was not until the 1960s though, that prenatal control of genetic disorders became a viable option. At present, accurate prenatal diagnosis of chromosomal anomalies is only available by obtaining fetal cells through an invasive procedure, such as amniocentesis or chorionic villous sampling (CVS). Due to the fact that both procedures are associated

with a risk of miscarriage (1% and 2%, respectively) they are currently applied only to a small group of women, which are in a higher risk of having an offspring with a chromosomal defect in comparison to the general population. In order to determine this high risk group, some screening approaches have been developed during the years. The aim of the currently available screening tests is actually to identify, with the highest possible sensitivity and specificity, the women who should be offered the invasive procedure. Nowadays, such a screening is provided by using the family history, the maternal serum screening and the ultrasonography. Every time a test is carried out, the background risk is multiplied by the test factor to calculate a new risk which becomes afterwards the background risk for the next test. This process is called sequential screening. Although screening tests are not diagnostic, they can indeed alter the odds. It is a fact that although the risk of any individual 36 years old is higher than that of a 26 years old one, there are so many more pregnancies in the 26 years old group that, from a population perspective, most abnormalities (approximately 70%) occur in the

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'low risk' population.

At present, prenatal control of fetal chromosomal anomalies is obtained by invasive procedures which entail a risk of miscarriage. That is the reason why the indication for CVS or amniocentesis is restricted to defined high-risk groups according to the up-to-date available screening tests. The available screening tests do not have 100% sensitivity and there is always a small false positive rate too.

In 1893, Schmorl identified trophoblastic cells in lung tissue of women dying of eclampsia, but not in women dying of other causes. Evidence for the presence of fetal cells in maternal circulation was first presented by Douglas *et al.* in 1959. Kleihauer *et al.* in 1957 detected fetal cells in the blood of pregnant women by staining the cells for fetal haemoglobin. In 1964, Clayton *et al.* reported the presence of immature red cells in the maternal circulation, in an increasing percentage as gestational age advanced, by using a staining method for fetal haemoglobin. In 1969, Walknowska *et al.* found the XY pair of chromosomes in maternal blood cells of a pregnant woman carrying a male fetus and Y-chromatin-positive cells in maternal blood of women carrying males. When cell sorting techniques became available in the 1970s, isolation and characterization of fetal cells was feasible. The first research group which used PCR to detect the presence of fetal cells in the maternal blood was Lo *et al.* (1998). In 1990, Bianchi *et al.* enriched the maternal blood specifically for fetal nucleated red blood cells (NRBCs) and analyzed these cells with PCR techniques. All the knowledge until now confirms the scarcity of the circulating fetal cells, counting 1-6 cells ml⁻¹ of maternal blood (Sekiwaza *et al.*, 2002; Tsui *et al.*, 2002), and constitutes a significant challenge for their detection. Until now, numerous protocols of enrichment procedures and sophisticated methods of identification have been developed. Initially, efforts to enrich fetal cells in maternal blood were based on density gradient centrifugation, but with marked contamination from maternal cells. Later, fetal specific markers were used, either with FACS (fluorescence-activated cell sorting) or with MACS (magnetic-activated cell sorting) in order to capture these cells and to confirm with additional markers their fetal identity. Today, PCR, FISH (fluorescence *in situ* hybridization) and cytogenetic analysis are used for the identification of the fetal cells and the prenatal control of inherited diseases.

TIMING OF FETOMATERNAL TRANSFER, TYPE AND NUMBER OF FETAL CELLS

In contrast to the traditional knowledge that placenta is an impermeable barrier which prevents any communication between maternal and fetal blood, multiple studies have already showed that intact fetal cells and fetal nucleic acids circulate freely in the maternal blood. Several investigators have reported the presence of Y chromosomal DNA in maternal blood samples as early as six weeks of gestation (Sekiwaza *et al.*, 2002). These data were accurate, since the population that had been used consisted of women who conceived with *in vitro* fertilization, so the gestation time could be measured accurately.

Fetal cells in maternal blood are very rare and a major issue is to define the actual number of circulating cells. Several studies have focused on the targeting of fetal cells and the definite establishment of their existence in the maternal blood. The detected cells were of different types. To date, five types of fetal cells have been isolated/detected from maternal blood: trophoblasts, lymphocytes, granulocytes, nucleated erythrocytes, and progenitor cells (Zhong *et al.*, 2000). These cell types could potentially be used for prenatal control.

According to Poon *et al.* (2000), the frequency of nucleated red blood cells in maternal blood is estimated at 1-10 cells ml⁻¹, while the frequency of fetal cells among them is estimated at 1-6 cells ml⁻¹ (Sekiwaza *et al.*, 2002). Thus, about 20 cells of fetal origin are expected to be isolated from 20 ml of maternal blood sample. In view of this scarcity, it is necessary to determine the exact number of these cells, and the ideal timing for blood sampling. Earlier sampling would be preferable for early prenatal control, but on the other hand it is important to ensure collection of a sufficient number of cells. Data from various studies showed that the number of fetal cells, whatever their type, increases in the maternal circulation with the advancement of gestation (Bianchi *et al.*, 1990; Sekiwaza *et al.*, 2002). Another problem that all investigators encounter is to determine specific, fetal cell markers which allow the optimal separation and identification only of fetal cells. A third issue is that some of this fetal material remains in the maternal circulation for years after delivery (up to 27 years), a phenomenon that was first described by Bianchi's group (Cameron *et al.*, 2002).

Numerous reports suggest different numbers of

fetal cells and DNA amount, not only in various gestational ages, but also in pathologic pregnancies. It is already known (Bianchi *et al.*, 1990) that the number of fetal cells in the maternal blood increases in fetal aneuploidy, pre-eclampsia, preterm labour, invasive placentation, hyper emesis gravidarum and polyhydramnios.

This increase in the fetal cells number may result from abnormalities in the placental barrier, secondary vascular changes or development of villous oedema (Bianchi *et al.*, 1990). A steady state of fetal cells in maternal blood may or may not exist. Even bimanual pelvic examination (squeezing the uterus prior to blood sampling) could cause transient passage of fetal cells into maternal blood (Zhong *et al.*, 2000).

Maternal blood sampling and testing with triple density gradient centrifugation, incubation with CD71 antibody, MACS and FISH from women who ought to have CVS, was successfully carried out in order to identify numbers of nucleated red cells with three-signal nuclei (Bianchi *et al.*, 1992). Three-signal nuclei were present in 5% of the enriched cells from 61% of cases with Down's syndrome and in none of the normal pregnancies, a similar finding with the sensitivity of the second trimester screening. For a cut-off of 3% of three-signal nuclei, the sensitivity for Down's syndrome was 97% and the false positive rate was 13%.

Combination of an increased number of circulating fetal cells and free fetal DNA in maternal blood could potentially be applied as an additional screening method for assessing the risk for fetal aneuploidies (Ng *et al.*, 2003). Of course, the techniques that have to be applied in order to achieve these measurements are much more complicated in comparison with the traditional serum screening. Nicolaides *et al.* (1999) suggested carrying out FISH on maternal blood enriched for fetal cells only for the high-risk group, after screening by combining maternal age, nuchal translucency and maternal hCG and PAPP-A. Thus, by performing FISH for fetal cells to the high-risk population, the need for invasive procedures would potentially be reduced to 1% of the whole population (those with 3% of three-signal nuclei and those with no positive signal of fetal haemoglobin).

TYPES OF ISOLATED FETAL CELLS IN THE MATERNAL BLOOD

1. Trophoblasts

Placentation gives rise to two major areas of intimate feto-maternal contact. In the inter-villous spaces, syn-

cytotrophoblasts are bathed by maternal blood. Cytotrophoblasts mingle directly with maternal tissues by invasion into decidua and myometrium to form the endovascular cytotrophoblast. As a result, any cell which becomes detached from the trophoblast layer may simply pass into maternal circulation.

Trophoblasts were the first cells to be identified in the maternal circulation, because of their large size, their unique morphology, and intimate contact with the maternal blood (Hawes *et al.*, 1994). They are crucial for the development and function of the placenta.

The syncytial trophoblasts are multinucleated cells, found in placenta villi, and are in direct contact with the maternal tissue. These cells may be carried to the lungs via the inferior vena cava, where they may be trapped within the pulmonary capillaries and eventually become destroyed. However, it is possible that few trophoblasts may escape from this net and finally reach the peripheral circulation from where they can be isolated and established in culture.

The cytotrophoblasts are nucleated cells that invade the uterine wall and its spiral arteries and can end up into the maternal circulation. This feto-maternal traffic may be a normal pregnancy event, which is influenced by some factors, like pre-eclampsia, eclampsia, manual removal of placenta, advancing gestation and parturition.

One of the differentiating features of the trophoblasts is their expression of cytokeratins, as opposed to all haematopoietic cells and endothelial cells of blood vessel origin, which are cytokeratin negative. For the first time in 1984, trophoblasts were isolated from maternal blood at varying stages of pregnancy (based on separation with monoclonal antibodies against H315) by Covone *et al.* (1984) and later by Mueller *et al.* (1990). This verified that five monoclonal antibodies to cytokeratins were specific for fetal tissue. After maternal blood was exposed to two out of the five monoclonal antibodies, the isolated fetal cells were subjected to PCR for detection or not of Y sequences. Fetal sex was correctly identified in all seven males and six out of seven females. Using cells separated by immunomagnetic lymphocyte depletion and flow-sorting, Cacheux *et al.* (1992) detected 47 XXY-type cells by FISH. Of 1387 examined nuclei, 14 (1%) showed two Y-specific domains and 45 (3.24%) nuclei had one Y-specific domain. Alternatively, trophoblast cells could be identified by hybridization of oligonucleotide probes complementary to HLA-G mRNA, a non-polymorphic major histo-

compatibility antigen, whose expression is relatively specific to placental tissues. Recently, Vona *et al.* (2000) reported a novel method for the isolation of large cells among a mixed population of cells (ISET). This approach has been demonstrated to be applicable for the isolation of trophoblasts from maternal blood. After ISET application, trophoblasts were captured by laser microdissection based on their cytomorphological and immunohistochemical characteristics (Rossa *et al.*, 2003).

However, there are some drawbacks, which prevent us from being optimistic for the use of trophoblasts for prenatal control. First, very small numbers of cells are present in the first trimester, when it would be ideal to perform prenatal control. Second, most of the trophoblasts that are released in the maternal circulation quickly become trapped and cleared in the pulmonary circulation and only rarely remain in the peripheral circulation. Third, it is known from the CVS experience that in 1% of syncytiotrophoblasts, the phenomenon of mosaicism has been observed and the placental karyotype differs from that of the fetus. Also, syncytiotrophoblasts are multinucleated cells that might complicate the application of FISH. Actually, several initial reports of isolation of trophoblastic cells were later demonstrated to be maternal leukocytes that had absorbed trophoblast agents. Other investigators, though, have managed to detect fetal Y chromosome sequences by PCR and FISH in isolated trophoblastic sprouts from maternal blood (Bianchi, 1995).

2. Lymphocytes

In the early 1970s, many studies documented the presence of male metaphases in the peripheral blood cultures of women who were carrying male fetuses. Neither the syncytiotrophoblast nor the cytotrophoblast expressed paternally derived HLA-A, B or DR antigens. In contrast, fetal leukocytes have been shown to express polymorphic HLA class I and II antigens from as early as 12 weeks of gestation (Bianchi, 1995). During pregnancy, primipara may develop anti-human leukocyte antigen HLA antibodies, which are directed against paternal HLA antigens inherited by the offspring.

Flow-sorting techniques were added to enrich the presence of fetal leukocytes bearing unique, paternally inherited human leukocyte antigens (Bianchi, 1995). Couples with father positive but mother HLA-A2-negative were studied. To elaborate, leukocytes

from maternal blood were first separated by Ficoll-Hypaque gradient and then subjected to fluorescence-activated cell sorting for HLA-A2-positive cells. Fluorescence-stained cells were scored visually for the presence or absence of Y chromatin. Among 12 pregnancies that resulted in male infants, five showed Y-chromatin cells (range from 0.3 to 1.6% of sorted cells). Among the seven mothers delivering infants whose lymphocytes failed to react with anti-HLA-A2 antiserum, none showed Y-chromatin cells. Those lymphocytes can be isolated and later identified by molecular techniques.

Identification of Y-chromosome containing cells was a method by which male cells of presumptive fetal origin were distinguished from maternal female cells (Wessman *et al.*, 1992). Accordingly, conventional karyotyping and quinacrine Y chromosome fluorescence staining techniques have been used to identify embolic fetal leukocytes. In conventional karyotyping, lymphocytes are stimulated to divide with a mitogen and the cell cycle is arrested in metaphase by the addition of colchicine. Chromosome spreads, stained with Giemsa, are examined under the light microscope. Male mitoses in maternal blood were detected from as early as the 8th week of gestation. These mitoses were probably derived from fetal lymphocytes because of their morphology and response to mitogens. Although cytotrophoblasts can divide and differentiate, neither themselves nor syncytiotrophoblasts, terminally differentiated cells, respond to mitogens. Quinacrine binds to nuclear chromatin at interphase and metaphase to give brilliant fluorescence of band Yq12 on the long arm of the Y chromosome. In contrast to conventional karyotyping, large numbers of cells may be rapidly screened. However, only a proportion (between 30 and 50%) of interphase male leukocytes exhibit fluorescence. In addition, fluorescence of autosomes and absence of fluorescence in lymphocytes taken from normal males, are acknowledged sources of error.

The isolation of fetal leukocytes has not found wide applications, firstly because fetal lymphocyte cell surfaces are not inherently different from their maternal counterparts. Separation strategies based on HLA difference is impossible when paternal and maternal HLA antigens are shared. Secondly, this approach is furthermore limited by the fact that isolated lymphocytes may respond to mitogens that are used to produce the necessary metaphases for karyotyping. Thirdly, in some cases, fetal lymphocytes can persist in the maternal circulation for many years

after pregnancy. Bianchi (1999) flew sorted cells based on hematopoietic stem cell markers and found Y-chromosome-specific sequences in six out of eight non-pregnant women who previously had a male child and were six months to 27 years postpartum. The persistent cells were either lymphoid or myeloid progenitors that expressed CD34 or both CD34 and CD38. In one woman, male CD4+ cells were detected. The women that were included in this study were healthy and had no history of blood transfusions. This study led to the speculation that normal pregnancy can lead to a physiological state of low-grade microchimaerism in a woman. The hypothesis was tested that fetal cell microchimaerism plays a role in the higher incidence of autoimmune disease that occurs in women years after childbearing. A blind study demonstrated significantly increased amounts of male (presumed fetal) DNA detectable in the peripheral blood of women who suffered from scleroderma, compared with their healthy sisters or normal controls (Bianchi, 1999). These data suggest that fetal microchimaerism resulting from pregnancy, labour, or delivery, plays a role in the pathogenesis of scleroderma. Probably, a major fetomaternal transfusion occurring at the time of delivery includes some fetal cells with proliferation potentials. These cells can migrate to lymphopoietic organs and start to proliferate when there is antigenic similarity between fetus and mother. A response of graft-host takes place in some cases and may result in the development of an autoimmune disease. The postpartum persistent fetal cells may be related to the etiology of autoimmune disorders that have higher incidence in women and have onset years after childbearing.

3. Granulocytes

Fetal granulocytes are the least studied among the whole target cell types. In 1975, Zilliacus *et al.* estimated that fetal granulocytes comprised 0.02 - 0.04% of the mononuclear cell samples taken from 19 pregnant women during the second and third trimesters. Wessman *et al.* (1992), using techniques of *in situ* hybridization, demonstrated that 0.26% of the maternal mononuclear cells became hybridized to a Y chromosomal probe. He used Ficoll-Paque density gradient centrifugation followed by FISH with a Y-specific probe that recognizes heterochromatic Yq (Rossa *et al.*, 2003). Y-specific cells were recovered from eight pregnant women, seven of whom gave birth to male infants. Bianchi (1999) described a me-

thod, which enabled the enrichment of fetal granulocytes captured by a combination of monoclonal antibodies, anti-CD71, antiglycophorin A and anti-CD36. Yet, the problems of the longevity and the persistence of the cells in subsequent pregnancies prevent application of the above methods (Steele *et al.*, 1996).

4. Fetal haematopoietic progenitor cells

Recently, the presence of fetal haematopoietic cells expressing the CD34 surface antigen in maternal circulation has been demonstrated (Little *et al.*, 1997). Under appropriate culture conditions, this cell population can be amplified, thereby providing material for further genetic analysis of the fetal genome (Jansen *et al.*, 2000). The emerging consensus based on studies that focused on quantification of fetal cells in the maternal circulation, is that the number of fetal cells increases in the maternal circulation with the advancement of pregnancy. A significant two-fold increase in the mean number of fetal CD34+ cells was observed from the first to the second trimester. Accordingly, sampling after the 12th week of pregnancy should increase the feasibility of detecting fetal cells in maternal blood samples. Another approach might be sampling at two times, one in the first and one in the second trimester, thereby the overall probability of detecting fetal cells is increased. Guetta *et al.* (2004) examined the presence of fetal CD+34 cells in 30 samples of women carrying male fetuses. Application of triple-density gradient centrifugation, magnetic activated cell sorting (MACS) technology, identification of the cells with FISH and PCR enabled the study of CD+34 cells in each trimester and within individual samples. Male/fetal cells were detected in 8/10 first trimester samples, 9/9 second trimester samples and 10/11 third trimester samples. The number of fetal CD+34 cells in 10 ml of maternal blood increased by two-fold from the first to the second trimester and decreased in a similar level in the third trimester (3/6/3 cells). Several reports have been published regarding the efficacy of *in vitro* culture for expansion and enrichment of fetal progenitor cells, isolated from the maternal circulation that can be accomplished under certain conditions. Under appropriate conditions, these cells can proliferate in culture, are more frequent in fetal blood than in peripheral adult blood, and also proliferate more rapidly in culture compared with those of adult origin (Eridani *et al.*, 1998). Seeding of the positive fractions in semi-solid methylcellulose medium or in liquid culture re-

sulted in improved gender prediction and increased yields of fetal cells available for analysis. In the study of Guetta *et al.* (2003), a 2.4-fold increase in the number of CD+34 cells was detected by FISH following liquid culture.

The major drawback of using these progenitor cells for analysis is that a small number of residual cells (1-2 cells per 20 ml of maternal blood sample) appear to persist from one pregnancy to another. A second serious problem is the lack of specific cell markers for CD+34 cells. Gender-based detection with FISH or PCR is possible in the case of a male fetus. However, this approach does not provide an adequate solution, because CD+34 cells from female fetuses cannot be distinguished from maternal CD+34 cells.

5. Fetal nucleated erythrocytes (NRBCs) and technical challenges

Perhaps the best characterized fetal cell type found in maternal circulation is the erythroblast, a nucleated red blood cell that migrates from the fetal circulation to the maternal blood. In the Kleihauer-Betke technique, acid elution of adult haemoglobin (HbA) from adult erythrocytes renders them “ghosted” and unstable. Larger fetal erythrocytes, which contain fetal haemoglobin (HbF), are resistant to such treatment and remain clearly stained. With this technique, fetal erythrocytes have been detected in maternal peripheral blood samples from as early as eight weeks of gestation. Tremendous interest about the fetal nucleated erythrocyte and its precursors as a target cell type has been developed in the last years for several reasons. They are mononuclear, abundant in the fetal blood during the first trimester (Bianchi, 1999) when haemopoiesis occurs mainly in the yolk sac; they are well-differentiated, they express unique antigens such as the transferrin receptor (Ganshirt-Ahlert *et al.*, 1993), they produce unique fetal haemoglobin chains such as ϵ , ζ and γ (Bianchi, 1999) and finally they have a short life-span. Nucleated erythrocytes have a full complement of nuclear genes. If fetomaternal transfusion is reflective of fetal blood, a 1000:1 red cell to white cell ratio is present. Up to date, no problem has arisen with persistence of nucleated erythrocytes into the next pregnancy. Generally, the fetal nucleated red cells have characteristic nuclear morphology, roundness, gamma globin staining intensity of the cytoplasm and peripheral brightness of the stained cytoplasm that makes them distinguishable

from the maternal cells (Dong *et al.*, 2004).

NRBCs are extraordinarily rare in maternal circulation. In order to be used for detection of fetal aneuploidy enrichment, protocols are necessary to be applied. Two approaches are possible: either “positive” selection of NRBC or “negative” depletion of unwanted maternal cells. Some separation techniques combine both approaches. At present, no consensus exists regarding the best way to isolate fetal cells from maternal blood. Each method has advantages and disadvantages with respect to fetal recovery, cost and time of separation. An important concept of the separation of the NRBCs is the yield (actual number of fetal cells recovered) and purity (percentage of fetal cells relative to maternal cells in the enriched sample). Many approaches have been designed to recover fetal cells from maternal blood. These include density gradient centrifugation, carbonic anhydrase inhibition, magnetic activated cell sorting, immunomagnetic bead separation and micromanipulation of individual cells (Bianchi *et al.*, 1996).

Most investigators begin with a 20 to 40 ml venous blood sample. An initial enrichment step facilitates removal of many maternal non-nucleated erythrocytes, either through density gradient centrifugation or exposure to lytic buffers. Differential density gradient separation is based on the segregation of maternal and fetal cells into different solutions depending on their density characteristics (Troeger *et al.*, 1999a). Subsequent “purification” can be performed by means of various cell separation techniques, such as FACS (fluorescence-activated cell sorting) (Bianchi *et al.*, 1993) and MACS (magnetic-activated cell sorting) (Busch *et al.*, 1994; Ganshirt-Ahlert *et al.*, 1994). In FACS, fetal cells bound to the fluorescence conjugated antibodies are sorted by flow cytometry. For MACS, typically, maternal cells are depleted through their interaction with antibody-conjugated to magnetic beads, against non-fetal antigens with or without additional enrichment of fetal cells with antibodies against a fetal cell marker (Busch *et al.*, 1994). Genetic analysis of the identified fetal cells has relied primarily on two techniques, fluorescence *in situ* hybridization (FISH) using chromosome specific probes and polymerase chain reaction (PCR), to amplify uniquely fetal gene sequences. FISH has had an enormous impact on this field because it does not require the presence of a dividing cell. Therefore, the major fetal cell conditions associated with an abnormality in chromosome number can be readily detected, including all autosomal trisomies (Ganshirt-Ahlert *et al.*,

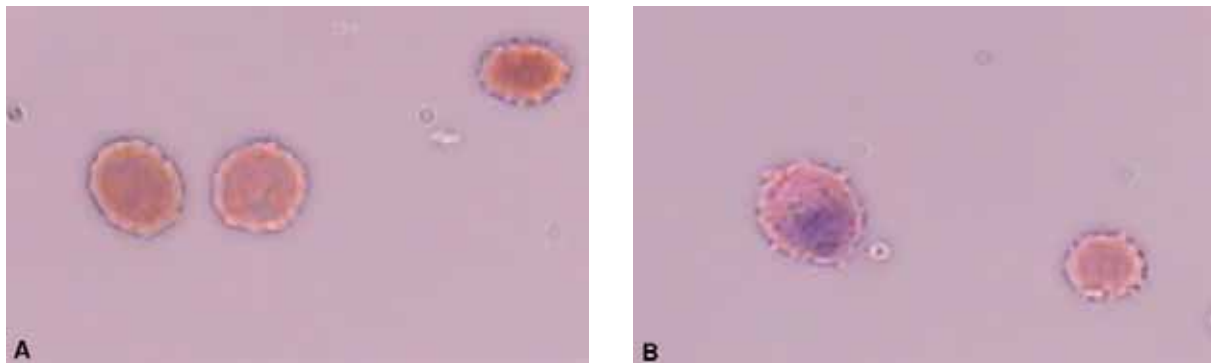


FIG. 1. A. Three proerythroblasts ($\times 1000$) enriched from the maternal blood stained with MAY-GRUNWALD-GIEMSA. B. One proerythroblast and one smaller late normoblast ($\times 1000$) enriched from the maternal blood stained with MAY-GRUNWALD-GIEMSA.

1993), some of the sex chromosome abnormalities, and triploidy (Bianchi, 1999). The development of PCR significantly affected this field, because of the scarcity of fetal cells in a maternal sample, but no longer is a limiting factor. Initially Y chromosome specific PCR was used to prove the existence of fetal cells in maternal blood (Bianchi *et al.*, 1990, 1994; Lo *et al.*, 1990). Subsequently, PCR was used to prove the presence of paternally inherited fetal genes that were absent in the mother, including β globin mutations (Bianchi, 1999), Rhesus D (Geifman-Holtzman *et al.*, 1996), Rhesus C and HLA DR (Bianchi, 1999).

Bianchi *et al.* (1997) were the first who focused on fetal erythrocytes. Erythrocyte frequency in maternal blood was estimated to be 1-10 cells ml^{-1} (Troeger *et al.*, 1999b). In trisomy 21, this number seems to increase (Bianchi *et al.*, 1992). They used FISH and PCR in order to estimate the number of fetal cells in maternal blood. Both techniques showed less than one fetal cell per 100,000 nucleated maternal cells in the first trimester and about one fetal cell per 10,000 nucleated maternal cells at term. Because of the low number, nucleated red blood cells (NRBC) need to be enriched from other cells with different features, usually by combining separation methods such as density gradient centrifugation, FACS or MACS and charge flow separation. There are reports of successful prenatal control of fetal aneuploidy and monogenic hereditary disorders based on analysis of fetal-derived NRBCs (Bianchi *et al.*, 1992; Zhong *et al.*, 2000). The published techniques are laborious. Attempts to enrich NRBC have concentrated on FACS, based on the use of specific antibodies that recognize selective cell-surface or intracellular markers (Bianchi *et al.*, 1990). Subsequently, MACS was introduced

based on trapping cells labelled with an iron-antibody complex to a magnetic surface (Ganshirt-Ahlert *et al.*, 1994). For this purpose specific antibodies have been developed. Erythroblasts are often identified by using anti-CD71 antibody, which recognizes the transferrin receptor (Ganshirt-Ahlert *et al.*, 1994), but unfortunately this antibody recognizes some other fetal nucleated cells (fetal leukocytes) as well as some maternal cells, and finally a mixture of fetal and non-fetal cells is obtained (Rossa *et al.*, 2003). Furthermore, only about half of the erythroblasts in maternal blood are of fetal origin (Troeger *et al.*, 1999b). The antibody to the γ chain of globin has been widely used, but this type of chain also was recognized in the maternal nucleated blood cells (Zheng *et al.*, 1995). Although antibodies that recognize embryonic globin chains such as ζ or ϵ are specific for fetal cells, the expression of these globin molecules diminishes as

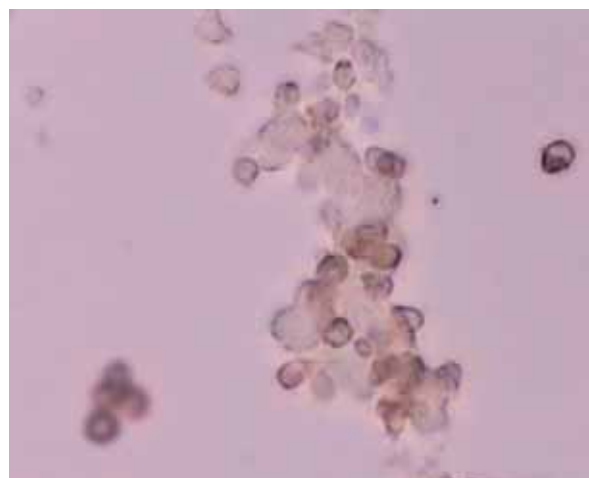


FIG. 2. Enriched red blood cells ($\times 1000$) from maternal blood stained with antibody to Glycophorin A after magnetic activated cell sorting (MACS).

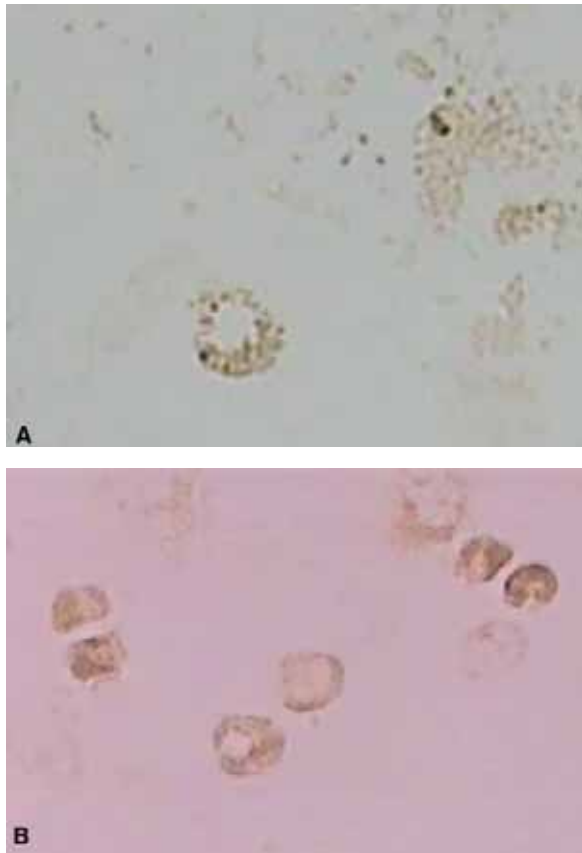


FIG. 3. A. Nucleated red blood cell ($\times 1000$) after enrichment from maternal blood stained with antibody to γ chain of haemoglobin. B. Nucleated red blood cells ($\times 1000$) after enrichment from maternal blood stained with antibody to γ chain of haemoglobin.

gestation progresses (Choolani *et al.*, 2001). Specifically, the expression of the gene encoding ζ globin declines dramatically after 6-7 weeks of gestation and although ϵ globin expression decreases later, it is present in less than half of all fetal nucleated red blood cells at 11-12 weeks of gestation and is essentially undetectable at 15 weeks (Choolani *et al.*, 2001). Other markers used for isolation of NRBCs are the thrombospondin receptor CD36 (Bianchi *et al.*, 1993), glycoprotein A (Troeger *et al.*, 1999a) and fetal globin chains γ or ϵ (Zheng *et al.*, 1995). Individuals with certain disorders of haemoglobin may demonstrate persistence of fetal haemoglobin, such as in carriers of β -thalassaemia mutation, and an increased number of maternal gamma-positive cells (Figs 1, 2, 3).

6. Free DNA

The potential utility of free fetal DNA in maternal plasma was ignored until reports demonstrated tu-

mor-specific extracellular DNA fragments in the plasma of cancer-developing patients (Chen *et al.*, 1996). Prompted by these reports, Lo *et al.* (1997) investigated the existence of a similar phenomenon in pregnancy. They have demonstrated surprisingly high concentrations of fetal DNA (3.4-6.2%) in the total maternal plasma DNA, with the mean fetal DNA concentrations increasing by 12-fold over the course of gestation. When results were expressed as copies of SRY (Lo *et al.*, 1997), a single copy of Y chromosome-specific sequence, it appeared that significantly more fetal DNA than fetal cells was present in the mother. According to Lo *et al.* (1997), male DNA was not detected in the plasma when the prior child was male, but the current pregnancy was female. Therefore, DNA must eventually be cleared from the circulation.

The finding of this new biological phenomenon could provide new possibilities for non-invasive prenatal testing. One explanation about the presence of free fetal DNA in the maternal blood is that there is continuous leakage or transfer of fetal cells across the placenta, but the maternal immune system rapidly destroys this material leaving free DNA in the plasma. An alternative explanation is that there is active remodelling of the placenta at the fetal/maternal interface, with continuous cell lysis and direct release of fetal DNA into the maternal circulation (Bischoff *et al.*, 2005). In addition, apoptosis of fetal cells may occur during the development (Bianchi, 1998). To address the question whether apoptotic fetal erythroblasts are the main source of the cell-free fetal DNA in maternal blood, Zhong *et al.* (2002) studied samples from pregnant women with normal and abnormal pregnancies carrying male fetuses. In the same samples, they enumerated fetal cells in maternal whole blood by both FISH with X and Y probes and single-cell PCR and then measured levels of cell-free DNA. There was no correlation between the number of fetal cells and the amount of free fetal DNA in the normal pregnancies, and the pregnancies affected by pre-eclampsia or by preterm labour. Thus, due to the fact that fetal erythroblasts are very rare, it is unlikely that they account for both the volume and the turnover of cell-free DNA in the maternal circulation. Bianchi (2004) tried to answer the question, whether apoptotic fetal erythrocytes could eventually disintegrate and release their DNA after phlebotomy and also, whether cytokines and maternal immune cells could destroy the fetal erythrocytes and release fetal DNA. They measured the amount of Y chromo-

somal DNA present as fetal marker and the beta-globin DNA as a marker of total (maternal plus fetal) DNA. Measurements at first and third trimesters and over 24-hour periods did not show any increase of fetal DNA.

It seems that the placenta is the most likely source of circulating fetal DNA due to its size and cellular activity. Many studies have already confirmed the relationship between amount of free DNA in maternal circulation and advancing gestation (Honda *et al.*, 2002; Chan *et al.*, 2003). The kinetics of fetal DNA in maternal circulation also suggests that the placenta is the dominant source of DNA. By measuring the clearance of circulating fetal DNA after delivery most women had undetectable levels of circulating fetal DNA by two hours postpartum (Lo *et al.*, 1999). Perhaps the most convincing and direct evidence comes from the fact that placental mRNA molecules are readily detectable in maternal plasma. Although the source of free DNA and RNA in maternal plasma is uncertain, there are strong indications that these nucleic acids are derived from trophoblast breakdown. Furthermore, the presence of RNA transcripts for choriogonadotropin and placental lactogen sequences indicates that the RNA is, at least in part, placentally derived (Jouni *et al.*, 2003). It is important to note that fetal DNA sequences have been detected in several maternal body fluids, including amniotic fluid, maternal urine, maternal cerebrospinal fluid and maternal peritoneal fluid (Bianchi, 2004). The concentration of cell-free fetal DNA in the amniotic fluid compartment is approximately 200 times higher than that in the maternal plasma. This has led to some speculation regarding the possibility of a concentration gradient, leading to the direct transfer of DNA molecules across the placental membranes.

Fetal DNA might be technologically less challenging than identification and isolation of fetal cells. In fact, free fetal DNA, which progressively increases during pregnancy, has been estimated to account for 3.4 to 6.2 % of the total DNA in the maternal blood (Rossa *et al.*, 2003). In addition to DNA sequences, RNA sequences are present in the maternal circulation. They are surprisingly stable, persisting for at least 24 hours after delivery (Poon *et al.*, 2000; Tsui *et al.*, 2002). The reliable detection of fetal RNA could pave the way for future applications in fetal gene expression profile.

Calculations have revealed that fetal DNA is liberated by a rate of 2.24×10^5 copies min^{-1} into the maternal circulation and fetal DNA is detected in the

maternal plasma as early as 32 days of gestation (Bianchi, 2004; Wataganara *et al.*, 2004).

Other authors disagree with Lo *et al.* (1999) and provide evidence for detecting male DNA sequences in the plasma of non-pregnant women who gave birth to sons some years before, ranging from 22 to 36% (Invernizzi *et al.*, 2002; Lambert *et al.*, 2002). They suggest that, fetal cells persist at postpartum and may potentially contaminate the plasma, resulting in the apparent finding of fetal DNA persistence. The most likely interpretation of these results is that plasma samples were processed by a one-step centrifugation protocol without a second centrifugation step, which has been shown to be inadequate for the removal of cells from the plasma.

The ability to measure fetal cell-free DNA from maternal blood has led to many clinical applications. In particular, applications have been reported for the assessment of sex-linked disorders (Bianchi, 1995), fetal RhD status (Lo *et al.*, 1993), achondroplasia and myotonic dystrophy (Bianchi, 1995) or paternally-inherited chromosomal aneuploidy (Lo *et al.*, 1993; Bianchi, 1995). New approaches have been developed to enable the non-invasive assessment of autosomal recessive disorders, including congenital adrenal hyperplasia, β -thalassemia and cystic fibrosis (Chiu *et al.*, 2002; Gonzalez-Gonzalez *et al.*, 2002). The prediction of fetal RhD status based on DNA analysis has attained such accuracy that its use has been introduced in clinical practice (Costa *et al.*, 2002). Honda *et al.* (2002) demonstrated 100% sensitivity in the detection of 40 male fetuses at seven weeks of gestation using conventional PCR; the same level was achieved at five weeks of gestation using real-time PCR.

Bianchi (2004) suggested that in pregnant women carrying fetuses with trisomy 21, the concentration of fetal DNA was twice as much as in normal pregnancies. They also used the measurements of the fetal DNA to the second trimester quadruple screening test and showed that at a 5% false positive rate, the detection rate for Down's syndrome increased from 81 to 86%. Potentially, the fetal DNA quantification could be used as a screening for fetal aneuploidy (Farina *et al.*, 2002). Another clinical application is the possibility of prediction of pre-eclampsia development. Many studies have demonstrated that there is a five-fold increase in the amount of fetal DNA in samples obtained from women with symptomatic pre-eclampsia. Also, fetal DNA levels were elevated in women who developed pre-eclampsia (Leung *et al.*, 2001; Hahn & Holzgreve, 2002). Increased levels of

fetal DNA in maternal blood could also be a marker for preterm delivery. According to Leung *et al.* (1998), fetal DNA levels in maternal blood are gradually increased in normal pregnancies (Lo *et al.*, 1998) but sharp increase after 32 weeks of gestation may reflect changes that herald impending delivery. Lo *et al.* (1998) found significantly higher fetal DNA concentrations in women who had spontaneous preterm labour before 34 weeks of gestation. Fetal DNA levels were also increased in women with placenta previa (Sekiwaza *et al.*, 2002) and hyperemesis (Sekiwaza *et al.*, 2003), but not in fetuses with intrauterine growth restriction.

Despite the increasing number of potential applications, the existence of fetal DNA sequences among the background of maternal DNA, results in the restriction of maternal plasma analysis to the detection of the male associated sequences. It is a major limitation the fact that mother and fetus share, on average, half of their genomic sequences. Thus, it is only possible to detect uniquely fetal DNA sequences that are paternally inherited.

CONCLUSIONS

At present, prenatal control of fetal chromosomal anomalies is obtained by invasive procedures which entail a risk of miscarriage. For this reason, the indication for CVS or amniocentesis is restricted to defined high-risk groups according to the up-to-date available screening tests. These tests do not have 100% sensitivity and also there is always a small false positive rate.

All these facts make the need for developing non-invasive approaches for prenatal control in the low-risk population, more than necessary. An alternative approach would be to separate the rare fetal cells circulating within the maternal blood for antenatal genetic analysis. If chromosome and single gene analyses could be accomplished without invading the uterus, prenatal control could be offered to all pregnant women without any consideration of their presumptive risk of having an affected fetus.

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