60 Prenatal diagnosis of fetal abnormality using fetal cells in maternal circulation

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INTRODUCTION

The presence of rare fetal cells as well as of cell-free fetal DNA (cffDNA) and mRNA in the maternal circulation over the course of gestation has been quite recently established. The possibility of performing an early noninvasive prenatal diagnosis on fetal cells isolated from maternal peripheral blood is an important goal for modern obstetric care (1–4). Thus, fetal material can be isolated and potentially tested for genetic analysis without the need of sampling by invasive procedures as amniocentesis and chorionic villus sampling (CVS).

The types of fetal cells discovered in maternal blood are various and include lymphocytes, erythroblasts, hematopoietic stem and progenitor cells, mesenchymal cells, and trophoblasts (5,6). The main advantage of using fetal cells rather than cffDNA is that they potentially offer a source of the whole fetal genome and that could be sampled in a noninvasive way for a prenatal detection of fetal aneuploidies (7). Nevertheless, the recovery and analysis of fetal cells from maternal blood is complex, and the sensitivity is still low because of the scarcity of these cells in the maternal circulation and of the lack of universal morphologic or immunocytochemical fetal markers that allow the isolation of fetal cells without contamination of maternal cells.

In this review, we summarize the current state of noninvasive prenatal diagnosis using fetal cells isolated from maternal blood during pregnancy.

DISADVANTAGE OF PRENATAL DIAGNOSIS BY INVASIVE PROCEDURES

Currently, prenatal diagnosis of fetal genetic traits relies on invasive procedures such as amniocentesis and CVS. These tests carry a small but significant risk of fetal miscarriage, and for this reason they are limited to only those women at increased risk of having a fetus with genetic abnormalities. In a recent systematic review of complications related to genetic amniocentesis and CVS, Mujezinovic et al. (8) searched on the Medline database for articles published after January 1995 reporting data for at least 100 women with singleton pregnancies with genetic amniocentesis and CVS carried out transabdominally. They found 29 articles for amniocentesis and 16 for CVS. After the statistical analysis, results demonstrated that the relative risk for fetal loss before 24 weeks was 0.6% for amniocentesis and 1.3% for CVS. They concluded that although the pregnancy loss risk is relatively low, lack of adequate controls in most studies tends to underestimate the true added risks of the procedures. Therefore, only a limited number of pregnant women with maternal age of 35 years or older and/or those having an abnormal prenatal screening should undergo invasive prenatal procedures. A recent retrospective Italian study was carried out by

accessing the results of cytogenetic analysis of amniotic fluid in women with less than and greater than 35 years and performed during years 1995 to 1996 (9). This study reported results of 88,965 amniocentesis and compared the frequencies of chromosomal abnormalities in the two groups of women. The authors demonstrated that the incidence of fetal genetic abnormalities at the amniocentesis is independent from the age of the pregnant women; the results confirmed the age dependency for aneuploidies (>35 years), whereas it did not confirm the age dependency for structural chromosomal abnormalities. These data provide together with other previous findings (10) important information about the actual prenatal counseling and its possible modifications due to the need to recommend noninvasive prenatal tests to all pregnant women.

For all these reasons, new noninvasive prenatal approaches are necessary.

Observation that intact fetal cells can enter and circulate within maternal blood has raised the possibility of noninvasive access to fetal genetic material that would allow the prenatal diagnosis of fetal chromosomal and fetal monogenic disorders.

FETAL CELLS IN MATERNAL CIRCULATION: AN ALTERNATIVE TO NONINVASIVE PRENATAL DIAGNOSIS

The main approaches have principally focused on the detection and genetic analysis of fetal cells and trophoblasts circulating in maternal blood and on the analysis of cffDNA and fetal mRNA in maternal plasma or serum.

History of Fetal Cell Discovery in Maternal Blood

The existence of fetal cells in maternal circulation was reported in 1893 by Schmorl (11), who first described the appearance of fetal trophoblasts in the lung of women who died of eclampsia. For many years, the existence of fetal cells in maternal blood was considered controversial. In 1959, Douglas et al. (12) showed circulating trophoblasts in maternal blood, but the proof of circulating fetal cells in maternal blood during pregnancy was obtained in 1969 from Walknowska et al. (13) reporting observations of 46,XY metaphases in a low number of maternal blood lymphocytes isolated from women carrying a male fetus.

Despite rising interest, no consensus existed by the mid to late 1970s when Herzenberg and colleagues (14) first isolated fetal cells from maternal blood using a fluorescence-activated cell sorting (FACS) and they assumed the fetal origin of these cells for the presence of a paternal allele not present in the mother. Other investigators made similar observations until the 1990s when the fields have begun to take a more optimistic turn with the advent of several new molecular genetic

technologies such as fluorescence in situ hybridization (FISH) and polymerase chain reaction (PCR).

For the first time, Bianchi and colleagues (15) identified fetal erythroblasts by the detection of Y chromosome sequences in male pregnancies after a FACS enrichment and PCR analysis. The ability to select the fetal erythroblasts and to couple it with FISH using appropriate chromosome-specific probes permitted Bianchi and other authors to identify fetal aneuploidies (16–18). Later, other successful results were obtained using trophoblasts and fetal stem progenitor cells (19,20).

Finally, the discovery of cfDNA by Lo et al. (21) and more recently fetal RNA by Poon et al. (22) in maternal plasma provided other new perspectives for fetal genotyping exploitation and placental gene expression, which could have diagnostic importance for fetal pathology.

Fetal Cell Trafficking in Pregnancy

Although the placenta was traditionally seen as a barrier that separate mother and fetus, many studies have demonstrated that a variety of fetal cells and cfDNA and mRNA circulate into maternal blood throughout pregnancy and that the trafficking of this material is a frequent phenomenon occurring during every pregnancy (23). It has also been observed that the trafficking between the mother and the fetus occurs in both directions. The same bidirectional traffic seems also to take place with plasma DNA, because maternal DNA has been found in cord blood of about 30% of samples tested (24).

The entity of the trafficking of the fetal cells depends by many factors such as complicated pregnancies and by the modes of releasing them in maternal circulation. This is due to their location inside the placenta and, as a result, different cell types are released by different modes (25).

Appearance of Fetal Cells in Maternal Blood

Data indicate that the timing appearance of fetal trafficking into maternal circulation exists (17,26,27). In accordance with placental growth, the interface between fetal and maternal tissues expands, such that more fetal cells may cross the placental barrier in the early stages of gestation. Some studies provided evidences that fetal cells are present in the maternal circulation in early gestational samples. The existence of circulating fetal material was documented by the detection of Y chromosome–specific DNA sequences in pregnant women bearing male fetuses from 8 to 12 weeks of gestation (28). In 1995, Thomas and colleagues (29) provided evidence that Y DNA fetal cell equivalents from cellular fractions were present in peripheral blood of two pregnant women after in vitro fertilization program as early as the 5th and 6th weeks of gestation, respectively.

Frequency of Fetal Cells in Maternal Blood

The major limitation that continues to overwhelm the clinical use of fetal cells is their extremely low number into maternal circulation, considered not yet sufficient to perform an accurate genetic analysis.

Until now, there is no consensus about the exact number of fetal cells circulating in the maternal blood because it depends on different factors such as the fetal cell type and the form of

cellular enrichment and identification techniques used, the weeks of gestation at sampling, and the presence of aneuploid pregnancies and/or pregnancy-related disorders.

Many attempts have been pursued to determine the precise number of these cells in order to individuate the best target cells for developing noninvasive prenatal testing and the best timing for their recovery. Considering their extremely low number and the absence of a specific fetal cell marker, most research groups have proceeded to apply various cellular enrichment procedures before trying to identify and count the fetal cells; consequently, only one cell category is usually isolated and examined leaving other fetal cells uncounted. Besides, it is likely that after a cellular enrichment, a large number of these rare fetal cells are lost in any case.

Therefore, few studies have striven to quantify the absolute number of all categories of fetal cells without prior enrichment. Bianchi et al. (30) determined the number of fetal-cell DNA equivalents present in the maternal whole blood samples using a quantitative PCR method aimed to identify fetal cells and Y chromosome–specific sequences as fetal marker. The mean number of male fetal-cell DNA found varied from 0.1–91 Y-cell equivalents in 16 mL of maternal blood with an average of 19 Y-cell equivalents. Using a real-time PCR analysis, Ariga et al. (31) surveyed 20 pregnant women with male fetus during the entire pregnancy and found that the best time for recovering fetal cells from maternal circulation is between 10 and 12 weeks of gestation. They also reported that the level of Y signals was higher in plasma than in cells except in these two weeks of gestation where cellular amount of fetal material was higher than that in plasma. Other studies confirmed these results. In particular, Zhong et al. (32), using a novel robust real-time quantitative PCR assay, demonstrated that the number of fetal cells ranged approximately from 0.5 to 2.3 fetal cells/mL of whole blood. All the data, obtained without enrichment and using quantitative PCR assays, demonstrated that it is possible to isolate approximately 1 fetal cell/mL of whole maternal blood.

To actually count cells rather than cell equivalents, another strategy was used that was aimed to estimate the total fetal cell number by the fetal genetic identification using the FISH or primed in situ labeling (PRINS) analyses without enrichment procedures. The most important study in this field was conducted by Hamada et al. (26) and more recently by Krabchi et al. (33). Krabchi visualized and counted fetal nuclei using, as fetal marker, the chromosome-specific probes for X and Y chromosomes, in pregnancies bearing male fetuses. They estimated that the total number of male fetal cells/mL of maternal blood varied from two to six cells with a median value of 4 fetal cells/mL of maternal blood within a group of normal pregnancies using both FISH and PRINS.

An elevated number of fetal cells in maternal blood have been observed in some pregnancy-related disorders and as pregnancies affected by fetal trisomies, pre-eclampsia, eclampsia, intrauterine growth restriction, or polyhydramnios with respect to normal pregnancies (25). Several studies have suggested that an abnormal structure of the placenta may result in an increased fetal cell trafficking to maternal circulation. Thus, an alteration of the placental barrier might explain this high number.

It has been reported that a high number of fetal cells are present in maternal blood when the fetus is aneuploid. In two consecutive studies, Krabchi and coworkers (34,35) noted a significant increase in total number of fetal cells in pregnancies carrying aneuploid fetuses. The methodological approach was similar to their previous study; the only difference was in the use of appropriate chromosome-specific probes in accordance with the chromosome to be visualized in the fetal nuclei, and aneuploidies were detected when an extra autosomal signal was observed. In the case of fetus with trisomy 21, the authors found the number of fetal cells three to five times higher than that from a normal pregnancy. The range varied from 6 to 32 fetal cells/mL of whole maternal blood with an average of 12.87 fetal cells. Similar results were obtained in the second study performed by the same group (35) in pregnancies affected by fetuses with trisomies 18 and 13. Also if the number of fetal trisomic cells was slightly less than those from fetus with trisomy 21, it has been observed as an increase of two to five times than that in normal pregnancies.

These studies, designed to detect all categories of fetal cells using cell-type-independent markers, such as aneuploidies and Y chromosome, have allowed to reach important information about the number of cells and the fact that these cells are consistently found in the blood of all women either with normal or pathologic pregnancies. Therefore, fetal cells can be reliably identified using FISH analysis in the diagnosis of fetal aneuploidies.

Enrichment, Identification, and Analysis Techniques

The absence of specific fetal antigens and the small number of fetal cells in maternal blood are the two principal caveats limiting their use in noninvasive prenatal diagnosis of fetal aneuploidies since the biologic samples to be investigated have a wide abundance of maternal cells creating a confounding background for genetic analysis.

For this reason, many attempts have been performed to isolate or enrich fetal cell populations by using numerous strategies in order to find biologic and antigenic differences between the mother and the fetus.

The most frequently used methods to enrich fetal cells from whole maternal blood are constituted by multistep procedures; usually the initial step is a density gradient separation followed by FACS or magnetic-activated cell sorting (MACS) technologies. While the first step is aspecific and allows only to reduce the abundance of maternal cells, the second step is specific since it is based on an antigen-antibody recognition using specific antibodies for a cell category of interest. Other technologies used for the enrichment of particular fetal cell population are micromanipulation based on morphologic nucleated red blood cell (NRBC) identification under microscopic observation of stained slides (36); ISET (isolation of epithelial tumor cells) technology, which allows to isolate trophoblasts on the basis of cell size by filtration (37); galactose-specific lectin-based enrichment, a method specific for isolation of fetal NRBCs, which are rich in cell-surface galactose and therefore they can be easily adsorbed to slides containing galactose sites (38); and laser microdissection and pressure catapulting, which can isolate a cell type discriminating it by morphology, fluorescence, or transfection markers (39).

Since the enrichment methods supply fetal cell samples still contaminated by maternal cells, scientific community has focused on the research of a specific fetal cell marker for their identification. Until now, fetal cell markers widely used are fetal hemoglobin, as a protein marker and Y chromosome, as a genetic marker. It has been shown that fetal hemoglobin is not a particularly relevant marker because it is partially present in maternal cells. Fetal cell identification based on Y chromosome by using cytogenetic (FISH) and biomolecular (PCR) techniques is an effective approach but it is limited to women carrying male fetuses.

For this reason, non-fetal gender-dependent approaches have been studied to distinguish fetal cells from maternal cells. Samura and coworkers (40) have experimented a PCR system based on analysis of microsatellite marker sequences (short tandem repeat), while the research group of Hultén (41) has detected differences in the length of telomeres in maternal versus fetal cells. The possibility of discriminating fetal and maternal cells by using these methodologies could allow the selection of fetal cells by the methods of cell isolation described above.

However, the markers used until now have shown to be rather unspecific since they are present also on a significant subpopulation of maternal blood cells.

Therefore, until now it is not possible to obtain a pure population of fetal cells, and their identification and genetic analysis are complicated by the high presence of maternal blood cells.

The genetic analysis of fetal cells has been mainly performed by PCR and FISH technologies. FISH can mainly analyze fetal gender and aneuploidies while PCR permits analysis of mutations in single-gene disorders, as well as fetal gender, rhesus blood type, and Mendelian diseases.

Since Price et al. (17) reported the first case of fetal trisomy 21 diagnosed by isolation of NRBCs from maternal blood, the FISH method has been used for noninvasive prenatal diagnosis of fetal aneuploidies, as well as fetal gender.

However, as long as new technologies for the isolation of pure fetal cell population will not be developed, the genetic analysis will be always hampered by maternal cell contamination.

TARGET CELLS FOR NONINVASIVE PRENATAL DIAGNOSIS

Leukocytes

Fetal leukocytes were the first type of fetal circulating material identified and enriched from maternal circulation. Fetal leukocytes were isolated by FACS with antibodies against paternally derived HLA-A2 antigens, and fetal gender and HLA type were successfully predicted (42). Despite these initial good results obtained, noninvasive clinical applications were considered unfeasible, principally due to the lack of monoclonal antibodies specific to fetal leukocyte antigens. Consequently, there is the necessity of performing HLA typing of both parents prior to flow sorting (1). Further limitation was their potential longevity. Some studies have shown that fetal leukocytes could persist in maternal blood for a period of up to 6 years postpartum, creating a state of long-term fetal microchimerism (1). This increases the risks of obtaining cells from previous pregnancy and therefore produces a confounding factor for prenatal diagnosis.

Trophoblasts

Trophoblasts are the first fetal cells that have been documented to enter into the maternal circulation at a very early stage of gestation, approximately from the 6th week (43).

Trophoblast invasion involves two types of cells, interstitial trophoblasts, and endovascular trophoblasts. The majority of circulating trophoblast material is constituted by interstitial trophoblasts that are deported passively into the maternal circulation by the shedding of syncytial fragments. The release of trophoblast material from placental villi is due to an apoptotic mechanism of normal turnover of this epithelium. Therefore, multinucleated syncytiotrophoblast cells cannot be recognized as circulating fetal cells because the structure is not as a cell but rather as a fragment of a multinucleated syncytium. These syncytial fragments can be detected in the uterine vein and peripheral circulation, but their isolation appears difficult since they are immediately trapped and destroyed by maternal pulmonary circulation as a consequence of their large size.

The other and predominant cell type of trophoblasts that circulates in the maternal blood is the HLA-G-positive uninucleate endovascular trophoblast. The cells, located in the lumen of spiral arteries, are in direct contact with maternal blood (25,44).

Since the release of trophoblast material occurs through an apoptotic mechanism of the epithelium turnover, a major source of cfDNA circulating in maternal blood is possibly of trophoblastic origin, and although the quality of this DNA is limited (because it is apoptotic), the amount is much higher and is suitable for fetal genomic analysis by PCR-related methods (45).

Trophoblasts have been isolated from maternal blood by several different methods related to surface antigen expression, for example, HLA-G, and cell size and proof of fetal origin were accomplished with FISH and PCR-related methods (44). However, the use of these cells for noninvasive prenatal diagnosis has encountered several difficulties. Common to all circulating fetal cells studied until now is their extremely low number in maternal blood. This is principally due by the fact that multinucleated syncytiotrophoblast cells are rapidly cleared by maternal pulmonary circulation (46) and by the lack of specific monoclonal antibodies against trophoblast surface antigens that allow the isolation of a pure cellular fraction without the contamination of maternal cells. Furthermore, the syncytiotrophoblast cells are multinucleated (2–80 nuclei) and not suitable for chromosomal analysis when FISH is used. Another disadvantage of using these cells is the extra embryonic origin that could imply a discrepancy between fetal and placental karyotypes because trophoblasts could exhibit confined chromosomal mosaicism in around 1% of cases (47).

However, despite these obstacles, the HLA-G-positive uninuclear endovascular trophoblasts appear to be the most amenable kind of trophoblast cell type for the purpose of noninvasive prenatal diagnosis. The HLA-G antigen recognized by monoclonal antibody (McAb) G233 is expressed only by endovascular trophoblasts, and using this antibody for immunocytochemical identification followed by XY-FISH, van Wijk et al. (48) demonstrated the presence of these fetal cells in maternal blood. Moreover, the same authors

established the frequency of these cells on average of about one fetal trophoblast expressing HLA-G per mL of maternal blood between 9 and 13 weeks of gestation. Furthermore, the same isolation and detection procedure has been used for prenatal diagnosis of Down syndrome and allowed confirmation of the presence of a partial hydatidiform mole (49). Hawes et al. have also accurately detected a fetal beta-globin mutation in trophoblasts present in maternal blood. Vona et al. (37) developed a strategy called ISET that is able to enrich circulating trophoblasts from maternal blood since they are larger than the peripheral blood cells. The isolated cells are individually microdissected with a laser microscope and their fetal origin was confirmed by genotyping using short tandem repeat markers. Furthermore, this method was applied to 12 pregnant women at risk of having a child affected by spinal muscular atrophy and 3 mothers were identified who were carrying an affected fetus (50).

In order to overcome the problem of the low number of these cells, Guetta et al. (19) studied the possibility to expand *in vitro* the trophoblasts expressing HLA-G cells in order to improve their number and possibly enable metaphase chromosome analysis. After sorting, the cells were analyzed directly or after culture. The confirmation of fetal origin was achieved by XY-FISH in samples of pregnant women carrying male fetus predicting fetal gender. The authors reached a significant fivefold increase in the yield of fetal cells compared with the sample directly analyzed. The gender prediction was successful in 93%. This approach might represent an important progress in this field.

Fetal Erythroblasts or NRBCs

NRBCs are mononuclear and relatively well-differentiated cells (51).

They are abundant in the fetal circulation during early pregnancy since they are the first hematopoietic cell lines produced during fetal development (52). They have a short life span compared with fetal lymphocytes given their limited proliferative capacity (53), making them unlikely to persist after pregnancy.

Fetal NRBCs carry a representative complement of fetal karyotype without mosaic characteristic, unlike trophoblast cells, and are consistently present in maternal blood during pregnancy. Kleihauer et al. (54) were the first to describe this effect in 1957, by developing a staining procedure specific for fetal hemoglobin, and successively Clayton et al. (55) confirmed the observation in 1964. However, only in 1990 Bianchi et al. (15) provided the definitive proof of fetal origin of erythroblasts by isolating them from maternal blood and enriching them using FACS with antibodies specific for transferrin receptor (CD71) and by evidencing the presence of male DNA.

This last discovery was important since in that period it was thought that NRBCs were rare in adult peripheral blood (56) and consequently the most of erythroblasts present in maternal circulation should have been of fetal origin. However, cell enrichment systems developed later demonstrated that a large population of NRBCs circulate during pregnancy, but most of them are of maternal origin (57). This evidence was confirmed by others, such as Troeger et al. (58), which using single-cell PCR analysis on single micromanipulated NRBCs identified by

May-Grunwald–Giemsa staining, demonstrated that only half of NRBCs in maternal blood were of fetal origin.

Nevertheless, the characteristics of NRBCs make them particularly suitable for the development of noninvasive prenatal diagnostic tests as proved by others, who assessed the potential of these cells by using a general setup based on enrichment, isolation, and genetic analysis as described above for the other cell types.

From the first isolation and enrichment methods developed by Bianchi et al. (15), other methods were designed based on density gradient centrifugation and on the presence of specific erythroblast surface antigens (17,59,60). These approaches based on the presence of specific surface antigens such as CD71, CD36 (thrombospondin receptor), and GPA (glyphorin A) allowed fetal NRBC isolation from blood samples of all pregnancies and not only from HLA-informative couples, such as for the lymphocytes. Among these surface antigens, CD71 was the most utilized and in particular the research was focused on the development of several anti-CD71 monoclonal antibodies and on the optimization of protocols for the isolation of NRBCs from peripheral maternal blood. An attempt was conducted by Alvarez (61), who developed a specific erythroblast antibody, named 2F6.3, and compared it with a commercial anti-CD71 antibody by using blood samples from pregnant women suffering from pregnancy-induced hypertension and in a control group of pregnant women without clinical features suggesting an increased risk of developing pre-eclampsia. They demonstrated that the antibody 2F6.3 succeeded in isolating a significantly higher number of erythroblasts with less maternal cell contamination than the commercial antibody in both women with pregnancy-induced hypertension and the control group.

More recent strategies of NRBC enrichment were based on the adsorption of these cells to slides containing galactose sites by galactose-bearing conjugation via soybean agglutinin, a galactose-specific lectin (62), and on microfluidic approach that consisted of microfluidic chip size-based cell separation and a magnetic device for hemoglobin-based cell isolation (63).

The possibility to enrich fetal NRBCs from maternal blood opened new perspectives of genetic analysis for noninvasive prenatal diagnosis. The main technologies used for genetic analysis were FISH (64) and PCR (65), as for the other cell types. Another molecular-cytogenetic approach employed with NRBCs was the comparative genomic hybridization (66), which allows the analysis of copy number changes (gains/losses) in the DNA content of a given subject by using fluorescently labeled DNA in order to detect unbalanced chromosome changes.

The development of all these strategies could improve the NRBC utilization in noninvasive prenatal diagnosis, even if their genetic analysis shows considerable challenges, given their limited number.

Many attempts have been performed in order to quantify fetal erythroblasts present in maternal blood because they are considered one of the most suitable target cells for developing noninvasive prenatal tests. The first studies performed in order to establish the number of fetal erythroblasts in maternal blood were carried out by Bianchi et al. (15) and Price et al. (17) analyzing flow-sorted erythroblasts on the basis of CD71-positive cells and using PCR and FISH

techniques to identify Y sequences. They obtained a ratio of fetal erythroblasts to maternal cells of $1:10^7$ to $1:10^8$ showing the reliability of isolating fetal cells from maternal blood although their number was extremely low. Moreover, the study conducted by Price et al. gave the first demonstration of the feasibility of performing prenatal detection of fetal aneuploidies on flow-sorted erythroblasts and chromosome-specific probes.

However, some recent results revealed that the analysis by FISH of fetal erythroblasts enriched from maternal blood gave low levels of diagnostic sensitivity due to the inability of detecting fetal erythroblasts by this technique. This thesis was explained by the demonstration that NRBCs have an abnormally dense nucleus with an apoptotic character, which renders it impervious to hybridization by FISH probes (62).

This finding did not dissuade the researchers from the study of the potential implications of NRBCs in noninvasive prenatal diagnosis since many clinical trials have been performed by using this kind of cells.

Hematopoietic Stem Cells and Progenitor Cells

As the other typologies of circulating fetal cells, hematopoietic stem cells and progenitor cells (HSPCs) enter into maternal circulation after accidental breakage of the villi, where it is supposed are encapsulated, or by conditions that promote exposure of the fetal endothelium and subsequent vessel breeching (25).

In reference to phenotype, stem cells express different antigenic surface markers, but the antigen mainly expressed in several stem cells lineage is the “hematopoietic” CD34 antigen. Therefore, hematopoietic CD34+ cells are the most representative cells in stem cell population. The CD34+ antigen, an integral membrane glycoprotein of 90 to 120 kD, functions as a regulator of hematopoietic cell adhesion to stromal cells of the hematopoietic microenvironment (67,68).

First-trimester fetal blood contains a higher frequency of hematopoietic CD34+ stem cells and the number circulating in fetal blood peaks in the second trimester. Hematopoietic CD34+ stem cells together with the mesenchymal stem cells represent about 0.4% of circulating cells in fetal blood during the first trimester of pregnancy in humans (23). Several reports have demonstrated the presence of circulating fetal hematopoietic CD34+ stem cells (fetal CD34+ cells) in maternal blood during pregnancy. Besides, it has been demonstrated that these cells have a clonogenic capacity of colony formation, since this type of cell can be induced to divide and proliferate in vitro. The clonogenic capacity of isolated fetal CD34+ cells from maternal circulation has been demonstrated through the formation of typical colonies such as fetal colony-forming unit—erythroid (CFU-E), erythroid burst-forming units (BFU-E), and granulocyte–erythrocyte–megakaryocyte–monocyte colony-forming unit (CFU-GEMM) by several authors (6,20,69). For this reason, among circulating fetal cells, fetal CD34+ cells appear to be one of the most promising candidate cell of fetal origin for noninvasive prenatal diagnosis. By this way, it is possible to increase the quantity of fetal material, overcoming the scarcity of the cells, for a reliable genetic analysis.

The first attempt to demonstrate the presence of these cells and their number in maternal circulation during pregnancy

was done by Hall and Williamsons (70), who developed a purification method using an antibody directed against CD34; fetal cells were present in a range of $1/4.75 \times 10^6$ to $1/6 \times 10^7$ of the nucleated cell fraction of maternal blood. Successively, Little et al. (6) quantified fetal CD34+ cells present in maternal blood of women bearing a male fetus. After a FACS and a Y chromosome-specific probe identification, they detected a range of 0 to 7 male fetal CD34+ cells in 20 milliliter of maternal blood. More recently, other investigators (71,72) established the number of these cells by using the MACS enrichment approach and the CD34 antibody coupled with the FISH analysis and the Y chromosome-specific probe. The results of the studies were very promising because they confirmed the presence of these cells in maternal blood, also if the number was low as that observed for the other categories of circulating fetal cells.

Several groups have conducted studies for identification of these cells present in maternal blood and their potential expansion.

The first evidence of feasibility of culturing fetal erythroid cells with clonal properties was achieved by Lo et al. (73). They demonstrated the selective enhancement of fetal erythroid cells with a purity rate of 0.01% to 0.25% after a coculture of male fetal liver-derived hematopoietic cells, with a ratio of fetal/maternal cells ranged from 1/400 to 1/9500.

After this pioneering study, other investigators, using different antibodies against fetal clonogenic cells and semisolid medium for their expansion, confirmed the capacity of expandability of fetal cells isolated from maternal blood. Valerio et al. (69) used peripheral blood from pregnant women and enriched erythroid cells by MACS coupled with a biotin-labeled erythropoietin antibody. They identified fetal colonies after cultivation reaching a fetal colony purity rate of 18%.

Later, fetal CD34+ cells in maternal blood were quantified in first-trimester samples by Little and colleagues (6). These authors studied the frequency of CD34+ cells of fetal origin in maternal blood and their in vitro proliferation. After 5 days of culture, a slight but significant expansion of fetal cells was observed. The number of cultured fetal cells ranged from 0 to 35 fetal cells in 20 milliliter of maternal blood. The fetal origin was confirmed using FISH and probes specific for chromosomes X, Y, and 21.

A further definitive confirmation of the clonogenic potential of fetal CD34+ cells was acquired by our group (20). We cultured in a semisolid culture medium, unenriched peripheral blood mononuclear cells, and enriched hematopoietic CD34+ cells by MACS from maternal blood of women bearing male fetus. Cells were then cultured for 14 days and frequencies of the expanded fetal cells were established by the use of XY-FISH. The numbers of BFU-E/CFU-E and CFU-GM colonies obtained by enriched cellular fraction were increased by 33 and 16 times respectively to unenriched cellular fraction. By FISH, we identified a suitable number of male fetal cells and a good fetal/maternal ratio even in normal pregnancies. Others studies from Tutschek et al. (74), Campagnoli et al. (75), and Guetta et al. (72) have positively raised the interest about the prospect of using fetal cell culture expansion for genetic testing. They showed, with different methodological approach, the successful clonal expansion of

fetal cells combining micromanipulation and fluorescent PCR or FISH technique alone. In particular, Guetta et al. (72) demonstrated a significant 2.4-fold increase in the number of fetal cells detected by FISH, after culture with respect to samples directly analyzed after CD34+ cell sorting.

In contrast to these attempts, others have not confirmed the potential for generating pure fetal hematopoietic colonies. Chen et al. (76) cultured erythroblasts from 16 maternal blood samples in a semisolid medium with and without prior enrichment by MACS using an erythropoietin antibody. They were not able to demonstrate a conclusive fetal colony by PCR for either Y-specific sequences or autosomal microsatellite markers.

In the studies of Zimmermann et al. (77) and Manotaya et al. (78), the demonstration that maternal blood contains fetal clonogenic erythroid cells failed; they were not able to expand fetal cells because, after culture, any colonies among those isolated and analyzed by PCR and FISH technique were of fetal origin.

Unfortunately, a direct comparison of results among all these contrasting studies is not possible principally due to the differences in the methods employed for sample processing and analysis utilized by different authors.

Several authors have reported that some types of circulating fetal cells, in particular cells with stem cell-like properties, as the fetal CD34+ cells, can persist in the maternal circulation for decades after delivery, creating a state of long-term fetal microchimerism (23,79–81).

This fact has, to date, limited their use in the development of noninvasive testing, as they could still be present in the maternal circulation and potentially create a confounding factor for prenatal diagnosis. In other words, incorrect interpretation of the prenatal tests in parous women could be possibly due to circulating fetal cells from previous pregnancies.

Many researchers have focused their attention on this observation. Recently, we performed a study to verify whether the culture expansion could increase the number of these persisting CD34+ cells from previous pregnancies and whether their expanded number could interfere with a subsequent prenatal diagnosis (82). The results demonstrated that the presence of cells from previous pregnancies does not affect prenatal fetal diagnosis in current pregnancies.

Clinical Trials for Detection of Fetal Aneuploidies

The main advantage of using fetal cells enriched from maternal blood with respect to cfDNA is that they potentially offer a source of complete fetal genetic material (7) for a noninvasive prenatal diagnosis; this is an essential prerequisite for genetic diagnosis of the main fetal pathologies, as the fetal trisomies, which cannot be easily met via the analysis of cfDNA. For this reason, the use of fetal cells appears the only way for reaching the goal of the detection of fetal aneuploidies in a noninvasive manner.

Several investigators have developed and pursued strategies for detecting fetal aneuploidies within fetal cells isolated from maternal blood. For the first time, in the year 1992, Elias et al. (18) diagnosed a fetal trisomy 21. One year later Gänshirt-Ahlert et al. (83) confirmed the feasibility to diagnose fetal aneuploidies on enriched fetal erythroblasts.

They correctly diagnosed 5 cases of trisomy 18 and 10 cases of trisomy 21. After these first observations, several other investigators confirmed this diagnostic approach and also demonstrated that it is possible to diagnose several other fetal chromosomal disorders as fetal trisomy 13, fetal triploidy, and sex chromosome mosaicism.

Reports of identification of aneuploid fetal cells in "clinical" series are present in the scientific literature and results varied widely on the consistency with which they may be found in maternal blood (Table 1).

The National Institute of Child Health and Human Development Fetal Cell Isolation Study was the first prospective multicenter clinical project, started in 1994, with the aim of examining the feasibility of using fetal erythroblasts from maternal blood for the detection of fetal aneuploidies (84). The study was performed enrolling 2744 pregnant women considered at high risk for fetal aneuploidies. The NRBCs were isolated from maternal blood and enriched in part by FACS and in part by MACS technology and their interphase nuclei were analyzed by FISH using probes specific for 13, 18, 21 X, and Y chromosomes. The data of FISH analysis were compared with fetal karyotype obtained by amniocentesis or CVS. From these findings, it was evident that target cell recovery and fetal cell detection were better obtained using MACS than FACS technology. Blinded FISH assessment of samples from women carrying singleton male fetuses found at least one cell with an X and Y signal in 41.4% of cases with a false-positive rate in gender detection of 11.1%. The diagnostic sensitivity in fetal aneuploidy diagnosis was 74.4%, with a false-positive rate ranging between 0.6% and 4.1%. The results of this clinical trial were not excellent, since the sensitivity in noninvasive prenatal diagnosis of fetal aneuploidies was comparable with that of a single marker prenatal serum screening. A possible limitation of this study could have been multiple processing protocols, which could have affected the diagnostic sensitivity in fetal aneuploidy detection.

Another study, carried out to analyze the potentiality of fetal trisomy detection by using fetal NRBCs from maternal blood (85), was performed on a large scale by testing a unique standardized protocol, overcoming in this way the limitation of the previous trial. The authors enrolled 230 pregnant women at 10 to 14 weeks of gestation, isolated NRBCs by

triple-density centrifugation and by anti-CD71 magnetic cell sorting and performed FISH analysis for fetal trisomy detection. Fetal karyotype was normal in 150 cases and abnormal in 80 cases, including 36 cases with trisomy 21. From the analysis of the study results, it was evident that the cutoff setting was of crucial importance for establishing the diagnostic performance of the protocol. In fact, while using a 21 chromosome-specific probe, three-signal nuclei were present in at least 5% of the enriched cells from 61% of the trisomy 21 pregnancies and in none of the normal pregnancies, for a cutoff of 3% of three-signal nuclei, the sensitivity for trisomy 21 was 97% with a false-positive rate of 13%. Similar values were obtained in trisomies 18 and 13 using the appropriate chromosome-specific probe. Therefore, this study showed that the examination of fetal NRBCs from maternal blood could provide a noninvasive prenatal diagnostic test for trisomy 21 with the potential of identifying about 60% of affected pregnancies.

Although the research group of Al-Mufti performed a trial using a standardized protocol, it is clear that it did not reach optimal results. This fact could be explained by the poor efficiency of MACS enrichment protocol. In fact, a study carried out by Parano et al. (86) achieved promising results employing a novel procedure for NRBCs separation, which involved direct micromanipulator isolation of histochemically identified fetal hemoglobin-positive nucleated cells to obtain fetal NRBCs of high yield and purity. Using this technique, followed by cell-by-cell multicolor FISH analysis of purified fetal erythroblasts, they were able to detect Down syndrome, Klinefelter syndrome, and Patau syndrome, in 33 pregnant women. The procedure provided complete concordance with the results of amniocentesis.

The results obtained from this last study were encouraging, but clinical trials on large scale using this approach were not performed, most probably because this protocol would have been too much complex and time consuming to be introduced in clinical routine.

Therefore, until now the relative rarity of fetal NRBCs in maternal blood and the difficulty to establish their fetal origin remained the two principal problems hindering the successful spreading of noninvasive prenatal diagnosis based on fetal cells in maternal blood.

Table 1 Principal Reports Present in the Scientific Literature Focusing on Prenatal Diagnosis of Fetal Abnormalities Using Fetal Cells in Maternal Circulation

Date	Author	Target cell	Methodology	Aneuploidies diagnosed	Detection rate (%)
1994–2002	Bianchi	NRBCs	MACS or FACS + FISH	Trisomies 13, 18, and 21	74.4
1999	Al-Mufti	NRBCs	MACS + FISH	Trisomies 13, 18, and 21	60
2001	Parano	NRBCs	Direct micromanipulator isolation of histochemically identified hemoglobin-positive nucleated cells + FISH	Trisomies 13, 21, and Klinefelter syndrome	100
2000	Valerio	Hematopoietic progenitor cells	Double-density gradient centrifugation + culture + FISH	Trisomy 18	100
2001	Coata	Hematopoietic CD34+ cell	MACS + culture + FISH	Trisomies 18, 21, and Klinefelter syndrome	100
2006	Di Renzo	Hematopoietic CD34+ cell	MACS + culture + FISH	Trisomies 13, 18, and 21	100 ^a

^aData not published.

To test the feasibility of using expanded fetal stem and progenitor cells isolated from maternal circulation in clinical practice, some researchers performed a study aimed to verify their applicability in the detection of fetal trisomies by interphase cytogenetic diagnosis. As it occurs for the other fetal cell types, detection of aneuploid cells is facilitated by the increased number of fetal cells detectable in the mother bearing a chromosomally abnormal fetus.

In several original reports, Valerio et al. (69,87,88) demonstrated the practicability to sort fetal hematopoietic progenitor cells from the maternal blood of all aneuploid pregnancies and successfully expand them in culture. By this way, the authors obtained an acceptable fetal cell purity that allowed them to diagnose all the cases of aneuploid

pregnancies investigated. It was possible to detect six fetal trisomy 21, three fetal trisomy 18, and one trisomy 13. Fetal trisomies were documented by FISH analysis with appropriate chromosome-specific probes and confirmed by fetal karyotype.

Another study adding possibility of culturing fetal CD34+ cells was obtained by our group. We confirmed the feasibility of utilizing fetal CD34+ cells isolated from maternal blood between 11 and 16 weeks of gestation and before any invasive procedures, for the prenatal diagnosis of fetal aneuploidies (20,71). In the first report we were able to diagnose two fetuses with trisomy 21 and we found a considerable number of trisomic fetal cells after expansion, with a ratio of 1/34 and 1/12 between fetal to maternal cells (Fig. 1). In the second

Figure 1 The figures show fetal nuclei presenting a trisomy 21 indicated by white arrows. In the nuclei, the three red signals represent chromosome 21.

Figure 2 (A1 and A2) The figures show fetal nuclei presenting a trisomy 18 indicated by white arrows. In the nuclei, the three red signals represent chromosome 18. (B) The figure shows a metaphase presenting a trisomy 18.

study, we correctly detected three fetal trisomies 21, one fetus with Klinefelter syndrome and one fetal trisomy 18. For the first time, we were able to document the fetal origin of the expanded cells in culture by a fetal metaphase of trisomic nuclei from the fetus with a trisomy 18 (Fig. 2).

Since 2006, our group has been performing a clinical trial with estimated completion date on 2010. The purpose of this study is to validate our developed combined methodology for noninvasive prenatal identification of fetal aneuploidies and fetal gender with blood samples from pregnant women who are scheduled to undergo an amniocentesis or CVS procedure. The obtained results are then comparing with those obtained by fetal karyotype. The enrollment criteria of pregnant women are very strict and include only those women at high risk for fetal chromosomal abnormalities, providing written informed consent.

The study design involves the isolation of fetal CD34+ cells from maternal blood, the in vitro cell expansion, and FISH analysis for recognizing and counting chromosomes 13, 18, and 21 and sex chromosomes.

Our preliminary results, based on the analysis of 500 blood samples, have shown a sensibility of 100% for the identification of each studied trisomy, while the specificity have varied from 94% and 96% for trisomy 13 and 21 respectively and to 100% for the trisomy 18.

In the same context, we evaluated whether the effect of the delay in the initial fetal cell enrichment processing of maternal blood samples from blood sampling could compromise the ability to recover fetal cells. We analyzed 45 maternal blood cells after one or more days from blood sampling. We observed a significant reduction of the CD34+ cells with respect to those samples immediately processed (usually within 1 hour). The reduction of these cells was more than 50% and this was very dangerous for our test because the fetal cells of our interest are present in this subset of cells. Consequently, we obtained a very low sensibility and specificity of the test (less than 90%), with false-positive and false-negative results (data not yet published) in the detection of fetal trisomies. From this observation, it is essential to process immediately the maternal blood samples (within 1 hour from the sampling) in order to avoid the loss of fetal cells when we have to utilize them for the noninvasive genetic tests.

CONCLUSION

Since the demonstration of fetal cells and fetal nucleic acids in the maternal circulation, the aim of many research laboratories around the world has been to isolate fetal material for applying to genetic analysis. Nowadays, we may state that fetal nucleic acids can be reliably utilized for rapid gender detection (89–91), RhD status (92,93), and some monogenic disorders, but the application for the detection of aneuploidies although positively attempted (94–96) is still far behind. Instead the use of fetal cells and particularly erythropoietic stem cells looks as the most promising approach for the detection of aneuploidies at least limited to chromosomes 13, 18, 21, X, and Y, which represent the vast majority of fetal chromosomopathies amenable of a meaningful prenatal diagnosis. Our studies and our preliminary results are positively showing that in this direction we are pretty close to offer a reliable noninvasive diagnostic test.

However, the definitive answer will come when we will be able to develop a surface monoclonal antibody toward a fetal specific antigen, which will allow us to obtain a population of fetal cells free of maternal background to which genetic analysis can be applied.

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