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# On fetomaternal hemorrhage

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# On fetomaternal hemorrhage

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klokke 16.15 uur

door

Denise Marta Vera Pelikan

geboren te Groningen in 1972

## Promotiecommissie

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*Aan mijn ouders*

*Voor Emma en Michiel*

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# General Introduction

1

Fetomaternal hemorrhage (FMH) is a serious complication of pregnancy. Substantial FMH may lead to life-threatening anemia in the fetus or newborn. In addition, exposure of Rhesus (Rh) D negative women to small amounts of fetal Rh D positive red cells during pregnancy or delivery may result in sensitization. Also, increased fetomaternal cell exchange may lead to persistence of fetal cells in the mother. Whether this phenomenon has consequences for multiparous women later in life is not exactly known. Therefore it is of clinical importance to identify potential risk factors for the occurrence of FMH in pregnant women, to develop new techniques to detect fetal cells and to study their potential to survive in the mother.

## 1. History

Transplacental passage of fetal cells into maternal blood is a common phenomenon in pregnancy and delivery. Cells from fetal origin were first recognized in 1893 by Schmorl,[1] who identified trophoblast cells in lung capillaries of women dying of eclampsia (figure 1). In 1957 Kleihauer, Betke and Braun first demonstrated the presence of fetal cells in the maternal circulation by application of the acid elution principle to identify fetal erythrocytes.[2] This method is based on the fact that fetal hemoglobin (HbF) is more resistant to acid elution than adult hemoglobin (HbA).

Accurate detection and quantification is important as FMH is related to many obstetrical disorders and invasive procedures and may lead to critical complications such as fetal exsanguination and red cell immunization. The Kleihauer-Betke test has been of key importance in the study of transplacental passage of fetal red cells, providing better understanding of the cause and possible strategies to prevent hemolytic disease of the newborn. Despite the worldwide use of the Kleihauer-Betke test for the detection and quantification of FMH, this test may suffer from poor reproducibility mainly due to several modifications made to the original method. So far, many investigators have focussed on the development of more reliable methods for FMH quantification using flow cytometry[3-8] and polymerase chain reaction (PCR).[9-11] Also, fetal cell detection (trophoblast cells, nucleated red blood cells) has become an important research target for the purpose of non-invasive prenatal diagnosis, as an alternative for chorionic villus sampling and amniocentesis.[12,13] Over the last decade the persistence of fetal cells in maternal tissues and the possible role of microchimerism in the pathophysiology of autoimmune disease have been investigated.

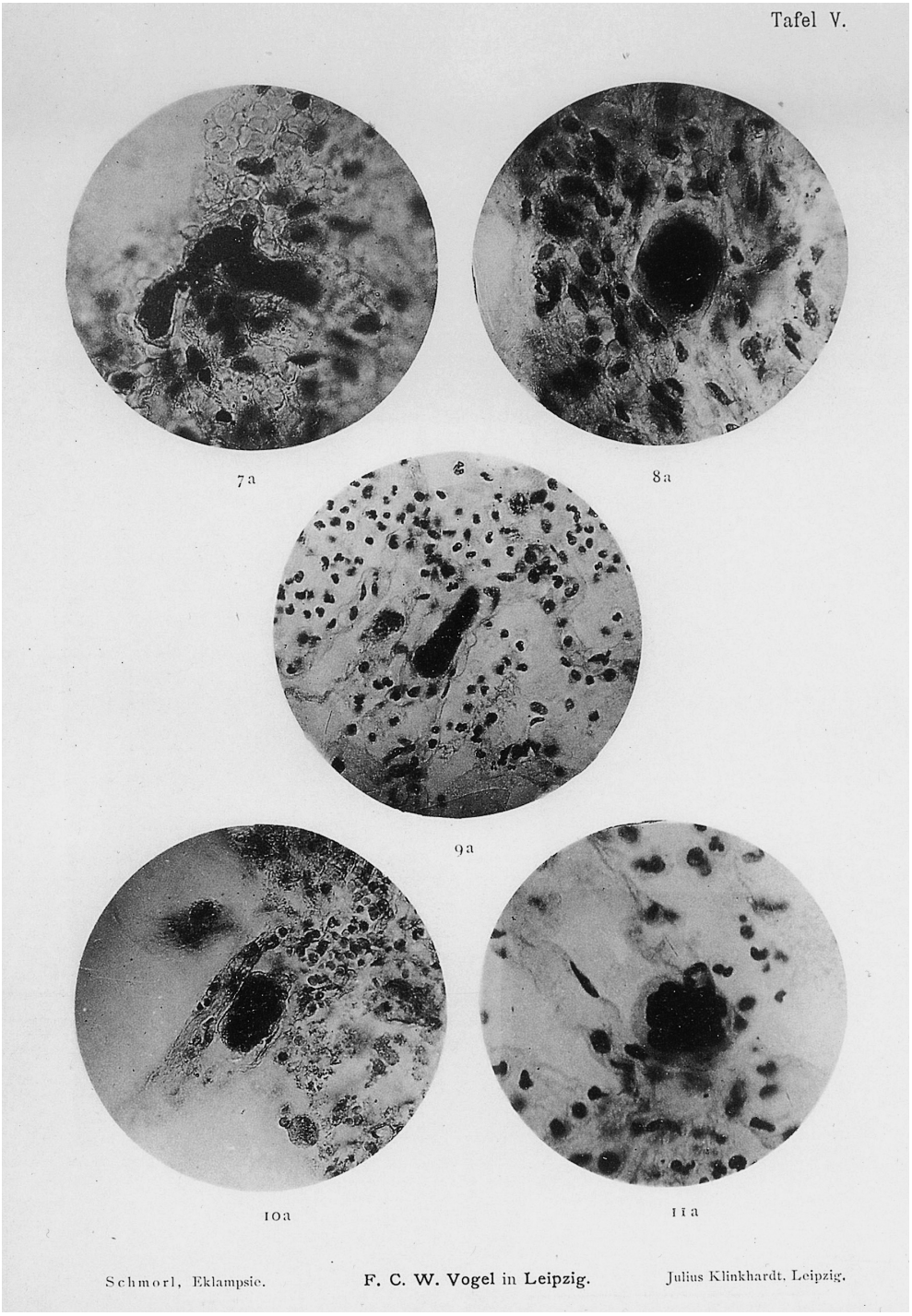


**Figure 1** - Trophoblast cells in lung capillaries of women dying of eclampsia as described by Schmorl in 1893. Leiden University Library (reference 1363 C30).

## 2. Biological basis of fetomaternal cell trafficking

The fetal circulation is separated from the maternal circulation by the placental barrier allowing exchange of metabolic and gaseous products. The basic idea that there is a placental barrier was already formulated at the beginning of the 18th century by John and William Hunter who injected liquid wax into the uterine artery and discovered that the wax did not appear in the fetal circulation.[14] The placental barrier prevents large intermingling of fetal and maternal blood, but does not maintain an absolute integrity and small amounts of fetal blood may enter the maternal circulation. This phenomenon, called fetomaternal cell trafficking, is being studied extensively at the moment.

### *2. 1. The placental interface*

#### 2.1.1. Anatomy: development of the placenta and its circulation

After fertilization of the ovum by a sperm cell in the Fallopian tube, the formed zygote undergoes a series of rapid mitotic cell divisions known as cleavage. The zygote is cleaved into a number of small cells and forms the blastomere. About three days after fertilization subsequent cell divisions of the blastomere result in the formation of the morula, which enters the urterine cavity. At day 4 the morula is converted into a blastocyst consisting of (1) an inner cell mass, the embryoblast, which gives rise to the embryo, (2) a blastocyst cavity, and (3) an outer cell layer, the trophoblast, which will form the embryonic part of the placenta. Four to five days after fertilization the blastocyst attaches to the endometrium. The villous trophoblast layer then differentiates into the cytotrophoblast, which is mitotically active and into the syncytiotrophoblast, which rapidly transforms into a large, multinucleated mass without distinguishable cell membranes. The extravillous trophoblast invades the endometrial epithelium and underlying stroma and by the end of the first week the blastocyst is superficially implanted. The functional layer of the endometrium in pregnancy is called the decidua, which is the maternal component of the placenta. In the second week of human development a lacunar network is formed in the syncytiotrophoblast and opening of uterine vessels into these lacunae establish the beginning of the uteroplacental circulation. At the end of the second week primary chorionic villi are formed. The fetal component of the placenta is formed by the chorionic plate, from which the chorionic villi arise. Maturation of the villous tree into secondary and later tertiary villi containing chorionic vessels which connect to the embryonic circulation, results in a primitive fetoplacental circulation by the end of the third week of embryonic development.[15]

The large surface area of the chorionic villi which are bathed in maternal blood that enters the intervillous space, enables exchange of nutrients and other substances between the embryonic and maternal circulation. The two circulations are separated by the so-called placental barrier, which consists of the following layers: (1) a continuous layer of syncytiotrophoblast cells, (2) an initially (in the first trimester) complete, but later on (second and third trimester) discontinuous layer of cytotrophoblast cells, (3) a trophoblastic basal lamina, (4) connective tissue derived from the extra-embryonic mesoderm, and (5) the fetal endothelium.[16] The different layers of the placental barrier are depicted in figure 2. Throughout pregnancy the placental barrier becomes progressively thinner while simultaneously fetal blood flow and blood pressure increase as the villous tree enlarges.[17]

Particularly in the third trimester and during labor small microscopic disruptions of the placental barrier allow fetal cells and other fetal blood components to leak into the intervillous space and thus enter the maternal circulation.

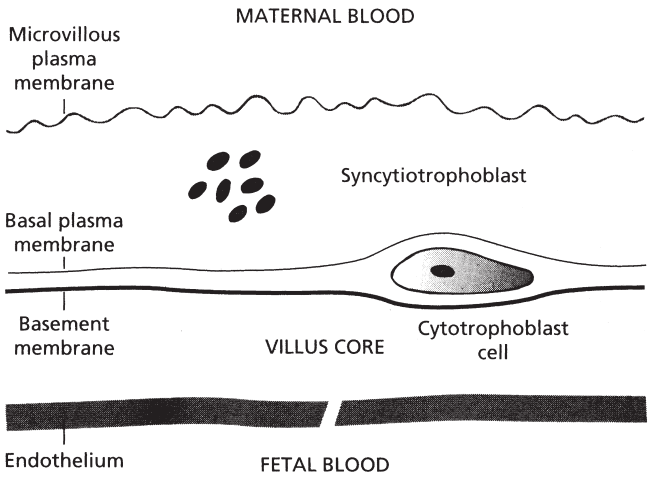


**Figure 2 -** The major components of the placental barrier between maternal and fetal blood near term (after Glazier et al. 1999).[18]

### 2.1.2. Physiology: maternofetal exchange and other placental functions

The placenta has three main activities: (1) transfer, (2) metabolism, and (3) endocrine functions. The major function of the placenta is to allow diffusion of oxygen and nutrients from maternal to fetal blood and diffusion of carbon dioxide

and excretory products from the fetus back to the mother. Other substances such as immunoglobulin G, various drugs, steroid hormones and certain viruses potentially causing fetal infection, are known to pass the placental barrier. Exchange of various substances across the placental barrier occurs by means of simple and facilitated diffusion, active transport and pinocytosis.[19] In the early stage of pregnancy, placental permeability is relatively low. The total surface area of the villi is still small and the villar membranes have not yet reached their minimum thickness. The permeability increases progressively in the second and third trimester. At term permeability decreases again due to aging, calcifications and infarction. Other important functions of the placenta are synthesis of glycogen, cholesterol and fatty acids early in pregnancy and production of human chorionic gonadotropin, estrogen and progesterone, which are essential for the continuance of pregnancy.[15] Also, the placenta and the fetal membranes protect the fetus against infection.

## *2.2. Fetal hematopoiesis*

### 2.2.1. Production of embryonic and fetal hematopoietic cells

Hematopoiesis in the embryo is first demonstrated in the yolk sac during the 3rd week after fertilization.[20-22] Together with angiogenesis and the formation of a cardiovascular system, embryonic hematopoiesis is one of the first processes established after implantation of the blastocyst and is needed for survival and growth of the embryo.[23] Hematopoiesis is a continuous process of proliferation and differentiation of hematopoietic stem cells (HSCs) into lineage-specific progenitor cells (erythroid, myeloid and lymphoid) and mature blood cells (erythrocytes, macrophages, platelets and leucocytes).[24] A schematic overview of hematopoietic cell development is given in figure 3.

The first blood cells observed in the embryo are large nucleated erythroid cells, which emerge from blood islands in the yolk sac. These cells have been termed “primitive”. Erythropoiesis in the yolk sac ends by the 11th week of gestation.

The next major site of erythropoiesis is the liver and finally the bone marrow. At various stages of fetal development hematopoiesis can be divided into three overlapping periods: mesoblastic, hepatic and myeloid (figure 4). Hepatic hematopoiesis, which takes place in the liver, starts at 5 weeks after fertilization.[20,25] This organ is the primary source of erythroid cells from the 9th to the 24th week of gestation. Erythropoiesis also occurs to a lesser amount in connective tissue, kidney, spleen, thymus and lymph nodes.

The final stage of hematopoiesis, which starts in the bone marrow of the 10- to 11-week embryo, mainly takes place in the bone marrow of the long bones.[26-28]

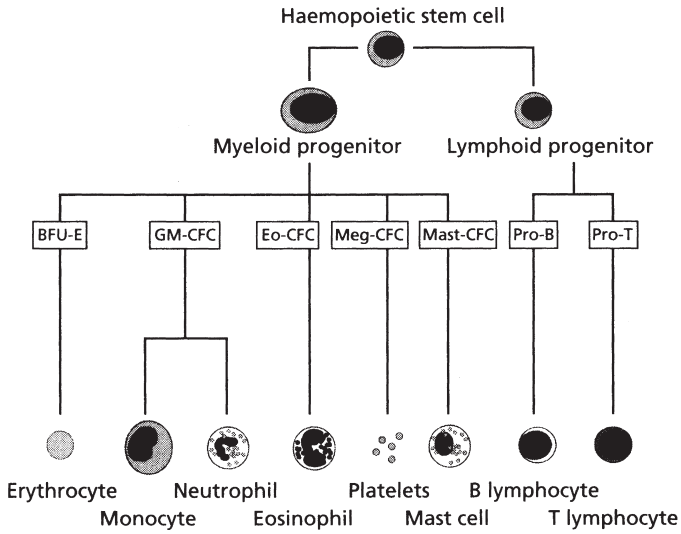


**Figure 3 -** A schematic overview of hematopoietic cell development (after Brown et al. 1999).[40]

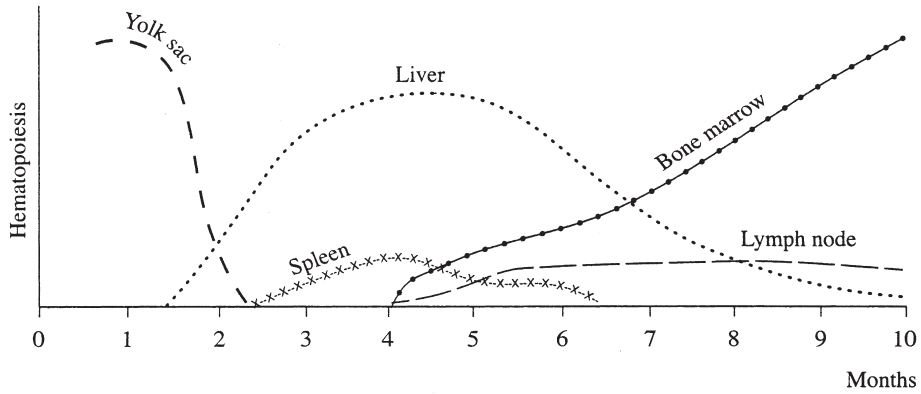


**Figure 4 -** Hematopoiesis in the fetus (after Kelemen et al. 1979).[41]

The bone marrow is the major site of hematopoiesis after 24 weeks of gestation. Hematopoiesis in the yolk sac is distinct from hematopoiesis in the liver and bone marrow as it appears to be restricted to the formation of two lineages: erythroblasts and macrophage progenitors. The “definitive” hematopoiesis includes erythropoisis, myelopoiesis and lymphopoiesis.[26] It is now widely assumed that “primitive” hematopoiesis takes place in the yolk sac and that “definitive” hematopoiesis has its origins in the aorta/gonad/mesonephros (AGM) region.[29-31] From this site stem cells first colonize the fetal liver[32-34] and later the bone marrow.[35] Recent reports provide evidence that hematopoietic progenitors derived from the yolk sac are partially

responsible for “definitive” hematopoiesis and are believed to seed the fetal liver generating the first definitive blood cells that rapidly emerge from the liver.[36-38] This process allows survival of blood cells until AGM-derived hematopoietic stem cells can emerge, colonize the fetal liver and differentiate into mature blood cells. The lymphoid progenitors, which originate from the bone marrow, further differentiate into B lymphocytes and T lymphocytes. The B and T lymphocytes are referred to as mononuclear cells (MNCs). The earliest cells of the B lymphocyte lineage can be detected in the fetal liver and continue to be present in bone marrow throughout life. T cells require a period of differentiation in the thymus and are subdivided into helper T cells, cytotoxic T cells and suppressor T cells.[39]

### 2.2.2. Fetal blood composition

The composition of fetal blood changes during development. The hemoglobin (Hb) level and hematocrit (Hct) are 10.9 ± 0.7 g/dl and 35 ± 3.6%, respectively at 15 weeks of gestation, increase to 13.4 ± 1.2 g/dl and 42 ± 3.3% at 26-30 weeks of gestation, and 16.5 ± 4.0 g/dl and 45-50% at term.[42,43] The mean corpuscular volume of fetal erythrocytes decreases from 134 at 18 weeks to 118 fl/cell at 30 weeks.[42] Embryonic and fetal erythropoiesis is characterized by the presence of large amounts nucleated erythroid cells in the fetal circulation. During development the number of nucleated erythroid cells decreases and the number of enucleated erythrocytes increases.[42] The total white blood count increases from 2.0x $10^9$/L at 16 weeks to 5.2 x $10^9$/l at 29 weeks of gestation[44-46] with a majority of lymphocytes and 5 to 10% neutrophils.[42] Further, small numbers of granulocytes, macrophages, mast cells are present in fetal blood at 8 weeks of gestation. Platelets are present in fetal blood at 8 weeks of gestation. The platelet count gradually increases during fetal development reaching values at term comparable to adults. In addition to mature fetal blood cells, significant numbers of circulating progenitor cells are present in fetal blood including pluripotential stem cells.[47-50]

### 2.2.3. Synthesis of fetal hemoglobins

The human hemoglobin is a conjugated protein consisting of four haem groups and four globin chains. The first erythroid cells, the primitive erythrocytes, which are large and nucleated, predominantly contain the early embryonic hemoglobins Gower I ($\zeta_2\varepsilon_2$), Gower II ($\alpha_2\varepsilon_2$) or Portland I ($\zeta_2\gamma_2$). Definitive erythrocytes are smaller and enucleate and switch from embryonic hemoglobin (HbE) to HbF ($\alpha_2\gamma_2$). Towards the end of pregnancy the amount of HbF ($\alpha_2\gamma2$) slowly decreases and is gradually replaced by HbA ($\alpha_2\beta_2$).[51] The HbF constitutes 90 to 95% of the total hemoglobin in the fetus until 36 weeks of gestation. In adults, hemoglobin is mainly of the $\alpha_2\beta_2$ type with low levels of $\alpha_2\delta_2$. In some individuals persistence of the HbF ($\alpha_2\gamma2$) is found

after birth in low percentages.[52] Adult erythrocytes containing small amounts of HbF are termed “F cells”. The expression of hemoglobins during fetal development is depicted in figure 5.

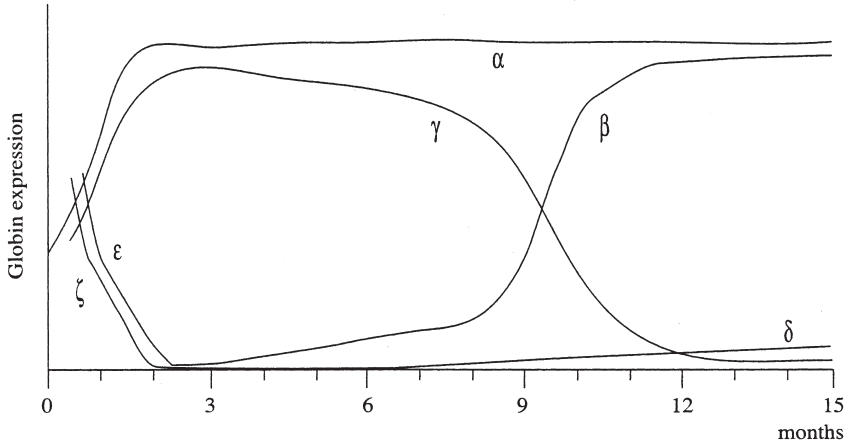


**Figure 5 -** Expression of hemoglobins during fetal development

### 2.2.4. Blood group antigens on fetal red blood cells

The production of blood group antibodies has led to the identification of numerous red cell antigens and phenotypes. Blood group antigens on red blood cells (RBCs) are inherited, polymorphic carbohydrate and protein structures located on the surface of RBC membrane. More than 250 different RBC antigens are known, which have been assigned to 29 blood group systems.[53] Several blood group antigens are not expressed or only weakly expressed on fetal RBCs. For example the antigens $Le^a$, $Sd^a$, Ch, Rg and AnWj are not expressed on fetal RBCs in term umbilical cord blood. The A, B, H, P1, I, $Le^b$, $Lu^a$, $Lu^b$, $Yt^a$, Vel, $Do^a$, $Do^b$, $Gy^a$, Hy, $Jo^a$, $Xg^a$, Kn and Bg antigens are weakly expressed on fetal RBCs at term as compared to adult RBCs. In contrast, the i and LW antigens are more strongly expressed on fetal than adult RBCs.[53]

The ABO blood group system was the first system described and remains the most significant one in transfusion medicine. RBCs are typed as A, B, AB or O. Individuals who lack either the A or B antigen on their RBCs make anti-A or anti-B antibodies. These antibodies are can cause severe hemolysis. However, ABO incompatibility only rarely causes hemolytic disease of the newborn, presumably because the A and B antigens are expressed on fetal RBCs late in pregnancy and because anti-A or anti-B antibodies are not only bound to fetal erythrocytes but also to other fetal tissues that express the A and B antigens. The Rh blood group system is the second most important blood group system after ABO. Rh D incompatibility is

the most common cause of hemolytic disease of the fetus and the newborn and also of major importance for hemolytic transfusion reactions. More than 45 antigens in the Rh system are known and the most important are D, c, C, e and E.[54,55] Expression of the Rh D antigen on fetal RBCs as early as 38 days after conception has been reported.[56]

### *2.3. Fetal cells and tissue detected in the maternal circulation*

#### 2.3.1 Fetal cell types and other fetal blood components

After the identification of trophoblast cells in the pulmonary circulation of woman dying of eclampsia,[1] Walnowska et al. identified the Y chromosome in lymphocytes isolated from blood of pregnant women carrying a male fetus in 1969.[57] In 1979 Herzenberg et al. demonstrated the identification of fetal leukocytes by their surface expression of the paternally inherited HLA-A2.[58] It was not until the late 80's of the past century that research groups worldwide gained interest in harvesting fetal cells from maternal blood.

The development of methods for isolation and detection of fetal cells from maternal blood has evolved as an important research area for the purpose of non-invasive prenatal genetic testing.[59,60] Dependent on the gestational age various fetal cell types and blood components can be detected in the maternal circulation, such as trophoblast cells, fetal red cells and their precursors, fetal leukocytes and their precursors, stem cells of fetal origin, cell-free fetal deoxyribonucleic acid (DNA) and fetal plasma proteins.

*Trophoblast cells*

From research performed in recent years it has become evident that a large amount of trophoblast cells is deported into the maternal circulation by the shedding of syncytial knots.[61,62] This typical mode of release of trophoblast material is known to be an apoptotic mechanism of normal turnover.[16,63] Another source for trophoblast cells in maternal blood is the pool of extravillous trophoblast.[64] Trophoblast cells, which are from fetal origin, have been identified using various markers. The first reports on trophoblast identification in maternal blood described the use of a monoclonal antibody against a syncytiotrophoblast-specific antigen (H315).[65] However, subsequent work showed that this method was less specific as a result of absorption of the H315 antigen by maternal cells.[66] Later, more specific methods were described using markers such as the human placental lactogen[67] and HLA-G.[68,69] In normal pregnancy intact trophoblast cells appear to be very rare in maternal peripheral blood, probably due to the fact that they are filtered out by the maternal pulmonary circulation.[70] Increased numbers of trophoblast cells are found in patients

with pre-eclampsia.[71,72]

*Fetal red blood cells*

The majority of fetal cells that are detected in maternal blood are mature enucleated erythrocytes. Direct microscopic visualization of fetal red cells in maternal blood was first demonstrated by Kleihauer et al. in 1957 using a technique based on the resistance of HbF to acid elution.[2] Fetal red cells have been identified in the maternal circulation in the first trimester.[73-76] Both the frequency and the volume of these cells increase as pregnancy progresses.[77,78] Other techniques to identify mature fetal erythrocytes are fluorescence microscopy or flow cytometry using antibodies against HbF and HbA or against the Rh D antigen in case of blood group incompatibility.

The presence of fetal nucleated red cells (NRBCs) or erythroblasts has been demonstrated by research groups focusing on strategies for non-invasive prenatal diagnosis. Fetal NRBCs, derived from the yolk sac and the liver, are the predominant nucleated cell type in the fetal cell circulation in the first trimester.[26] In the second trimester fetal NRBCs account for 10% of the total population, whereas in adults they are quite rare. If fetomaternal cell trafficking occurs early in pregnancy, then fetal NRBCs are likely to be the main cell type detected in maternal blood. In 1990 Bianchi et al. were the first to report on fetal NRBC isolation from maternal blood.[12] Their method was based on flow-sorting of fetal erythrocytes from peripheral blood of pregnant women on the basis of CD71 (transferrin receptor) expression, the presence of fetal hemoglobin by acid elution and by PCR analysis using specific primers that amplified a section of the Y chromosome. Since then, many other study groups have detected fetal NRBCs in maternal blood using various combinations of fluorescence in situ hybridization, staining with antibodies against HbF, HbE or CD71.[13,79-86]

Studies on non-invasive prenatal diagnosis mainly focus on fetal NRBCs because their frequency is relatively high compared to other fetal cell types. In addition, they have a limited lifespan of approximately 120 days[87] and are therefore unlikely to persist between pregnancies.

*Fetal leukocytes*

The presence of fetal lymphocytes was first described by Walnowska and co-workers in 1969.[57] These investigators demonstrated the presence of a Y chromosome in mitogen-stimulated lymphocytes obtained from pregnant women carrying a male fetus. Other studies confirmed this finding by the use of similar techniques.[88,89] Detection of fetal leukocytes by the use of flow cytometry with antibodies against HLA-A2, which was only expressed on fetal cells and not on maternal cells, was demonstrated by Herzenberg et al. in 1979.[58] The detection of fetal granulocytes has

been described in a few studies. In 1975 Zilliacus et al. detected this cell type in the circulation of pregnant women in very low frequencies (0.02 to 0.04% of mononuclear cells).[90] Several years later, Wessman et al. isolated granulocytes from maternal peripheral blood samples using density gradient centrifugation and identified fetal granulocytes by fluorescence *in situ* hybridization (FISH) with Y specific probes.[91] Long-term survival and persistence of these cells has been described.[88,89,92]

To identify fetal leukocytes in maternal blood specific markers are needed to discriminate between fetal and maternal cells. One of the possibilities is to detect fetal lymphocytes on the basis of a paternally inherited human leukocyte antigen (HLA), which is absent in the mother. These inherited “antigens” are glycoproteins on the surface of cells encoded by a number of genes which constitute the major histocompatibility complex (MHC). The MHC molecules which are referred to as HLA in humans, were initially identified by their role in transplant rejection. Their physiological role is the presentation of antigens to T cells. The MHC genes exist in a large number of alleic forms in different individuals and thus exhibit polymorphism. The HLA-A and HLA-B were the first genetic loci encoding for HLA molecules described, followed by a third minor locus designated HLA-C. These genes on chromosome 6 encode the HLA class I molecules, which are present on all nucleated cells. The genes of the three sub-loci HLA-DP, HLA-DQ and HLA-DR code for HLA class II molecules.

The expression of HLA class II molecules is mainly confined to cells directly involved in immune responses, e.g. macrophages, B cells and activated T cells. By the lack of specific monoclonal antibodies against HLA and the low frequency of fetal leukocytes in maternal blood, it has been very difficult to detect and isolate fetal leukocytes on the basis of their HLA polymorphism.

However, with the application of specific monoclonal antibodies against a paternally inherited HLA antigen, it would be possible to identify a minor population of fetal leukocytes in maternal blood independently of the fetal sex.

*Fetal hematopoietic progenitor/stem cells*

The recent discovery that male fetal progenitor cells (CD34+ and CD34+/38+) were still present in maternal blood 27 years postpartum, demonstrated a long-term survival and persistence of fetal progenitor cells.[92] Other research groups have confirmed the persistence of fetal cells in maternal blood and other maternal organs.[93-96] The identification of circulating multipotent hematopoietic progenitors in first trimester fetal blood[48] and mesenchymal stem cells in first trimester fetal blood, liver and bone marrow,[97] has encouraged investigators to develop strategies to detect fetal progenitor cells in pregnant women. It is well recognized that in human pregnancies, fetal progenitor cells that express CD34+ are transferred into the

maternal circulation.[98,99] It is currently hypothesized that in order to persist in maternal blood and other maternal tissues these cells of fetal origin must have stem- cell-like properties.[100]

*Cell-free fetal DNA*

Since the first reports on the presence of fetal DNA in maternal serum and plasma by Lo and his colleagues in 1997[101] and 1998[102] many studies on cell-free fetal DNA have been published. This research group demonstrated high mean concentrations of fetal DNA in maternal plasma and serum at term using PCR analysis with specific probes to detect sequences of the Y chromosome. Significantly more fetal DNA was present in maternal plasma and serum than previous studies on the detection of intact fetal cells would indicate. Fetal DNA was detectable in as little as 10 ml of maternal plasma accounting for 3.4% of the total cell-free DNA in maternal plasma between 11 and 17 weeks of gestation.[102] Plasma samples obtained from women at term contained as much as 6.2% fetal DNA of the total circulating DNA. None of the women pregnant of a female fetus and none of the non-pregnant control women had detectable fetal DNA using amplification of Y chromosome sequences. Fetal DNA in maternal plasma can be detected as early as 5 weeks of gestation.[103,104] Lo et al. further investigated the clearance kinetics and turnover of fetal DNA from maternal blood.[105] In plasma samples obtained from women during labour, immediately after delivery and hours to days postpartum they showed that in most women fetal DNA was cleared within 2 h. The mean half-life for circulating DNA was 16.3 min, suggesting that large quantities of fetal DNA have to be liberated continuously into the mother to maintain a steady state.

To date, little is known about the molecular and biological characteristics of cell-free fetal DNA present in maternal blood. Several mechanisms have been described in literature to explain these findings.[106,107] Possible sources of cell-free fetal DNA could be 1) continuous leakage of fetal cells across the placental barrier that are rapidly destroyed by the maternal immune system, 2) active remodelling of the placenta at the fetomaternal interface with continuous cell lysis, 3) direct release of DNA of fetal origin into the maternal circulation.

The discovery of cell-free fetal DNA in maternal plasma and serum has opened a new perspective for the non-invasive prenatal diagnosis of fetal genetic traits and may be useful for the study of complications in pregnancy.

*Fetal plasma proteins*

Not only intact fetal cells and fetal DNA can be detected in maternal blood, but also other contents of fetal plasma, such as plasma proteins. One of the serum glycoproteins present in fetal plasma is the alpha-fetoprotein (AFP), which is

synthesized during fetal life mainly by the yolk sac and trophoblast early in the first trimester followed shortly thereafter by the fetal liver. In the human fetus the serum concentration of AFP peaks at 13 weeks of gestation (3-4 mg/ml), falls to about 50 μg/ml at term and significantly decreases after birth.[108] The serum AFP concentration in adults is approximately 5 μg/l. The primary roles of AFP are regulation of cell growth by controlling apoptosis, involvement in inflammatory reactions and immunoregulation.[109] The source of AFP in maternal blood is nearly all fetal.[110] Most of the maternal serum AFP transferred across the placenta is derived from fetal serum rather than from amniotic fluid. Although a small amount of AFP crosses the placenta by paracellular diffusion, the major fetal-maternal transfer of AFP is accomplished through bulk flow of AFP containing fluids driven by a hydrostatic gradient across the placental villi.[108]

Cytotrophoblast cells are known to synthesize AFP early in pregnancy, but at term the placenta does not synthesize AFP and with an intact placental barrier the presence of AFP in the placenta is a reflection of the mechanisms described above.[111,112] In normal pregnancy maternal serum AFP levels continue to rise until the 32$^{nd}$ week of gestation despite the decrease in fetal serum AFP throughout pregnancy.[108] After the 32$^{nd}$ week the maternal serum AFP level starts to decline until term.

Elevated levels of AFP in maternal blood during pregnancy are associated with multiple gestation, fetal malformations, such as neural tube defects, placental tissue damage and fetomaternal hemorrhage.[108]

Spontaneous or induced breakdown of the placental barrier will cause direct influx of AFP from the fetal to the mother, reflecting the volume of fetal to maternal bleeding. Also, destruction of trophoblast cells as a consequence of invasive prenatal diagnostic procedures in the first trimester may cause a release of AFP in the maternal circulation. Decreased levels of AFP are associated with chromosomal abnormalities, such as trisomy 13, 18 and 21.[108]

### 2.3.2. Time of appearance and frequency of fetal cells

Due to the very small fetoplacental circulating blood volume, it was originally assumed that only few cells were transferred from the fetus to the mother in the first trimester. However, several research groups demonstrated that fetal Y chromosomal DNA is present in maternal blood as early as 5 weeks of gestation.[103,113-115]

The fetal DNA detected early in the first trimester is most likely derived from trophoblast cells, shed into the maternal circulation during the ongoing process of placentation. Although during this stage of embryonic development the numbers of fetal hematopoietic cells that pass the placental barrier are expected to be very low, several studies have described the presence of fetal red blood cells in maternal blood

in the first trimester.[73-76]

Both the frequency of finding fetal cells and the volume of the cells increase as pregnancy progresses, particularly in the third trimester and during delivery.[114] At least half of the women have fetal red cells in their circulation after delivery, detectable by the Kleihauer-Betke test.[77,78] From another study using flow cytometry for fetal red cell detection in maternal samples postpartum, it was concluded that all women have a small but detectable amount of fetal red cells in their circulation postpartum.[116] Sebring and Polesky [117] reviewed the literature on the incidence of fetal cells in maternal blood in large series of women.[77,78,118-130] They concluded that the volume of fetal blood present in the maternal circulation is usually very small. In 74% of the women postpartum the fetal red cell volume was smaller than 0.025 ml, less than 0.05 ml was detected in 96%. The fetal red cell volume ranged from 1 to 15 ml in 3.7% and only 0.3% of the women had a red cell FMH larger than 15 ml. The reported frequency of fetal nucleated cells in maternal blood of normal pregnancies varies widely, ranging from 1 in $10^5$ to 1 in $10^8$ nucleated cells.[114,131,132]

Overall, factors that may influence the frequency of fetal cells detected in maternal blood include the gestational age at the time of sampling, the fetal cell type analyzed, and the accuracy of methods to enrich, identify and quantify the fetal cell population.[133] Further, the incidence of fetal cells in maternal blood is influenced by a number of biological parameters. Increased fetomaternal cell trafficking is observed in pregnancies with abnormal fetal or placental karyotype, complicated pregnancy and a number of invasive diagnostic and operative procedures. However, particularly in clinical settings where small amounts of fetal cells are likely to pass the placental barrier, e.g. spontaneous antepartum bleeding, ectopic pregnancy, chorionic villus sampling, first trimester termination of pregnancy, it remains difficult to quantify FMH due to the lack of sensitive methods.

### 2.3.3. Clearance versus persistence of fetal cells in maternal blood

Clearance or persistence of fetal cells in maternal blood strongly depends on the fetal cell type, the antigens exposed by fetal cells, and whether the fetal cells are progenitor cells capable of proliferation or mature hematopoietic cells.

Fetal erythoblasts and enucleated red cells are not capable of surviving in maternal blood. The clearance rate of fetal RBCs from maternal blood depends on a number of facts: the ABO and Rh compatibility, administration of anti-D immunoglobulin and the time of entrance in the maternal circulation. Results on the lifespan of ABO Rh compatible fetal RBCs described in literature showed different clearance rates. Some studies report a shorter lifespan compared to adult RBCs.[87,134] In two other studies a fetal RBC lifespan equal to adult RBCs was reported.[117,135] Differences in the observed lifespan of fetal RBCs in maternal blood are most likely

due to a varying age distribution of cells entering the maternal circulation.[117]

Many reports are available on the clearance rate of ABO incompatible fetal RBCs from maternal blood. Fetal RBCs are less often identified in postpartum maternal blood samples in case of ABO-incompatibility between mother and child.[78,118,121,122,124,127] In contrast, Ness et al. found no difference in the incidence of detectable FMH in ABO-compatible or -incompatible pregnancies.[136] The clinical symptoms and clearance rate of ABO-incompatible fetal RBCs both depend on the hemolytic potential of isoagglutinin, the amount of incompatible antigen on other tissues, the strength of the antigen on the incompatible RBCs, and the volume of incompatible blood.[117] Clearance of fetal cells requires mechanisms, such as the removal of fetal cells by the maternal immune system and apoptotic cell death as a consequence of inappropriate maternal environment. The fetus is considered as a semi-allograft and paternal antigens can elicit a maternal immune response. Also, proliferating fetal progenitor cells need specific cytokines for survival, which are available in fetal blood and other tissues, but not sufficiently apparent in maternal blood, leading to apoptotic cell death in maternal blood.[137] However, since the pioneer work of Bianchi et al. in 1996[92] and also from previously published work,[88,89] it is well known that a small number of fetal cells, resulting from fetomaternal cell trafficking are capable of survival, homing and proliferation and thus persistence in maternal blood and other maternal organs.[93-96]

### *2.4. Maternal to fetal cell trafficking*

Although it is now well recognized that fetal cells pass the placental barrier and are capable of persisting in maternal blood and other tissues, relatively little is known about the transfer of maternal cells to the fetal circulation. The results of a number of published studies suggest that fetomaternal cell trafficking is bidirectional. Socie et al. detected maternal cells in umbilical cord blood in 1 of 47 cases.[138] Another study by Hall et al. detected maternal cells in umbilical cord blood in 10 of 49 male umbilical cord blood samples using fluorescence in situ hybridization with X and Y chromosome specific probes.[139] Lo et al. found maternal cells in 16 of 38 umbilical cord blood samples.[140] Sekizawa et al. evaluated bidirectional transfer of plasma DNA through the placenta in patients undergoing Cesarean section.[141] Five patients had pre-eclampsia and 10 normal controls were included. Their findings indicate that cell-free fetal DNA is unequally transferred through the placenta and that the majority of the cell-free fetal DNA in maternal plasma is derived from villous trophoblast.

It has been suggested that maternofetal cell trafficking may be related to the development of inflammatory and autoimmune disorders during childhood or later in life, but the biological significance of maternal cell microchimerism is unknown to date.

## 3. Fetomaternal hemorrhage

### *3.1. Pathophysiology: breakdown of the placental barrier*

From population based studies it is clear that the presence of fetal red cells in maternal blood is a common phenomenon. However, the presence of a large amount of fetal cells in the maternal circulation is considered as pathological. A volume of more than 15 ml of fetal red cells (or 30 ml of fetal whole blood), which has been transferred to the mother, is usually defined as a large FMH.

That FMH could occur was first postulated by Dienst in 1905, who concluded that "eclampsia is nothing but a transfusion of incompatible blood of the child into the mother's circulation".[142] Fifty years later Chown serologically demonstrated a minor cell population of Rh-positive cells in a blood specimen derived from Rh negative women postpartum and concluded that the anemia observed in Rh positive neonates was due to FMH.[143] Since the direct microscopic visualization of fetal erythrocytes in maternal blood by Kleihauer et al.,[2] the study of transplacental passage of fetal cells and understanding of the cause of rhesus immunization have largely expanded.

Due to spontaneous or induced disruption of the placental barrier fetal plasma and blood cells including their precursors will leak into the maternal circulation.

Large FMH is a serious complication of pregnancy, which occurs in approximately 3 out of 1000 deliveries.[117,144] FMH may cause severe fetal anaemia, in some cases leading to fetal death due to exsanguinations.[145] A typical sinusoidal fetal heart rate pattern may be observed in cases with fetal anemia. Other serious consequences that may arise from fetal-maternal cell trafficking are red cell immunization,[146] in case of blood group incompatibility between mother and fetus, potentially affecting present and future pregnancies and platelet immunization.[147] As with fetomaternal cell trafficking various fetal cell types, such as fetal RBCs including precursors, fetal white cells including precursors, fetal stem cells, fetal DNA and other plasma components may be involved in large FMH, but it is unclear whether all fetal cell types and other fetal plasma components pass the placental barrier in proportion. Likely, relatively small cells have a high chance for passing the placental barrier.

### *3.2 Increased fetomaternal cell trafficking as a marker of disease*

Circulating fetal DNA has also been targeted as a marker for assessment of fetal and maternal well-being.[148] Increased fetomaternal cell trafficking has been observed in several clinical conditions and pregnancy-related complications. Elevated levels of fetal DNA as compared to uncomplicated pregnancies have been described in relation to pre-eclampsia, preterm labour, twin pregnancies and fetal chromosomal abnormalities. The best studied is pre-eclampsia, where failure of adequate trophoblast invasion due to an increased rate of apoptosis, apparently results in

increased levels of circulating fetal DNA in maternal plasma.[149-152] Elevated fetal DNA concentrations prior to the onset of clinical symptoms of pre-eclampsia and correlation with the severity of the clinical condition were also described.[150,152]

The finding of gradual increase in fetal DNA concentration in maternal serum as pregnancy progresses, has led to the hypothesis that such an increase may occur earlier in pregnancies complicated by pre-term labour. This has indeed been confirmed in case-control studies by Leung et al. and Farina et al.[153,154] In fetal chromosomal abnormalities levels of fetal DNA may also be increased. Using real-time PCR Lo et al. demonstrated a two-fold increase in fetal DNA levels for trisomy 21, compared to euploid cases.[155] Subsequent studies have supported these observations, although such an increase is not observed in trisomy 18.[156] Other pregnancy-related conditions related to increased levels of fetal DNA in maternal plasma recently described are invasive placentation,[157] hyperemesis gravidarum,[158,159] intra-uterine growth restriction,[160] and multiple gestation.[161]

Increased trafficking of fetal nucleated cells has also been described in pregnancies complicated by pre-eclampsia,[162,163] fetal growth restriction,[164] and trisomy 21.[165] Moreover, an increase of fetal cells in the maternal circulation preceding the onset of pre-eclampsia or intra-uterine growth restriction has been reported.[163] In addition, trophoblast cells were found to be increased in pre-eclamptic pregnancies.[166]

### *3.3. Potential risk factors for occurrence of fetomaternal hemorrhage*

Large FMH may occur spontaneously in previously uncomplicated pregnancy and delivery. However, a number of pathological conditions, such as abdominal trauma,[167,168] placental abruption,[117,169,170] or choriocarcinoma,[171,172] are identified as risk factors for the occurrence of significant FMH. In patients with third-trimester vaginal bleeding the incidence of FMH does not appear to be increased when compared to non-complicated controls or to other obstetrically complicated pregnancies.[173] In addition, a number of prenatal invasive procedures and obstetrical interventions during pregnancy and delivery that cause breakdown of the placental barrier place patients at risk. Chorionic villus sampling (CVS), an invasive intrauterine procedure for first trimester prenatal diagnosis, may induce FMH.[174-178] Since reliable quantification of small numbers of fetal red cells in the maternal circulation is difficult, FMH after CVS has been studied predominantly by measurement of the AFP concentration.[174-176,179-182] A significant increase of fetal red cells after chorionic villus sampling using the Kleihauer-Betke test has not been detected.[182,183] Elevated AFP levels following second trimester amniocentesis also have been reported.[184,185] Increased fetomaternal cell trafficking after first-trimester termination of pregnancy was described by Bianchi et al. using fetal nucleated cell detection by PCR

amplification of the Y chromosome.[186] Wataganara et al. found elevated cell-free fetal DNA concentrations shortly after surgical first-trimester termination of pregnancy.[187]

Further, cordocentesis,[188] cephalic version near term,[9] Cesarean section,[117,136189,190] manual removal of the placenta,[125,191] and complicated vaginal delivery[136] have been associated with an increased risk of FMH. Due to inadequate quantification of small numbers of fetal red cells in first trimester events, the large inter-observer variability of the methods, and the various formulas reported in literature to calculate the FMH volume,[3,5,146,190,192] identification of patients at risk for FMH as compared to uncomplicated pregnancy and delivery is difficult in clinical practice.

### *3.4. Assessment of fetomaternal hemorrhage*

Reliable detection of FMH is important in Rh negative women potentially at risk for red cell immunization and in all cases complicated by fetal or neonatal anemia. Both qualitative and quantitative tests are available for this purpose.

*Qualitative tests*

Several commercial diagnostic assays are available to detect the presence of fetal cells in maternal blood. After delivery of a Rh D positive child, all Rh D negative women should receive a single dose of 300 μg anti-D immunoglobulin, which is sufficient to clear 15 ml of Rh D positive cells (or 30 ml of whole blood).[193] When the FMH volume is larger than 15 ml, additional doses of anti-D immunoglobulin are required.

Qualitative tests to detect FMH are are available to screen Rh D negative women after delivery of a Rh D positive child. To be useful for clinical practice such test must be 100% sensitive to detect a FMH volume of 15 ml of Rh D positive cells in Rh D negative maternal blood. If positive, a quantitative technique is required to determine the exact FMH volume in order to administer the appropriate amount of anti-D immunoglobulin. Common diagnostic assays are the erythrocyte rosette test and the antiglobulin test. In the rosette test, reagent anti-D is added to a suspension of maternal Rh negative RBCs. During incubation, the reagent antibody binds to any fetal Rh positive RBCs that are present. Indicator Rh positive RBCs are then added to the test system. These indicator cells will bind with the anti-D present on the fetal Rh positive RBCs, forming rosettes around each antibody-coated fetal cell. After centrifugation and resuspension microscopic assessment of rosette formation is performed. In the antiglobulin test a suspension of RBCs is incubated with anti-D and anti-IgG. After centrifugation agglutination is assessed microscopically.

Three qualitative tests (the Microscopic $D^u$, the rosette test and the PEG $D^u$) were compared in a study by Bayliss et al. using spiked samples of Rh D positive cells from umbilical cord blood in Rh D negative adult cells and patient samples.[3]

No false positive results were obtained by any of the three methods within this study. Within this study the rosette test showed the highest sensitivity detecting fetal red cell percentages as small as 0.06% fetal RBCs in 80% of the test samples. Other studies on the use of qualitative test to detect FMH reported a poor sensitivity of the Microscopic $D^u$ to detect significant FMH (0.6% fetal RBCs)[194,195] and a poor specificity of the rosette test generating too many false positive results.[195,196]

*Quantitative tests*

The Kleihauer-Betke test has been used for decades to quantify the FMH volume in clinical situations.[2] Although this method has proven to be clinically useful in the detection of fetal red cells in maternal blood, a relative high inter-observer and inter-laboratory variability have been reported, most likely due to various modifications of the test and analysis of an insufficient number of microscopic fields.[3,4,5,197]

Over the last fifteen years flow cytometric assays using polyclonal antibodies directed against the human D surface antigen and monoclonal antibodies against HbF have demonstrated high sensitivity and statistical accuracy both in spiked and patient samples.[3-8] Flow cytometry is capable of detecting > 0.1% fetal cells in maternal blood, below this level it is considered insensitive. However, due to the presence of variable amounts of maternal F cells in certain patients, it is sometimes difficult to discriminate the fetal red blood cells from the maternal red cell population.[198,199] This problem may partially be solved by the use of two discriminating parameters.

Commercial kits for flow cytometric analysis containing combinations of antibodies have been available, for example the combination of antibodies against HbF and the so-called “i” antigen. The “i” antigen is present on fetal cells and disappears during the first year of life.[200] Red cells positive for both HbF and “i” are of fetal origin. In another commercial flow cytometric assay antibodies for dual labelling against HbF and carbonic anhydrase are combined. Carbonic anhydrases are zinc metalloenzymes involved in the process of gas exchange, acid-base equilibrium and secretion of iones. Erythrocytes contain the carbonic anhydrase I and II isoenzymes and are mainly expressed during adult life. Only small percentages of fetal cells contain carbonic anhydrase.[201] Although the use of two antibodies should theoretically improve the accuracy to define the fetal red cell population, a lower specificity of the second antibody may result in technical problems.

### *3.5. Estimation of the fetomaternal hemorrhage volume*

The Kleihauer-Betke test and flow cytometry are most frequently used to quantify the number of fetal red cells in maternal blood. The performance and restrictions of both methods have extensively been discussed in previous studies.

Generally, FMH is expressed as the proportion of the detected number of fetal red cells and maternal background red cells multiplied by 100%. The fetal red cell percentage is then transformed to a fetal red cell or fetal whole blood volume. Several formulas are used to calculate the FMH volume.[3,5,146,190,192] A maternal blood volume of 5000 ml, a maternal hematocrit of 0.35 and a newborn hematocrit of 0.50 are assumed in the following formula, which is frequently used to calculate the FMH volume.[192]

fetal blood volume (ml) = the fetal *whole blood* volume =
(maternal blood volume x maternal Hct x fetal red cell %) / newborn Hct

However, it is well known that the fetal and maternal hematocrit and the maternal circulating blood volume both depend on individual biological and pathophysiological factors such as gestational age and bodyweight. The use of several assumptions and various formulas for the calculation of the FMH volume inevitably leads to a certain degree of imprecision, which may result in the administration of an inadequate dose of anti-D immunoglobulin.

## 4. Implications of fetomaternal cell trafficking

Both small and large quantities of fetal cells entering the maternal circulation may have an effect on the maternal immune system and on future pregnancies through several pathways, which we will discuss briefly.

### *4.1. Red cell alloimmunization*

Maternal immunization to the Rh D antigen is a known cause of fetal and neonatal haemolytic disease. There is a relative high probability for Rh D negative women to sensitize to the paternally inherited Rh D antigen, given the fact that 85% of the Caucasian population is Rh D positive. Immunizations to other red cell antigens, like Kell, Rh c and Duffy, may also cause fetal and neonatal anemia, but are less frequently seen. Non-Rh D immunizations often result from incompatible blood transfusions.

In clinical situations that place Rh D negative women at risk for FMH and subsequent antibody formation, a relatively large dose of anti-D immunoglobulin is recommended. Guidelines for the appropriate and efficient management of women at risk in order to further decrease the incidence of Rh D immunization are available.[117,202-204] The routine administration of anti-D immunoglobulin to Rh D negative women after delivery of a Rh D positive infant, has decreased the risk of alloimmunization to approximately 2%.[205] Antenatal prophylaxis has reduced the

risk even further to 0.2%.[206] Despite well-organized prophylaxis programs, Rh D alloimmunization continues to occur as a serious complication of pregnancy.

Accurate quantification of small FMH is particularly important, considering the fact that there is a dose-dependent relation between the volume of Rh D positive red blood cells to which a Rh D negative person is exposed and the incidence of Rh D alloimmunization. A FMH volume as small as 0.1 ml or 0.006% fetal red cells may result in antibody formation.[207] In addition, very small amounts of FMH in pregnancy may evoke sensitization, which might result in detectable antibody formation in a subsequent pregnancy.[208] ABO incompatibility between mother and child confers some protection against red cell immunization because fetal red cells entering the maternal circulation usually are rapidly destroyed before they elicit an antigenic response.

### *4.2. Platelet immunization*

Neonatal alloimmune thrombocytopenia (NAITP) is caused by an immune-mediated process. Little is known about the pathophysiology and the natural history of NAITP. It is considered as the platelet equivalent of hemolytic disease of the newborn, but in contract to red cell immunization, NAITP is induced during the first pregnancy in more than half of the cases.[147]

Specific human platelet antigens (HPA) expressed by platelets and their precursors, endothelial cells, vascular smooth muscle cells and foreskin fibroblasts, may evoke a maternal immune response after sensitization, thus leading to NAITP. In affected pregnancies the fetus and neonate is at risk for an intracranial hemorrhage. NAITP has a high recurrence rate in subsequent, incompatible pregnancies.

### *4.3. Microchimerism*

As described earlier, different fetal cell types may enter the maternal circulation. Some cell types may persist in maternal blood and other organs for years.[88,89,92-96] This phenomenon is referred to as “microchimerism”, a long-term donor cell survival in a small proportion relative to the host cell number. The term microchimerism first appeared in literature, when Liegeois et al. reported a steady state low-level proliferation of allogeneic bone marrow cells in the mouse.[209] In 1981 the same group demonstrated the presence of allogeneic fetal cells in maternal tissue during and long after pregnancy also in mice.[210]

It is now known that microchimerism may result from blood transfusion, twinning, organ transplant and bidirectional cell trafficking during pregnancy. As hypothesized by Khosrotehrani and Bianchi, fetal cells capable of persisting in the mother must have stem-cell-like properties in order to persist after delivery in a maternal stem cell niche.[100] The implications of persistent fetal cells in maternal tissue have led to the

"bad microchimerism" theory, which was first proposed by Nelson et al. in 1996.[211] This theory suggested that the persistence of fetal cells after pregnancy may lead to a graft-versus-host like response in multiparous women, and that the maternal immune response to these cells may contribute to the development of autoimmune disease. Studies on the higher frequency of certain autoimmune diseases in women following their childbearing years were considered as evidence in the support of this hypothesis.[93,212] Subsequently, reports were published that conflicted with this theory.[94,213] The recently proposed theory of "good microchimerism" suggests that persistent fetal cells, instead of inducing a maternal immune response, survive in a maternal stem cell niche and provide a rejuvenating source of fetal progenitor cells in case of maternal tissue injury. They may home to the damaged organ and differentiate as a part of the maternal repair response.[100,214]

## Outline of this thesis

Since Kleihauer, Betke and Braun in 1957 demonstrated a technique for direct microscopic visualization of fetal erythrocytes against a background of maternal erythrocytes, based on acid elution of the adult hemoglobin and staining of the more acid resistant fetal hemoglobin, many studies on FMH detection using this method have followed. Due to several modifications of the Kleihauer-Betke test used to estimate the FMH volume, this test has suffered from subjectivity and imprecision. Other methods, such as flow cytometry, automated microscopy and PCR analysis, have been developed to improve reliability and precision of FMH quantification.

In this thesis we will focus on the development of an automated microscopic method to quantify FMH, the comparison of different techniques available for FMH quantification, the incidence of FMH in a number of clinical conditions and procedures, the biology of and the detection of fetal red and mononuclear cells in maternal blood following large FMH. The aim of the studies, described in detail in the following chapters, is summarized in short.

*Chapter 2*

Reliable detection and quantification of fetal red cells in maternal blood is important in routine obstetrical practice. Particularly in clinical settings where low numbers of fetal cells pass the placental barrier, FMH quantification is difficult. The manual Kleihauer-Betke test is widely used, but suffers from imprecision and subjectivity. This study was designed to investigate whether automated readout of Klaihauer-Betke stained slides can improve sensitivity and accuracy in spiked samples.

*Chapter 3*

Many techniques are available for detection and quantification of FMH. In this study we compare three techniques: manual and automated microscopic analysis of Kleihauer-Betke stained slides and flow cytometry in 44 clinical samples obtained from patients at risk for FMH. We will discuss diagnostic strategies to individualize the administration of anti-D immunoglobulin.

*Chapter 4*

In this study we investigated whether chorionic villus sampling results in a proportional increase of the alpha-fetoprotein concentration and fetal red cells in maternal blood in a group of 59 patients. The measurement of the AFP concentrations and detection of fetal red cells by automated microscopic analysis of Kleihauer-Betke stained slides before and after chorionic villus sampling were compared to quantify the FMH volume.

*Chapter 5*
The aim of this study was to investigate whether women undergoing Cesarean section are at risk for fetomaternal hemorrhage. Results obtained from the Kleihauer-Betke test and simultaneous measument of the alpha-fetoprotein concentration in maternal blood samples of 57 patients before and after Cesarean section were compared.

*Chapter 6*
In this case study we evaluated the clearance rates of alpha-fetoprotein, fetal red blood cells, and fetal MNCs from maternal blood postpartum following large FMH near term. For this purpose we developed a new approach to detect fetal MNCs using a staining with a monoclonal antibody directed against the paternally derived HLA-A2 antigen. We aimed to detect very low frequencies of fetal MNCs at different time intervals after delivery.

*Chapter 7*
In recently published article the incidences of large fetomaternal hemorrhage following Cesarean and vaginal delivery, were considerably higher than previously reported. In this chapter comment on the formula used to calculate the transfused fetal blood volume.

*Chapter 8*
The conclusions of the studies presented in this thesis and the future perspectives of fetomaternal cell trafficking are discussed in this chapter.

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# Improvement of the Kleihauer-Betke test by automated detection of fetal erythrocytes in maternal blood

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## Abstract

***Objective:*** Reliable detection and quantification of fetal red cells in maternal blood is important in routine obstetrical practice. The manual Kleihauer-Betke test (KBT) is widely used, but suffers from imprecision and subjectivity. This study was designed to investigate whether an automated readout of the KBT can improve the sensitivity and the accuracy.

***Methods:*** Glass slides containing different percentages of fetal red cells in adult blood ranging from 0.0001% to 1% were prepared and stained using acid elution. Automated microscopic analysis of all slides was performed, whereby detected fetal red cells were reviewed by two independent investigators. In addition, standard manual evaluation of the KBT was performed on the same slides by one investigator.

***Results:*** Within 10 replicate measurements of each slide the automated KBT showed high reproducibility and very small inter-observer and intra-observer variability. Typical coefficients of variation were 3-4% for fetal red cell percentages ranging from 0.001% to 0.1%. The automated KBT showed strong correlation between the theoretical and detected fetal red cell percentage ($r^2$=0.999). In the range from 0.0001% to 0.001% the standard KBT underestimated the fetal red cell percentage, whereas the automated KBT was very precise. The correlation between both methods was good ($r^2$=0.999).

***Conclusions:*** The automated readout of KBT stained slides provided improved accuracy of the fetal red cell detection in the range from 0.0001% to 1%, particularly when larger numbers of cells were analyzed.

## Introduction

A reliable method for identification and quantification of fetal red cells in maternal blood is necessary for routine evaluation of fetomaternal hemorrhage (FMH) in patients during pregnancy and after delivery. Even in uncomplicated pregnancy and delivery, small amounts of fetal cells occur in maternal blood.[1,2] It is well described that significant FMH can be detected in patients undergoing prenatal or obstetrical procedures, e.g. cephalic version, caesarean section, manual removal of the placenta, or in patients with placental abruption and abdominal trauma in pregnancy.[3,4]

Moreover, in the case of Rh D incompatibility between mother and fetus, fetal red cell detection and subsequent typing for the D-antigen is important, as Rh D positive fetal cells can induce an immune response in Rh D negative mothers. The anti-D alloantibody can be produced by Rh D negative mothers after exposure to Rh D positive fetal red cells during pregnancy. The binding of maternal anti-D to fetal red cells often leads to hemolysis, resulting in fetal anaemia, hydrops fetalis, and even fetal death. Reliable detection and quantification of FMH, therefore, is necessary to ensure the administration of an appropriate dose of anti-D immunoglobulin, given to prevent immunization of the mother.[5]

Also, in Rh D positive mothers suspected of FMH, it is important to have a reliable diagnostic test. FMH may cause severe anaemia of the fetus, and in some cases even followed by fetal death due to exsanguination.[4,6]

The Kleihauer-Betke test (KBT) is widely used for the detection of fetal red cells in maternal blood. This method is based upon elution of adult hemoglobin (HbA) from adult red cells, whereby the more acid resistant fetal hemoglobin (HbF) remains intact in fetal red cells. This remaining hemoglobin is subsequently visualised by staining with erythrosin.[7]

Although the manual KBT is used by most laboratories for the quantification fetal red cells in maternal blood, it is not accurate enough in the quantification of FMH.[8] This is partially due to statistical imprecision as a consequence of evaluating low numbers of cells. Both underestimation and overestimation of the fetal red cell count are reported.[9] In addition, there is a large inter-observer and inter-hospital variation in interpreting the results obtained from the KBT.[10-12]

Over the last ten years several studies reported the use of flow cytometric methods for fetal red cell identification in maternal blood using polyclonal antibodies to human Rh D surface antigen and monoclonal antibody to HbF. Flow cytometry theoretically provides better accuracy than the standard KBT in FMH in the range from 0.1% to 10%, mainly because large numbers of cells are evaluated using an objective method.[8,9,12-16]

We developed a new strategy for the evaluation of KBT slides using automated microscopy aiming at two goals: 1) to distinguish fetal and maternal red cells in an unbiased way, 2) to analyse a larger area of the slide as compared to the standard manual KBT, and thus be able to evaluate many more cells to improve accuracy.

The concept of automated microscopy to evaluate the presence of rare-events is well described.[17] The analysis of large numbers of cells in order to detect rare target cells occurs in various medical settings. We studied the suitability of this strategy to enumerate fetal red cells in artificially spiked adult samples, with the expectation that the subjectivity and imprecision of the standard manual KBT may be significantly improved by the automated analysis of large numbers of cells.

## Materials and methods

### *Blood samples*

Adult blood from healthy non-pregnant female volunteers and umbilical cord blood was collected in EDTA vacutainer tubes and stored at 4 °C for no longer than 24 hours. Red cell counts in adult blood and cord blood samples were determined using a cell counter (Becton Dickinson, Franklin Lakes, NJ) and dilution with phosphate-buffered saline (PBS) was applied to equalize red cell counts. Artificial mixtures of 1%, 0.1%, 0.01%, 0.001% and 0.0001% fetal red cells in ABO Rh-matched adult blood were prepared. Positive and negative controls consisted of 100% adult blood and 100% cord blood, respectively.

### *Staining protocol of the slides*

Hundred µl of each artificial mixture was mixed with 100 µl PBS. Conventional blood smears were prepared on glass slides using 2.5 µl of this diluted suspension. Each slide contained approximately 5.4 x $10^6$ red cells. The slides were air-dried for 30 minutes at room temperature and fixed in 80% ethanol for 5 minutes. After rinsing with tap water, they were placed in citrate buffer at 37 °C for exactly 5 minutes and rinsed again. The citrate buffer consisted of 750 ml 0.1 M $C_6H_8O_7.H_2O$ and 250 ml 0.2 M $Na_2HPO_4.2H_2O$; pH 3.20 to 3.30 at 37 °C. The slides were counterstained with Papanicolaou dye (hematoxylin solution, Merck Diagnostica, Darmstadt, Germany) for 3 minutes at room temperature and rinsed in tap water. Finally, the slides were placed in Sørensen buffer for 5 minutes and stained with erythrosin B (Merck Diagnostica, Darmstadt, Germany) for 5 minutes at room temperature. The Sørensen buffer consisted of 400 ml 0.067 M $Na_2HPO_4.2H_20$, 340 ml 0.067 M $KH_2PO_4$ and 260 ml distilled water. The pH of this solution is 6.9 at room temperature. After rinsing with tap water, the slides were air-dried at room temperature for at least 2 hours. The

slides were stored in a slide box at room temperature until evaluation. After staining, standard manual and automated KBT were performed. In addition, we performed a manual counting in order to verify the total number of fetal red cells detected by automated KBT.

***The standard manual KBT***

The standard KBT was evaluated by a single investigator. The number of fetal red cells was counted in 400 subsequent microscopic fields (i.e. 4 rows) with a 40x objective according to a published method.[18] According to this method considerably more than the prescribed 2000 cells are counted. Adult F cells and fetal red cells were distinguished by the intensity and intracellular distribution of the pink staining. The pale, eluted adult red cells and the slightly pink adult F cells were classified as negative. Only bright pink stained cells were classified as fetal red cells. The percentage of fetal red cells was calculated by dividing the number of fetal red cells by the total number of red cells. The total number of red cells within 400 fields was estimated by the following formula: average number of red cells in 3 different fields (40x objective) x 400 fields.

***The automated KBT***

Preparation of the slides for automated microscopy

The same slides used for the manual KBT were embedded in Fluoromount embedding medium (Gurr, BDH Limited Poole, UK) and sealed with a cover glass the day after staining to ensure complete dehydration of the slides.

System description

For automated microscopy, a MDS 1 image analysis system (Applied Imaging Corporation, Santa Clara, CA) was used. An extensive description of the system and the procedure is published elsewhere.[19] In brief, the system consisted of a microscope (Olympus BX-60) providing bright field and fluorescence capabilities, a trinocular head, and 10x, 20x, and 40x objectives. This microscope was equipped with an automated scanning stage (Maerzhauser Co, Wetzlar, Germany), a 7-position transmission filter wheel, and an automated focus drive (TOFRA). All these devices were controlled by microstepping motor controllers (Intelligent Motion Systems, Marlborough, CT). Images were acquired by a CCD camera with light integration capability (COHU 4910, Cohu, Inc, Poway, CA) and a custom built frame grabber board, which included a 10-bit analog to digital converter and frame averaging ability. A personal computer (Dell Poweredge sp590-2, Round Rock, Tx) was used to control all microscope functions, to perform image acquisition and processing, and to perform user interface functions. Images were visualized on a Nanao T2.17 display.

### Scanning and finding parameters

Software settings were defined that, after every three fields, the system automatically corrected its focus. Bright field analysis of the slides was performed using a green and red absorption filter. The computer-assisted microscope scanned the slide on the basis of preset characteristics (an algorithm). A two-step filter-switch method was developed to identify fetal red cells. First, a green absorption filter was applied to select all pink stained cells. Then, a red absorption filter was used to identify the hematoxylin-counterstained white blood cells. Events were only selected and stored in memory, when positive for green absorption and negative for red absorption. Optimal software settings for detection and analysis were defined on a set of test samples. The positive, fetal red cells were distinguished from pale adult cells by the intensity and intracellular distribution of the pink staining and cell size.

The automatically detected cells were stored in a database and relocated by the operator for direct microscopic inspection, since their coordinates on the slides were also recorded. Only bright pink cells were marked as fetal by the operator. Non-fetal cells and artefacts were marked as “unidentified alarms” (UA).

### Automated analysis of the slides

A fixed area of 1517 low-power (10x objective) fields (area surface 32535 μm x 21864 μm) was scanned. After automated analysis, each selection gallery was independently reviewed by two investigators. The percentage of fetal red cells was calculated by dividing the number of fetal red cells by the total number of red cells.

The total number of red cells within this area was estimated as follows.
The total number of red cells equals the average number of red cells in 3 different fields (10x objective) x 1517 fields. To correct for a known field overlap of 13%, the fetal red cell count was adjusted by using the following formula. The corrected fetal red cell count equals the fetal red cell count – (13/100 x fetal red cell count). A certain field overlap is inevitable, otherwise a small percentage of target cells located on the border of a microscopic field would be missed by the scanning device.

#### *Manual evaluation of the slides*

In order to verify the number of fetal red cells detected by automated KBT, manual evaluation of the entire area was performed by two investigators independently.

A conventional microscope was used with a 20x objective. The total number of fetal red cells within this area was counted.

#### *Validation studies*

The results obtained from automated microscopy were compared with the results from the standard KBT. Precision analysis of the automated technique was

determined by performing 5 replicate cycles of each concentration. In order to examine the inter-observer variability of the automated method, the selection gallery was reviewed by two investigators independently. The number of fetal red cells detected by automated microscopy was verified by manual screening of the same area by two investigators independently. The standard manual KBT was evaluated by a single investigator. The stained slides were coded in a manner that both the manual and the automated counting were performed in a blinded fashion. In order to investigate detection sensitivity and linearity, serial dilutions of cord blood into adult blood were prepared. Intra-observer and inter-observer variability were tested by calculating the absolute and percentage differences between two investigators.

Correlation study and precision statistical analysis were performed using Pearson's correlation coefficient and coefficient of variation (CV) calculations (Excel2000, Microsoft Inc, Redmond, WA).

## Results

### *Automated microscopy*

Slides containing different mixtures of adult blood and cord blood were analyzed within a period of two weeks. All slides were analyzed using the same software settings for automated analysis. An important feature contributing to precision and reproducibility of data analysis was the use of a two filter-switch to exclude white blood cells. Stained white blood cells have an absorption near that of stained fetal red cells and exclusion of these cells significantly reduced the total number of automatically detected events in the selection gallery.

A microscopic field containing 1 fetal red cell in the middle is shown in figure 1a (10x objective) and 1b (40x objective). An example of one page of the selection gallery is shown in figure 2. Only true fetal red cells are marked as "fetal" in the selection gallery, the non-fetal cells are marked as "unidentified alarm" (UA).

Preset parameters used for automated differentiation between fetal and adult red cells can be plotted in a histogram. In figure 3 the intensity of staining (x-axis) is plotted against the cell size (y-axis). It illustrates a bimodal distribution of selected cells even without interference of an operator. The larger cell population (blue) mainly consists of fetal red cells, whereas the smaller cell population (grey) represents the adult red cells. After visual evaluation of the selection gallery, this feature can be used for verification of the results.

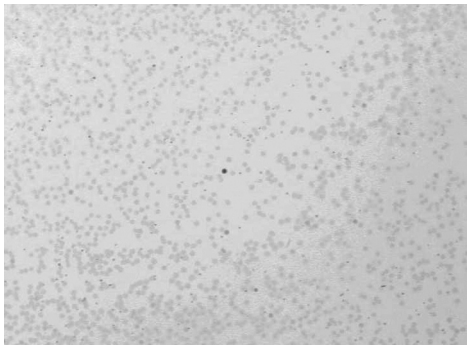

**Figure 1a** - Microscopic field after staining showing 1 fetal red cell (10x objective).

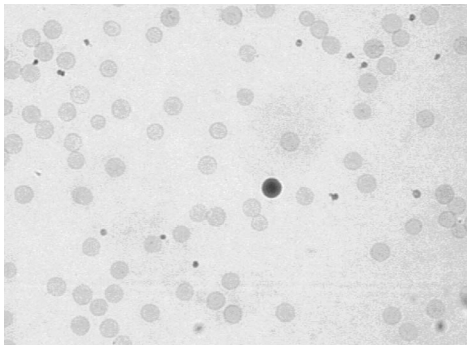

**Figure 1b** - Microscopic field after staining showing 1 fetal red cell (40x objective).

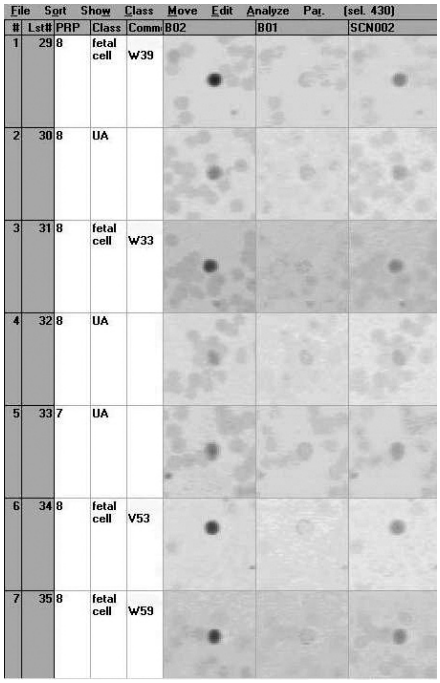


**Figure 2** - Example of a selection gallery containing 4 fetal red cells and 3 adult red cells (indicated as "UA").

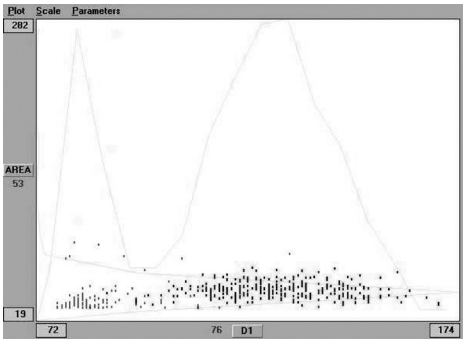


**Figure 3** - Intensity of staining (x-axis) plotted against the cell size (y-axis). Fetal red cells are displayed in grey and adult red cells in black.

**Table 1** - Inter-observer and intra-observer variability: number of detected fetal red cells grouped by dilution and by investigator.

| | Number of fetal red cells detected by automated KBT* | | | |
|---|---|---|---|---|
| Theoretical % of fetal cells | Investigator 1 | Investigator 2 | Absolute difference (inv1-inv2) | Percent** difference (inv1-inv2) |
| 0% | 0 | 0 | 0 | 0 |
| | 0 | 0 | 0 | 0 |
| | 0 | 0 | 0 | 0 |
| | 0 | 0 | 0 | 0 |
| | 0 | 0 | 0 | 0 |
| Mean | 0 | 0 | 0 | 0 |
| ± SD | ±0 | ±0 | | |
| 0.0001% | 3 | 3 | 0 | 0 |
| | 3 | 3 | 0 | 0 |
| | 3 | 3 | 0 | 0 |
| | 3 | 3 | 0 | 0 |
| | 3 | 3 | 0 | 0 |
| Mean | 3 | 3 | 0 | 0 |
| ± SD | ±0 | ±0 | | |
| CV (%) | 0 | 0 | | |
| 0.001% | 36 | 36 | 0 | 0 |
| | 35 | 36 | -1 | 2.8 |
| | 37 | 38 | -1 | 2.7 |
| | 38 | 39 | -1 | 2.6 |
| | 39 | 39 | 0 | 0 |
| Mean | 37 | 37.6 | -0.6 | 1.6 |
| ± SD | ±1.6 | ±1.5 | | |
| CV (%) | 4.27 | 4.03 | | |
| 0.01% | 278 | 277 | 1 | 0.4 |
| | 279 | 280 | -1 | 0.4 |
| | 265 | 264 | 1 | 0.4 |
| | 268 | 268 | 0 | 0 |
| | 286 | 286 | 0 | 0 |
| Mean | 275.2 | 275 | 0.2 | 0.1 |
| ± SD | ±8.6 | ±8.9 | | |
| CV (%) | 3.12 | 3.25 | | |
| 0.1% | 2540 | 2532 | 8 | 0.3 |
| | 2727 | 2711 | 16 | 0.6 |
| | 2667 | 2674 | -7 | 0.3 |
| | 2750 | 2765 | -15 | 0.5 |
| | 2634 | 2640 | -6 | 0.2 |
| Mean | 2663.6 | 2.664.4 | -0.8 | 0.03 |
| ± SD | ±83.1 | ±87.3 | | |
| CV (%) | 3.12 | 3.28 | | |
| 1.0% | 26018 | 26027 | -9 | 0.03 |
| | 25651 | 25638 | 13 | 0.05 |
| | 26347 | 26349 | -2 | 0.01 |
| | 26406 | 26394 | 12 | 0.05 |
| | 26108 | 26145 | 37 | 0.14 |
| Mean | 26.106 | 26110.6 | -4.6 | 0.02 |
| ± SD | ±301.2 | ±303.6 | | |
| CV (%) | 1.15 | 1.16 | | |
| Total no. of detected cells | 145424 | 145453 | 29 | 0.02 |
| Mean of detected cells | 4847 | 4848 | 1 | 0.02 |

*n=5 replicate measurements per dilution reviewed by investigator 1 and 2
**Percent difference = absolute difference/mean(investigator1, investigator2)

### *Study of reliability and validity of automated KBT*

Of each dilution, 5 replicate measurements were performed and stored in a separate selection gallery. Each gallery was reviewed by two investigators, resulting in 10 paired measurements per dilution. These results are shown in table 1.

The absolute and percentage difference between both investigators was calculated. No systematic difference was found between investigator 1 and investigator 2 within each dilution. Percentage differences between both investigators were relatively low and, as expected, even declined with increasing concentrations of fetal red cells. No fetal red cells were detected by either of the investigators when repeated measurements of the negative control sample were performed. Therefore, in further analysis, the data of both investigators were pooled. Manual counting of fetal red cells by two investigators within the same area as used for automated analysis corresponded with the results of automated analysis (data not shown).

For each dilution a mean, a standard deviation (SD), and a CV were calculated from the number of fetal red cells detected by automated KBT (table 2). The CV decreased with increasing concentrations of fetal red cells. A strong correlation was found between theoretical and detected concentrations of fetal red cells by automated microscopy ($r^2$=0.999), shown in figure 4.

### *Correlation of the automated KBT with the standard manual KBT*

The mean percentages of fetal red cells detected by automated microscopy and the manual KBT are given in table 2. In the range from 0.0001% to 0.001% the standard manual KBT underestimated the fetal red cell percentage, whereas the automated KBT was very precise (figure 4). In the range from 0.01% to 1%, the results of both techniques were comparable. The standard manual KBT as well as the automated KBT tended to underestimate the fetal red cell percentage in the higher ranges. Both methods correlated well ($r^2$=0.999).

**Table 2** - Comparison of the standard KBT and the automated KBT: theoretical fetal red cell percentage (%) versus detected fetal red cell percentage (%).

| Theoretical fetal red cell % | Manually detected fetal red cell % | Automatically detected fetal red cell % (CV) |
|---|---|---|
| 0 | 0 | 0 (0) |
| 0.0001 | 0 | 0 (0) |
| 0.001 | 0.000555 | 0.000044 (4.19) |
| 0.01 | 0.00972 | 0.000287 (3.19) |
| 0.1 | 0.0861 | 0.00268 (3.19) |
| 1 | 0.8361 | 0.0109 (1.34) |

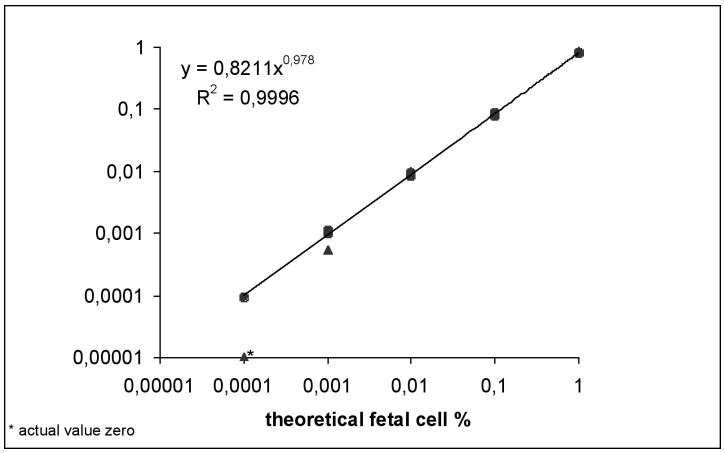


**Figure 4** - Quantification of fetal red cell percentage (%) on serially diluted mixtures of cord blood and adult blood measured by automated KBT (●) and standard manual KBT (▲)

## Discussion

This study shows that automated KBT is able to enumerate fetal red cells varying from very small to large amounts and therefore is suitable for routine clinical use in the assessment of FMH. Repeated measurements demonstrated high reproducibility and very small inter-observer and intra-observer variability (range of CV 0-4%). Compared to a carefully performed manual KBT, good correlation was found between both methods ($r^2$=0.999).

While the standard KBT may be sensitive in detecting fetal red cells, its accuracy in quantifying FMH is inadequate. Some authors report an underestimation of the fetal red cell count, whereas others report an overestimation.[8,11-13] Problems concerning the KBT are caused by lack of consistency in the methods used by various laboratories and subjectivity of the manual KBT resulting in large inter-observer and inter-laboratory variability.[10-12] As a consequence, alternative methods for the assessment of FMH have been pursued.

Changes in maternal serum alpha-fetoprotein in women undergoing chorionic villus sampling, medical abortion or amniocentesis and in uncomplicated pregnancies have been described.[20-24] An increase in maternal serum alpha-fetoprotein is associated with FMH, but its clinical usefulness is limited by the necessity of a pre-hemorrhage sample. Whether a rise in alpha-fetoprotein in maternal serum is proportional to the number of fetal red cells in maternal blood is not exactly known.

Good results in quantifying FMH have been achieved with flow cytometric methods using monoclonal anti-HbF or anti-D in FMH >0.1%.[8,9,12-15] However, in FMH

smaller than 0.1%, flow cytometry is considered inaccurate. The use of monoclonal anti-D is restricted to those cases where the mother is Rh D negative and the fetus is Rh D positive.[12,14] Although antibodies against HbF can be applicable in all cases suspected of FMH, in practice difficulties are observed in differentiating between true fetal red cells and maternal F cells. In comparison to the standard manual KBT and the automated KBT, flow cytometry is superior in speed, but automated microscopy offers the advantage that the image of each detected cell can be stored in memory, for subsequent visual verification, which allows for better detection sensitivity.

A previous study described the use of automated microscopy for detection of fetal red cells in maternal blood.[25,26] Mixtures of Rh D positive and Rh D negative red cells were stained with fluorescent immunobeads. This method demonstrated accurate detection of Rh D positive cells in the range from 0.001% to 1%. In contrast to our method this technique is only applicable in cases of Rh D incompatibility.

Our study was performed to demonstrate that automation of the KBT leads to better performance. However, the analysis procedure has not yet been optimised. For instance, the mean analysis time of one slide was relatively long. By adjusting the software we anticipate a final analysis time comparable to standard manual KBT and suitable for routine clinical use. The final version of the software will also perform an automated count of the total number of background cells in order to calculate the percentage of fetal red cells. In this study we counted manually all adult cells in three different low power microscopic fields and multiplied the average by the number of analysed microscopic fields (i.e. 1517), which inevitably leads to a certain degree of imprecision. Even with fully optimised software, some imprecision in quantifying FMH will remain due to the error in estimation of the total maternal blood volume.[6]

Further, within 5 replicate measurements of each sample, there was a moderate variation of the number of initially selected cells by automated analysis (data not shown). This is due to a varying ratio of selected adult F cells as a consequence of slightly different digitizer settings before each new scan. We preferred high sensitivity in this study in order not to miss any fetal red cells and therefore accepted a lower specificity. Reviewing all automatically selected cells, the adult F cells are recognized and removed from the selection by the operator. As shown in our results, these data are very consistent.

The method described in this paper can be helpful in future research concerning obstetric procedures and pregnancy complications. Particularly in clinical settings where small amounts of FMH occur, e.g. spontaneous antepartum bleeding, ectopic pregnancy, an automated approach can be helpful in quantifying FMH. Although guidelines for administration of anti-D immunoglobulin are available, it may also help in reaching consensus on the administration of the amount of anti-D immunoglobulin, which has not yet been reached.[5]

## Acknowledgments

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# Quantification of fetomaternal hemorrhage: a comparative study of the manual and automated microscopic Kleihauer-Betke tests and flow cytometry in clinical samples

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## Abstract

***Objective:*** Quantification of fetomaternal hemorrhage (FMH) in 44 patients at risk by the manual and automated microscopic analysis of Kleihauer-Betke stained slides and by flow cytometry.

***Methods:*** Blood smears were stained and evaluated manually according to the Kleihauer-Betke test (KBT). The same slides were used for automated microscopy. In addition, flow cytometry using anti-HbF immunostaining was performed.

***Results:*** FMH>0.1% was detected in 4 patients by manual and automated KBT and by flow cytometry. FMH was absent according to all three methods in 13 patients, whereas in 27 patients FMH<0.1% was detected either by manual or automated KBT or both. Moderate agreement was observed between the manual and automated KBT (weighted $\kappa$=0.56, 95% CI 0.33–0.78). Agreement between the manual KBT and flow cytometry was fair (weighted $\kappa$=0.40, 95% CI 0.15–0.66).

***Conclusions:*** Automated microscopic detection of fetal cells in clinical samples provided accurate quantification comparable to the manual KBT, both in small and large FMH. Flow cytometry was only capable of quantifying FMH>0.1%.

## Introduction

After exposure to fetal Rh D positive red cells during pregnancy and delivery, Rh D negative women are at risk of alloimmunization. In subsequent pregnancies, red cell alloimmunization may lead to severe fetal anemia, hydrops fetalis, heart failure and even fetal death. The routine administration of anti-D immunoglobulin to Rh D negative women after delivery of a Rh D positive infant, has decreased the risk of alloimmunization to approximately 2%.[1] Antenatal prophylaxis has reduced the risk even further to 0.2%.[2] However, despite well-organized prophylaxis programs, Rh D alloimmunization continues to occur as a serious complication of pregnancy. Reliable detection and quantification of fetal red cells in maternal blood, therefore, is important for the assessment of fetomaternal hemorrhage (FMH) in Rh D negative patients. In addition, a reliable diagnostic test is needed for Rh D positive patients suspected of FMH. Both in normal and pathological conditions, such as placental abruption or abdominal trauma, significant FMH may occur.[3,4] Also, invasive diagnostic tests and other obstetrical interventions are known for their potential risk of FMH.[5] Even in uncomplicated pregnancy and delivery, small amounts of fetal cells cross the placental barrier and can be detected in maternal blood.[6]

To prevent alloimmunization in Rh D negative patients, an appropriate amount of anti-D immunoglobulin has to be administered.[5] The frequently used standard dose of 300 μg anti-D immunoglobulin is sufficient to clear 15 ml of Rh D positive cells (30 ml of whole blood).[7] When the FMH volume is larger than 15 ml Rh D positive cells, additional doses of anti-D immunoglobulin are required.

The Kleihauer-Betke test (KBT), based on resistance of fetal hemoglobin (HbF) to acid elution, is widely used to determine the FMH volume.[8] Although this method has proven to be clinically useful in the detection of fetal red cells in maternal blood, some studies showed an unacceptable high inter-observer and inter-laboratory variability.[9-12] Over the last ten years several studies reported the use of flow cytometric assays for the quantification of fetal red cells in adult blood using polyclonal antibodies to human D surface antigen and monoclonal antibody to HbF. Flow cytometry (FCM) demonstrated high sensitivity and statistical accuracy in the detection and quantification of substantial FMH.[9,10,12-15] Recently, we developed an automated microscopic approach for the quantification of fetal red cells in artificially spiked adult samples of Kleihauer-Betke stained slides, to improve the accuracy and objectivity of the standard KBT.[16]

In the present study we compared the manual and automated microscopic analysis of Kleihauer-Betke stained slides and FCM using monoclonal anti-HbF in unselected clinical samples of patients at risk of FMH. Our main target was to investigate the quantitative performance of all three methods for FMH ranging from

very small to large volumes. Secondly, our objective was to provide arguments for a more reliable strategy for the administration of anti-D immunoglobulin, based on an accurate technique for the quantification of especially small FMH. For patients with <0.1% FMH, the dose of anti-D immunoglobulin could be adjusted taking a margin of safety into account, thereby reducing both costs and risk of viral transmission.

## Material and methods

### *Samples*

Blood samples from 44 patients, admitted to our obstetrical department and at risk of FMH, were collected in EDTA vacutainer tubes (Becton Dickinson, Ruthford, NJ). FMH detection was indicated either because of Rh D incompatibility or used as a diagnostic tool for the assessment of FMH. Samples were stored at 4 °C and processed within 48 hours of collection. ABO and Rh type matched umbilical cord blood and adult blood from healthy non-pregnant volunteers was collected in EDTA vacutainer tubes for the preparation of control samples. After washing with phosphate-buffered saline (PBS), artificial dilutions consisting of 0%, 0.0001%, 0.001%, 0.01%, 0.1%, 1%, 5% and 10% fetal red cells in adult blood were prepared and analyzed by manual KBT, automated microscopy and FCM.

### *Kleihauer-Betke test*

#### Preparation of the slides

Hundred µl of each sample was mixed with 100 µl PBS. Conventional blood smears were prepared on glass slides using 2.5 µl of this diluted solution. Fixation of the cells and elution of HbA were performed as described previously.[8,17] The slides were air-dried for 30 minutes at room temperature and fixed in 80% ethanol for 5 minutes. After rinsing with tap water, they were placed in citrate buffer at 37 °C for exactly 5 minutes and rinsed again. The citrate buffer consisted of 750 ml 0.1 M $C_6H_8O_7$. $H_2O$ and 250 ml 0.2 M $Na_2HPO_4.2H_2O$; pH 3.20 to 3.30 at 37 °C. The slides were counterstained with Papanicolaou dye (hematoxylin solution, Merck Diagnostica, Darmstadt, Germany) for 3 minutes at room temperature and rinsed again in tap water. Finally, the slides were placed in Sørensen buffer for 5 minutes and stained with erythrosin B (Merck Diagnostica, Darmstadt, Germany), for another 5 minutes at room temperature. The Sørensen buffer consisted of 400 ml 0.067 M $Na_2HPO_4.2H_2O$, 340 ml 0.067 M $KH_2PO_4$ and 260 ml distilled water. The pH of this solution was 6.9 at room temperature. After rinsing with tap water, the slides were air-dried at room temperature.

### Manual evaluation of Kleihauer-Betke stained slides

Fetal red cells were counted in 400 subsequent microscopic fields using a 40x objective.[17] Adult red blood cells, containing small amounts of HbF, were distinguished from fetal red cells by intensity and intracellular distribution of the pink staining. Only bright pink stained cells were classified as fetal. The fetal red cell percentage was calculated from the proportion of the detected number of fetal red cells and the estimated total number of background red cells in 400 fields.

### Automated microscopic analysis of Kleihauer-Betke stained slides[16]

After manual counting, the same slides were embedded in Fluoromount embedding medium (Gurr, BDH Limited Poole, UK) and sealed with a cover glass. An automated microscope (Olympus BX-60), equipped with a scanning stage (Maerzhauser Co, Wetzlar, Germany) and a MDS 1 image analysis system (Applied Imaging Corporation, Santa Clara, CA) was used. At least 500 low power fields (10x objective) were scanned with a two-step filter-switch method to identify fetal red cells by bright field analysis. A green filter was applied to select all pink stained cells and a red filter to identify and exclude the hematoxylin counterstained white blood cells. Optimal software settings for detection and analysis were defined on a set of test samples.

Automatically detected cells were stored in a database and relocated for direct microscopic verification. Only bright pink cells were selected as fetal. The fetal red cell percentage was calculated from the proportion of the detected number of fetal red cells to the estimated total number of background red cells (at least 1,000,000).

## *Flow cytometry*

### Staining protocol

The red blood cell (RBC) count of each patient sample was determined on a Sysmex K-1000 cell counter (Baxter Scientific, Chicago, IL) and adjusted to a final concentration of 2.5 x $10^6$ /μl with PBS. Approximately 2.5 x $10^7$ red cells (10 μl) were fixed in 1 ml freshly prepared, cold 0.05% glutaraldehyde in PBS, thoroughly mixed, and incubated at room temperature for 10 minutes. The samples were washed 3 times using 2 ml PBS supplemented with 1% bovine serum albumin (PBS/1% BSA). The cells were resuspended and permeabilized in 0.5 ml 0.1% Triton X-100 (Sigma, St Louis, MO) in PBS/1% BSA at room temperature for exactly 4 minutes, and then washed with 2 ml PBS/1% BSA. The cell pellet was resuspended in 0.25 ml PBS/1% BSA. Five μl of this suspension was incubated with 5 μl of fluorescein isothiocyanate (FITC)-conjugated anti-HbF antibody (Caltag, Burlingame, CA) and 35 μl PBS/1% BSA in the dark for 15 minutes at room temperature. The cells were washed twice in PBS/1% BSA and resuspended in 0.25 ml PBS/1% BSA. Flow cytometric analysis was performed within 4 hours. Control samples were included with each run.

### Flow cytometric analysis

A FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA) was used for the flow cytometric analysis. Sample acquisition was performed on a minimum of 100,000 cells, with collection of measures for forward scatter (FSC) and side scatter (SSC), log green fluorescence (525-nm band pass filter), and autofluorescence using log orange fluorescence (575-nm band pass filter). A light scatter threshold was set to exclude nonspecific signals from buffer contaminants and platelets. Data analysis was performed with CellQuest (Becton Dickinson, San Jose, CA). The region for analysis of fetal red cells was determined using a positive control sample. The artificial dilutions of cord blood in adult blood were used to fine-tune the flow cytometric assay with respect to gate setting and amplification.

### *Statistical analysis*

Correlation between results from patient and control samples, measured by the manual KBT, automated microscopy and FCM was performed using linear regression and Pearson's correlation coefficient (r) both after log transformation (SPSS 10.0 for windows, SPSS Inc., Chicago, IL). A coefficient of variation for each method was calculated by the performance of 5 replicate determinations of the control samples (Excel2000, Microsoft Inc., Redmond, WA). To assess agreement between methods in patient samples a weighted kappa ($\kappa$) was calculated (SAS, SAS Inc, Cary, NC). The value of $\kappa$ was assigned a degree of agreement as defined by cited literature.[18]

## Results

### *Patient samples*

Blood samples from 44 patients between 25 and 42 weeks of gestation, admitted to the Department of Obstetrics of the Leiden University Medical Center, were analyzed. The medical records of all patients and, when available, pathological reports were reviewed. Patients' characteristics are given in table 1. Substantial FMH, as detected by all three methods, occurred in 4 patients. One patient with obstructed labor underwent a ventouse-assisted delivery and fundal pressure. Another patient had a caesarean section, complicated by a difficult removal of the placenta. The third patient presented with an antepartum fetal death at term. Pathological investigation of the placenta revealed an intra-placental choriocarcinoma. The fourth patient had a placental abruption. A caesarean section was performed immediately. The infant was anemic (hemoglobin 3.0 g/dl) at birth, but recovered after a blood transfusion.

**Table 1 -** FMH grouped by obstetrical diagnosis in 44 patients.

| | | FMH | | |
|---|---|---|---|---|
| Diagnosis | n | 0%* | <0.1%** | >0.1%* |
| abdominal trauma | 9 | 3 | 6 | 0 |
| vaginal bleeding: | | | | |
| marginal placenta previa | 2 | 1 | 1 | 0 |
| preterm labor | 2 | 1 | 1 | 0 |
| unknown | 6 | 4 | 2 | 0 |
| antepartum fetal death: | | | | |
| choriocarcinoma | 1 | 0 | 0 | 1 |
| placental infarct | 1 | 0 | 1 | 0 |
| chorionic vein thrombosis | 1 | 0 | 1 | 0 |
| unknown | 2 | 1 | 1 | 0 |
| complicated delivery (fundal pressure) | 2 | 0 | 1 | 1 |
| manual removal of the placenta | 2 | 0 | 2 | 0 |
| placental abruption | 3 | 0 | 2 | 1 |
| caesarean section: | | | | |
| breech presentation | 3 | 1 | 2 | 0 |
| placenta previa | 2 | 1 | 1 | 0 |
| monochorionic twin pregnancy | 2 | 0 | 1 | 1# |
| dichorionic twin pregnancy | 1 | 0 | 1 | 0 |
| intra-uterine growth restriction | 1 | 0 | 1 | 0 |
| neonatal anemia: | | | | |
| monochorionic twin pregnancy | 1 | 0 | 1 | 0 |
| ventouse assisted delivery | 1 | 0 | 1 | 0 |
| unknown | 2 | 1 | 1 | 0 |
| Total | 44 | 13 | 27 | 4 |

* FMH detected by manual KBT, automated microscopy and flow cytometry

** FMH detected by manual KBT or automated microscopy only

\# complicated by difficult removal of the placenta

### *Distribution of fetal cell percentages between methods*

A comparison of the results from manual KBT, automated microscopy and FCM, divided into the categories 0%, <0.1% and >0.1% FMH, is given in table 2. All three methods were negative in 13 patients. FMH varying from 0.0001% to 0.1% was found in 27 patients either by automated microscopy (n=10) or manual KBT (n=3) or both methods (n=14). In these patients, FCM was unable to distinguish the signal of fetal cells from the background of HbF containing adult cells. In 4 patients FCM and both manual KBT and automated microscopy detected a FMH >0.1%, as shown in table 3. The FMH volume was calculated assuming a red cell volume of 1800 ml in pregnant women.[19] FCM tended to detect slightly higher fetal red cell percentages than the

manual KBT and automated microscopy. In one patient diagnosed with a placental abruption, FCM detected a substantially higher fetal red cell percentage than the manual and automated KBT.

***Agreement and correlation between methods***

The correlation between the expected and detected fetal red cell percentage measured in control samples by manual KBT, automated microscopy and FCM was good (r= 0.99, 0.99 and 0.96). The coefficient of variation for each method studied in 5 replicate determinations was less than 5% in the majority of control samples, except for the 0.0001% dilution the coefficient of variation was 14%. Calculations of agreement between methods in patient samples were based on the results given in table 2. Moderate agreement was observed between automated microscopy and the manual KBT (weighted $\kappa$=0.56, 95% CI 0.33–0.78). A comparison of the fetal red cell percentage measured by the manual KBT and automated microscopy after log transformation is illustrated in figure 1 (r=0.77). Agreement between FCM and the manual KBT was fair (weighted $\kappa$=0.40, 95% CI 0.15–0.66) and correlation (r) was 0.69.

**Table 2 -** A comparison of the results from manual KBT, automated microscopy and flow cytometry in 44 patient samples.

| | automated microscopy | | | flow cytometry | |
|---|---|---|---|---|---|
| manual KBT | 0% | <0.1% | >0.1% | 0%* | >0.1% |
| 0% | 13 | 10 | 0 | 23 | 0 |
| <0.1% | 3 | 14 | 0 | 17 | 0 |
| >0.1% | 0 | 0 | 4 | 0 | 4 |
| total | 16 | 24 | 4 | 40 | 4 |

* the cut-off value for a positive flow cytometric test result was ≥0.1%

**Table 3 -** A comparison of the fetal red cell percentage (%) and the FMH volume (ml) measured by manual KBT, automated microscopy and flow cytometry in 4 patients with substantial FMH.

| | manual KBT | | aut. microscopy | | flow cytometry | |
|---|---|---|---|---|---|---|
| sample | % | (ml)* | % | (ml)* | % | (ml)* |
| No. 1 (complicated delivery) | 0.27 | (4.9) | 0.25 | (4.5) | 0.3 | (5.4) |
| No. 2 (caesarean section) | 4.2 | (76) | 4.0 | (72) | 5.0 | (90) |
| No. 3 (choriocarcinoma) | 7.3 | (131) | 7.7 | (139) | 8.3 | (149) |
| No. 4 (placental abruption) | 7.0 | (126) | 8.0 | (144) | 13.0 | (234) |

* The volume of FMH (ml) was calculated, assuming a maternal red cell volume of 1800 ml

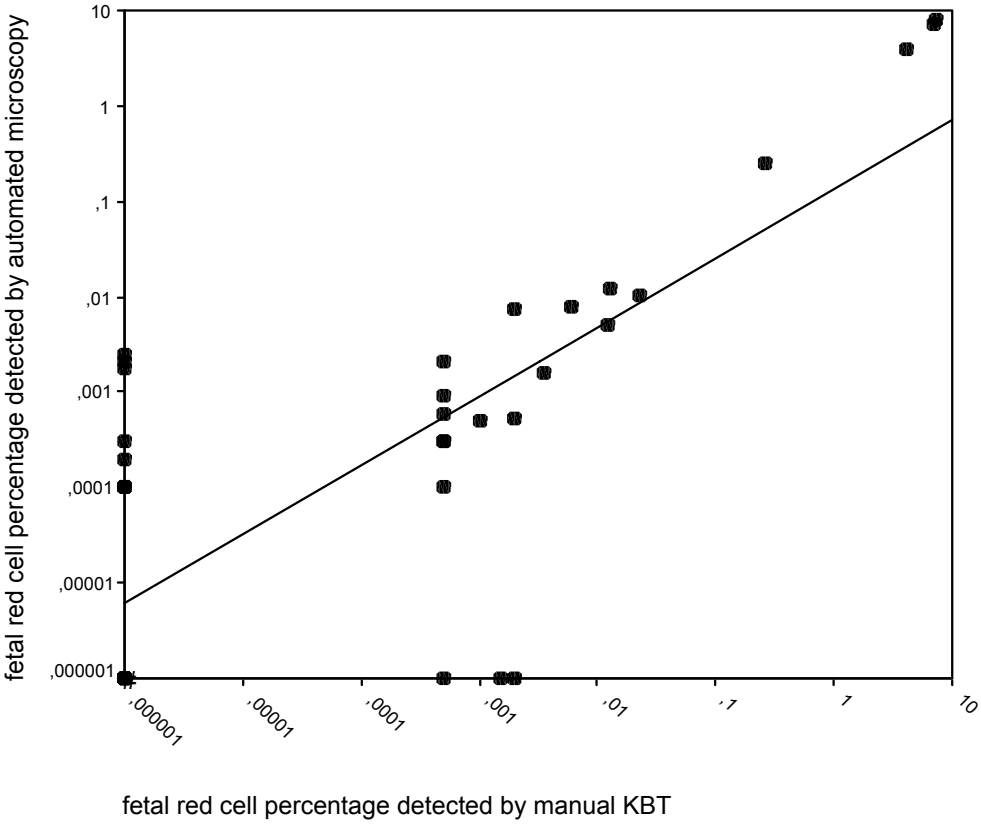


**Figure 1 -** A linearity study of the fetal cell percentage measured by manual KBT and automated microscopy in 44 patient samples (log y= -0.878+0.723log(x), r=0.77). The zero values were transformed to 0.00001% for the purpose of a logarithmic expression (#).

## Discussion

In the present study we quantified FMH in a high-risk patient group by manual and automated microscopic analyses of Kleihauer-Betke stained slides and by FCM. Moderate agreement was found between the manual and automated KBT, due to the differences between these methods in the smaller ranges of FMH. Fair agreement was observed between the manual KBT and FCM, mainly because of the insensitivity of FCM to detect FMH <0.1%.

In the majority of patients a FMH between 0% and 0.1% was detected. Large FMH was diagnosed in 4 high-risk obstetrical cases. In three of these patients with FMH ranging from 0.3 to 8.3% quantification was almost identical with the three methods. In one patient FCM detected a FMH of 13%, while both the manual and

automated KBT resulted in 7%. The implausible high level of FMH, as estimated by FCM, may be explained by a high maternal F cell percentage, which could not be discriminated from the fetal cell population, leading to an overestimation by FCM.

In a recent publication on the relation between third trimester bleeding and the occurrence of FMH >0.01% measured by the KBT, no significant increase of FMH was found compared to non-complicated pregnancies.[20] A FMH >0.01% was found in 4 out of 91 cases with vaginal bleeding. In our study, we detected a FMH between 0.001% and 0.002% by manual and automated KBT in 3 out of 10 pregnancies complicated by vaginal bleeding without signs of placental abruption. Vaginal bleeding due to placental abruption was diagnosed in 3 patients. One of these patients had a substantial FMH and in the other two patients a very small FMH was detected.

Over the past years a large proportion of the hospitals have abandoned the traditional KBT and now use flow cytometric assays for the quantification of FMH. This change is due to presumed unreliability of the KBT, mainly as a consequence of modifications of the test and analysis of an insufficient number of microscopic fields, leading to large inter-observer and inter-laboratory variability.[11,12] Publications on the performance of FCM using anti-HbF or anti-D have shown that FCM is capable of quantifying only FMH ≥0.1% with accuracy.[9,10,12,13,15] The relation between the manual KBT and flow cytometric assays strongly depends on the KBT method used. Both underestimation[9] and overestimation[10,15] of the fetal cell percentage by the KBT are reported. A study on the quantitative performance of the KBT, fluorescence microscopy and FCM with anti-D immunostaining in artificially spiked samples, demonstrated that the KBT is inappropriate in quantifying FMH in the range of 0 to 1% fetal cells, while fluorescence microscopy and FCM were accurate.[21] These findings are in contrast to our study of clinical samples, where FMH <0.1% was detected by the manual and automated KBT and not by FCM.

The reported differences may partially be explained by the fact that statistical precision is related to the number of fields evaluated and the varying thickness of the blood smear. If recommendations are followed and a sufficient number of microscopic fields is counted using a 40x objective, then, in our opinion even inexperienced laboratory staff can perform the KBT.[17]

Recently, we have shown that automated microscopic analysis of Kleihauer-Betke slides demonstrates high reproducibility, very small inter-observer and intra-observer variability and good correlation with the manual KBT.[16] The automated microscopic procedure, as performed in the present study, is capable of detecting both small and substantial FMH. In the very small range of FMH we found fetal red cells in the maternal circulation varying from 0.0001% to 0.1% in 27 patients either by automated microscopy or manual KBT or both.

There is consensus on the importance of diagnosing large FMH in clinically indicated cases such as severe fetal anemia and for the appropriate dosing of anti-D immunoglobulin. Accurate quantification of small FMH is particularly important, considering the fact that there is a dose-dependent relation between the volume of Rh D positive red blood cells to which a Rh D negative person is exposed and the incidence of Rh D alloimmunization, with volumes as small as 0.1 ml or 0.006% red cells resulting in antibody formation.[22] In addition, very small amounts of FMH in pregnancy may evoke sensibilization, which might result in detectable antibody formation in a subsequent pregnancy.[19] Consequently, the FMH volume is an important consideration in the risk of sensitization of Rh D negative women. Therefore, an accurate and standardized method capable of detecting <0.1% fetal red cells in the maternal circulation is needed. To overcome laboratory and observer variability, we have studied the application of an automated readout of manually stained Kleihauer-Betke slides. The software used for the automated analysis was primarily developed to show “proof of principle”.[16] After an automated, highly standardized Kleihauer-Betke staining procedure, algorithms for further fine-tuning of the analysis software may ultimately result in an accurate and highly sensitive procedure.

Despite all efforts, the estimation of the total maternal blood volume, will remain an unreliable factor in calculating the FMH volume. As recommended, a maternal red cell volume of 1800 ml is used for the calculation of the FMH volume. [3,19] However, the true red cell volume in pregnant women depends on biological and pathophysiological factors such as gestational age and the individual hematocrite level.

Although our study contains a small number of cases, the results indicate that the dosage of anti-D immunoglobulin might be further fine-tuned, especially for patients with FMH <0.1%. In our opinion, anti-D immunoglobulin should be restricted to those patients who need it. The administration of a relatively large dose of 300 μg to all Rh D negative patients who are at risk of alloimmunization after sensitizing events, may eventually lead to a future shortage of anti-D immunoglobulin, obtained from volunteers with high circulating antibody levels. In addition, considering cost-effectiveness and the fact that anti-D immunoglobulin is a blood product with a small potential risk of viral transmission (e.g. prion disease), one could discuss another strategy. With the application of a method more reliable in quantifying smaller volumes of FMH, the anti-D immunoglobulin dose could be adjusted to the detected FMH percentage including a margin of safety. The automated analysis of Kleihauer-Betke stained slides, which is not influenced by individual observers, is the preferable procedure for the quantification of FMH.

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# Fetomaternal hemorrhage in relation to chorionic villus sampling revisited

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*(Prenat Diagn, in press)*

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## Abstract

***Objective:*** To investigate whether chorionic villus sampling (CVS) results in a proportional increase of alpha-fetoprotein (AFP) and fetal red cells in maternal blood.

***Methods:*** Blood samples were collected before and after CVS. The AFP concentration was measured and supervised automated microscopy of Kleihauer-Betke slides was applied to quantify fetal red cells.

***Results:*** AFP analysis was performed in 53 paired samples and automated microscopic scanning in 59 paired samples. Median AFP concentrations before and after CVS were 12.0 μg/l (range 6.4–36.4) and 18.7 μg/l (range 8.2–668.9), respectively, indicating a significant increase ($p<0.0001$). Median numbers of fetal red cells detected before and after CVS were 0 (range 0–36) and 0 (range 0–31), respectively. No significant increase of fetal red cells was observed ($p=0.72$). The delta ($\Delta$) fetal red cells and the $\Delta$ AFP correlated poorly ($\rho=-0.22$, $p=0.11$). The amount of villi correlated moderately with the $\Delta$ AFP ($\rho=0.32$, $p=0.02$) and did not correlate with the $\Delta$ fetal red cells ($\rho=-0.11$, $p=0.43$).

***Conclusions:*** Although the AFP concentration after CVS was increased, no increase of fetal red cells was detected. These findings suggest that CVS results in a leakage of proteins due to placental tissue damage, rather than increased trafficking of fetal red cells.

## Introduction

Chorionic villus sampling (CVS), an invasive intrauterine procedure for first trimester prenatal diagnosis, may induce fetomaternal hemorrhage (FMH), leading to complications such as red cell and platelet immunization.[1-6] Even very small volumes of Rh positive blood may result in antibody formation in Rh negative individuals.[7] Since reliable quantification of small numbers of fetal red cells in the maternal circulation is difficult, FMH after CVS has been studied predominantly by measurement of the alpha-fetoprotein (AFP) concentration.[2-4,8-11]

AFP is a major serum glycoprotein synthesized during fetal life mainly by the yolk sac and the fetal liver. In the human fetus the concentration of AFP peaks at 13 weeks of gestation (3-4 mg/ml), falls to about 50 μg/ml at term and disappears after birth.[12]

Elevated levels of AFP in maternal blood during pregnancy are associated with fetal malformations, such as neural tube defects and with placental tissue damage. A significant increase of the AFP concentration after CVS has been reported in previous studies.[2-4,9-11] A positive correlation between the amount of villi removed and the AFP concentration increase was found in the majority of these studies.[2-4,9] In addition, the number of attempts was related to the magnitude of the AFP increase.[12] Although not proven, it is generally assumed that fetal red cells and AFP are being transferred into maternal blood in proportion. As CVS is performed by biopsy of villi, which are bathed in maternal blood, it is conceivable that fetal whole blood is being transferred and not only plasma. In the absence of a sensitive method to quantify very small numbers of fetal red cells, this has not been confirmed to date.

Recently, we developed an automated microscopic scanning procedure of Kleihauer-Betke stained slides capable of detecting one fetal red cell per 1,000,000 adult red cells.[13] We applied this technique to blood samples from patients before and after CVS and compared the results with the AFP concentration in the same samples. The aim of this study was to determine whether CVS results in a transplacental passage of proportional amounts of AFP and fetal red cells.

## Materials and methods

### *Patient samples*

In the period from September 2000 to November 2001 venous blood samples from 59 patients were collected before and after CVS. All patients gave their written informed consent in accordance with the Medical Ethical Review Board of our hospital. Patients were randomly included in the study without any selection. The

**Table 1** - Patient and procedure related characteristics

| | | mean (range) |
|---|---|---|
| maternal age (years) | | 38 (31 – 45) |
| gestational age (weeks) | | 11 4/7 (11 – 13) |
| amount of villi aspirated (mg) | | 27 (10 – 60) |
| | | n |
| mode | transcervical CVS | 56 |
| | transabdominal CVS | 3 |
| instrument | biopsy forceps | 53 |
| | canula | 3 |
| | aspiration needle | 3 |
| number of passes | 1 pass | 50 |
| | 2 passes | 9 |
| fetal karyotype | normal 46,XX | 31 |
| | normal 46,XY | 28 |

patient and procedure related characteristics are given in table 1. All pregnancies were cytogenetically normal. The biopsy forceps (De Elles Instruments, Surrey, UK) and the canula (Cook Instruments, Spencer, IN) were used for transcervical CVS. An aspiration needle (TSK Supra Access needle 0.90x120 mm, TSK Laboratory, Dublin, Ireland) was used for abdominal sampling. The first maternal blood sample was drawn 15 minutes before and the second 15-20 minutes after CVS. Blood samples were collected in vacutainer tubes containing ethylene diamine tetra-acetic acid (EDTA) and were processed within 3 hours of collection.

### *Alpha-fetoprotein measurement*

Two ml of each sample was centrifuged at 1200 *g* for 10 minutes. Plasma (0.5 – 1.0 ml) was collected and stored at -80 °C until further processing. After sample collection was completed, the AFP measurements were performed in a single run using a Chemiluminescent Microparticle Immuno Assay (Architect I-2000, Abbott, Hoofddorp, the Netherlands). The AFP concentration was expressed in μg/l.

### *Automated microscopic scanning of Kleihauer-Betke stained slides*

#### Preparation and staining of the slides

100 μl of each patient sample was mixed with 100 μl phosphate-buffered saline (PBS). Two blood smears of each sample were prepared on glass slides using 2.5 μl of the diluted solution per slide. The slides were air-dried for 30 minutes at room temperature and fixed in 80% ethanol for 5 minutes. After rinsing with tap water, they were placed in citrate buffer at 37 °C for exactly 5 minutes and rinsed

again. The citrate buffer consisted of 750 ml 0.1 M $C_6H_8O_7.H_2O$ and 250 ml 0.2 M $Na_2HPO_4.2H_2O$; pH 3.20 to 3.30 at 37 °C. The slides were counterstained with Papanicolaou dye (hematoxylin solution, Merck Diagnostica, Darmstadt, Germany) for 3 minutes at room temperature and rinsed again in tap water. Finally, the slides were placed in Sørensen buffer for 5 minutes and stained with erythrosine B (Merck Diagnostica, Darmstadt, Germany), for another 5 minutes at room temperature. The Sørensen buffer consisted of 400 ml 0.067 M $Na_2HPO_4.2H_2O$, 340 ml 0.067 M $KH_2PO_4$ and 260 ml distilled water. The pH of this solution is 6.9 at room temperature. After rinsing with tap water, the slides were air-dried at room temperature. For microscopic evaluation, the slides were embedded in Fluoromount embedding medium (Gurr, BDH, Poole, UK) and sealed with a cover glass.

### Automated microscopic analysis

An extended description of the automated scanning of Kleihauer-Betke stained slides has been published previously.[13] An automated microscope (Olympus BX-60), equipped with a scanning stage (Maerzhauser Co, Wetzlar, Germany) and a MDS 1 image analysis system (Applied Imaging Corporation, Santa Clara, CA) was used. A two-step filter-switch method was applied to identify fetal red cells by bright field analysis. A green filter was used to select all pink stained cells and a red filter to identify and exclude the hematoxylin counterstained white blood cells. Optimal software settings for detection and analysis were defined on a set of test samples.

Automatically detected cells were stored in a database and relocated for direct microscopic verification. Only bright pink cells were selected as fetal. An area of 561 microscopic fields (10x objective) per slide was scanned automatically. Two slides per patient sample were analyzed (1122 fields), containing approximately $2x10^6$ red cells in total. The slides were evaluated in a blinded fashion.

### *Statistical analysis*

The Wilcoxon signed ranks test (SPSS 10.0 for windows, SPSS Inc., Chicago, IL) was used to compare the median of observed values. Spearman's correlation coefficient ($\rho$) was used for correlation study (SPSS 10.0 for windows). The significance level was set at $p<0.05$. Data are displayed as line charts (Excel2000, Microsoft Inc., Redmond, WA).

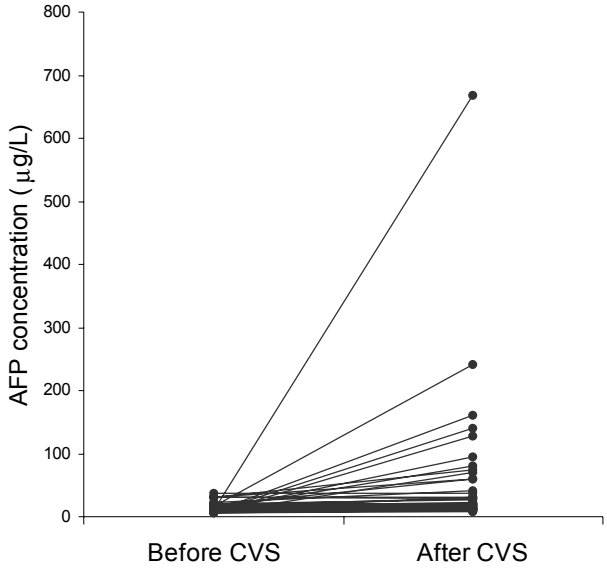


**Figure 1a -** AFP concentration in maternal blood before and after CVS in 53 patients

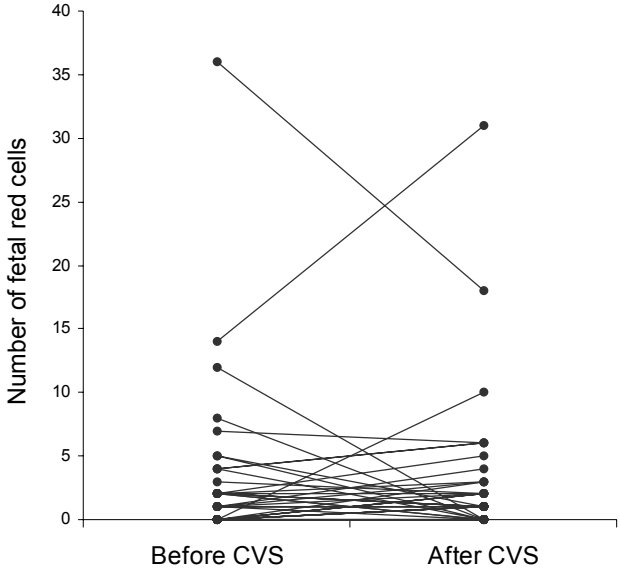


**Figure 1b -** Number of fetal red cells in maternal blood before and after CVS in 59 patients

**Table 2 -** The increase of AFP concentration and fetal red cells in maternal blood

| | patients (n=53) | |
|---|---|---|
| Δ AFP concentration (μg/l) | n | % |
| ≤ 0 | 8 | 15.0 |
| 1 – 10 | 30 | 56.6 |
| 10 – 50 | 7 | 13.2 |
| 50 – 100 | 3 | 5.7 |
| 100 – 200 | 3 | 5.7 |
| 200 – 500 | 1 | 1.9 |
| > 500 | 1 | 1.9 |
| | patients (n=59) | |
| Δ fetal red cells (n) | n | % |
| ≤ 0 cells | 41 | 69.5 |
| 1 – 5 cells | 16 | 27.1 |
| 5 – 9 cells | 0 | 0.0 |
| 10 – 20 cells | 2 | 3.4 |

## Results

AFP concentration was measured in 53 patients, 6 patients were excluded due to an insufficient sample volume. Paired slides were available from all 59 patients. The median AFP concentrations before and after CVS were 12.0 μg/l (range 6.4–36.4) and 18.7 μg/l (range 8.2–668.9), respectively, indicating a significant increase ($p< 0.0001$; figure 1a). The median numbers of fetal red cells detected before and after CVS were 0 (range 0–36), and 0 (range 0–31), respectively. No significant increase of fetal red cells was observed ($p=0.72$; figure 1b). The observed delta (Δ) AFP and Δ fetal red cell values grouped by level of increase (%) are given in table 2. The median Δ AFP concentration was 3.8 μg/l (range -0.9–657.9, mean 33.5, 95% CI [6.4–60.6]). The median Δ fetal red cells was 0 (range -18–17, mean -0.1, 95% CI [-1.2–1.0]). The correlation between the Δ fetal red cells and the Δ AFP was poor ($\rho=-0.22$, $p=0.11$). The amount of villi aspirated (mg) correlated moderately with the Δ AFP ($\rho=0.32$, $p=0.02$) and did not correlate with the Δ fetal red cells ($\rho=-0.11$, $p=0.43$). Both Δ AFP and Δ fetal red cells did not correlate with the number of passes ($\rho=-0.11$, $p=0.45$ and $\rho=0.03$, $p=0.83$, respectively).

## Discussion

Our results indicate that in first trimester CVS fetal red cells and AFP do not cross the placental barrier in proportion. With the application of an automated read-out of Kleihauer-Betke stained slides, which provides better sensitivity and statistical accuracy as many more cells are evaluated compared to the standard Kleihauer-Betke test, we were able to quantify red cell FMH following CVS with improved accuracy.[13] Due to the evaluation of low numbers of microscopic fields, the standard Kleihauer-Betke test is generally not capable of detecting fetal red cells in maternal blood in the range of 0.0001% to 0.001%. If fetal red cells enter the maternal circulation after CVS, it is most likely within this range. Particularly in clinical settings in which extremely small volumes of FMH may occur, an automated microscopic approach allowing the analysis of large numbers of cells for the detection of low frequencies of cells can be helpful. This technique developed on artificially spiked samples of fetal red cells in adult blood, demonstrated high reproducibility and precision with coefficients of variations $\leq 4\%$ and strong correlation between the theoretical and detected fetal red cell percentages.[13] In the present study, we preferred high sensitivity and therefore accepted a somewhat lower specificity. Software settings for detection and analysis were adjusted in order not to miss any fetal red cells. The positive cells found in the pre-CVS samples are most likely maternal F cells.

The significant increase of the AFP concentration found in the present study is in agreement with published reports.[2-4,9-11] Although we measured AFP in plasma as opposed to others measuring AFP concentrations in serum, our $\Delta$ AFP levels were comparable. The results of our study are concordant with a previous study on the quantification of FMH in relation to CVS in which increased AFP levels were found after CVS in the majority of the cases, while all Kleihauer-Betke tests were negative.[11] Thus, it may be suggested that the AFP rise in maternal blood is partially derived from another source. Since cytotrophoblasts are known to synthesize AFP early in pregnancy,[14] it is conceivable that the increase is caused by release of AFP into maternal blood due to destruction of cytotrophoblasts. The positive correlation between AFP and the amount of villi aspirated, also described by others,[2,4] would further support this theory. Although in our study no correlation between the number of passes and the $\Delta$ AFP or $\Delta$ fetal red cells was observed, possibly due to the small number of patients requiring multiple passes (n=9), this was described previously.[2,11] An alternative explanation of an increased AFP level without fetal red cell increase would be the possibility that when villi are broken by CVS, and red cells presumably spill into the maternal lacunae bathing the villi in vivo, most of the fetal red cells do not find their way into the maternal circulation. They are perhaps caught up in a

**Table 3 -** Results from the AFP analysis versus automated microscopy in 53 patients grouped by FMH volume

| | Δ AFP (µg/l) | | | |
|---|---|---|---|---|
| Δ fetal red cells | <4.7 µg/l (<10µl*) | 4.7-47 µg/l (10-100 µl*) | >47 µg/l (>100 µl*) | total |
| **<4 cells (<10 µl*)** | 29 | 14 | 8 | 51 |
| **4-40 cells (10-100 µl*)** | 1 | 1 | 0 | 2 |
| **>40 cells (>100 µl*)** | 0 | 0 | 0 | 0 |
| **total** | 30 | 15 | 8 | 53 |

* calculated FMH volume based on a maternal plasma volume of 3000 ml and a maternal red cell volume of 1500 ml

clot formation local to the site of spillage. Both explanations regarding a significant increase of AFP without fetal red cell increase after CVS place the use of AFP as a marker of FMH in doubt.

Given the lack of reliable clinical data, the issue of maternal antibody formation directly in relation to first trimester CVS is difficult to address. Although it is not exactly known how many fetal red cells are required to cause a primary response in Rh negative individuals, volumes as small as 100 µl of whole blood, but possibly even 10 µl, may result in antibody formation.[7] Based on our data we calculated the corresponding fetal whole blood volume crossing the intervillous space to the maternal circulation for both Δ AFP and Δ fetal red cells (table 3). We assumed that the fetal serum AFP concentration at 11 weeks of gestation is approximately 2 mg/ml, that the average fetal hematocrit is 30%, that the average maternal plasma volume is 3000 ml and that the average maternal red cell volume is 1500 ml.[2] The maternal plasma and red cell volume was calculated from a plasma volume of 2500 ml and a red cell volume of 1500 ml in non pregnant women with a body weight of 60-65 kg and an average increase of the plasma and red cell volume at 11 weeks of gestation of 18-20% and 1-2%, respectively.[15,16] The Δ AFP found ranged from -0.9 to 657.9 µg/l (median 3.8 µg/l), corresponding to -1.9 to 1409 µl of fetal whole blood (median 8.1 µl). In 8 of 53 patients the Δ AFP found was more than 47 µg/l, corresponding to a fetal whole blood volume of more than 100 µl. However, the Δ fetal red cells in these 8 patients ranged from -18 to 0 cells, indicating no fetal red cell transfusion after CVS. Considering a cut-off level of 10 µl fetal whole blood, or a Δ AFP of 4.7 µg/l, 23 patients would be at risk for sensitization. In these patients we detected a Δ fetal red cells ranging from -18 to 10 cells, which corresponds with volumes ranging from -45 to 25 µl fetal whole blood, when assuming a maternal red cell volume of 1500 ml. Overall, the Δ AFP was very small (≤ 10 µg/l) in 72% of the patients (table 2). In one

patient only, we found a Δ AFP (40 μg/l = 86 μl) and a Δ fetal red cells (10 cells = 25 μl) both within the range of 10-100 μl FMH. The largest Δ fetal red cells (17 cells = 45 μl) was found in combination with a very small Δ AFP (1.5 μg/l = 3.2 μl) in one patient and the largest Δ AFP (657.9 μg/l) without increase of fetal red cells was found in another patient. All 3 patients underwent an uncomplicated transcervical CVS by biopsy forceps and their pregnancies were uneventful.

Although our study group is relatively small with the majority of the patients undergoing an uncomplicated transcervical CVS performed by biopsy forceps, we found significantly higher AFP levels after CVS without increase of fetal red cells.

These observations confirm our hypothesis that the AFP rise is discordant with red cell FMH. In view of our findings, the risk of maternal immunization to a fetal red cell antigen, following CVS is extremely small, but not absent. Therefore, a dose of 50 μg anti-D immune globulin remains recommended to prevent immunization in Rh D negative patients.

## Acknowledgements

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# Fetomaternal hemorrhage in women undergoing Cesarean section

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## Abstract

***Objective:*** To investigate whether women undergoing Cesarean section (CS) are at risk for fetomaternal hemorrhage (FMH).

***Methods:*** In this prospective cohort study venous blood samples of 57 women were collected before and 15-20 minutes after CS by phlebotomy. Fifty women had a planned CS for various indications and 7 underwent an emergency CS because of fetal distress. The Kleihauer-Betke technique used to quantify fetal RBCs in maternal blood and measurement of the alpha-fetoprotein (AFP) concentration was performed on all pre- and post-CS samples. FMH was defined as the proportion of fetal and maternal RBCs multiplied by the assumed maternal RBC volume of 1750 ml.

***Results:*** A significant increase of fetal RBCs after CS ($p=0.036$) was found, while the AFP concentration was decreased ($p=0.028$). The correlation between the $\Delta$ fetal RBCs and $\Delta$ AFP was poor ($r^2=0.244$). A FMH <0.1 ml was found in 71.9% of the women. In 28.1% the FMH volume ranged from 0.1 to 4.8 ml. No specific risk factors for FMH were found.

***Conclusions:*** After CS fetal RBC numbers in maternal blood were increased, indicating that the procedure itself resulted in a small but detectable FMH volume in an unselected population undergoing CS. The AFP decrease was most likely explained by intravenous fluid supply in combination with per-operative blood loss resulting in dilution of maternal plasma.

## Introduction

Fetomaternal hemorrhage (FMH) may occur following obstetrical procedures, potentially leading to complications such as red cell and platelet immunization. The routine postpartum administration of anti-D immunoglobulin to Rh D negative women after delivery of a Rh D positive child has decreased the incidence of red cell immunization to approximately 2%.[1] Antenatal prophylaxis has reduced the risk even further to 0.2%.[2] Despite well-organized prophylaxis programs, Rh D immunization remains a matter of clinical concern, with many cases worldwide. One of the reasons for the continuity of this problem is failure to recognize clinical events that place women at risk for immunization and failure to administer the appropriate amount of anti-D immunoglobulin.[3] The standard dose of 300 μg anti-D immunoglobulin is sufficient to clear 30 ml Rh D positive whole blood (or 15 ml of Rh D positive RBCs).[4] When the FMH volume is larger than 30 ml, additional doses of anti-D immunoglobulin are required.

Clinical guidelines are provided for the appropriate and efficient management of women at risk in order to further decrease the incidence of Rh D immunization. Assessment of the FMH volume is recommended for all Rh D negative women at risk.[3,5-8] The Kleihauer-Betke test (KBT), based on resistance of fetal Hb (HbF) to acid elution, is widely used for this purpose.[9-11]

Normally, relatively few fetal cells are transferred into the maternal circulation, but any clinical situation that causes breakdown of the placental barrier will result in an increased transfusion of fetal cells. Although large FMH occasionally occurs after normal vaginal deliveries, traumatic deliveries such as Cesarean section (CS) are more likely to be associated with large FMH.[7,12,13]

Our aim was to assess whether CS contributes to the transplacental passage of fetal RBCs and alpha-fetoprotein (AFP) into the maternal blood in an unselected population. In addition, we tried to identify possible risk factors for FMH within this group of pregnant women.

## Material and methods

### *Patient samples*

In the period from May 2002 to August 2003 venous blood samples from 57 pregnant women undergoing CS at our obstetrical department were collected. The first maternal blood sample was drawn 1 hour before and the second 15-20 minutes after CS both by phlebotomy. Blood samples were collected in vacutainer tubes containing ethylene diamine tetra-acetic acid (EDTA) and were processed within 3 hours of

collection. All samples were analyzed for their content of AFP and fetal RBCs. All women gave written informed consent. The Medical Ethical Review Board of the Leiden University Medical Center approved the protocol (P01.016) for collecting maternal blood samples for research purposes. The medical records of all women were reviewed. The pregnancy and procedure related characteristics are given in table 1.

**Table 1 -** Pregnancy and procedure related characteristics of 57 patients

| characteristics | median (range) |
|---|---|
| maternal age (years) | 32 (21 – 42) |
| gestational age (weeks) | 38 2/7 (29 – 41) |
| birth weight (g) | 3250 (1035 – 4975) |
| placental weight (g) | 545 (250 – 980) |
| | patients (n) |
| rhesus positive | 44 |
| rhesus negative | 13 |
| spinal analgesia | 42 |
| combined spinal/epidural analgesia | 14 |
| epidural analgesia | 1 |

***Quantification of fetal RBCs***

The Kleihauer-Betke test (KBT) was used to quantify fetal RBCs in maternal blood. Of each sample 100 μl was mixed with 100 μl phosphate-buffered saline (PBS). Conventional blood smears were prepared on glass slides using 2.5 μl of this diluted solution. Fixation of the cells and elution of HbA were performed as described previously.[9,14] Fetal RBCs were counted in 400 subsequent microscopic fields using a 40x objective. The total number of maternal RBCs in 400 microscopic fields was estimated by counting the number of background cells in 5 different fields. All slides were evaluated in a blinded fashion. Negative and positive control samples consisting of spiked samples of fetal RBCs in adult RBCs were included.

The fetal RBC numbers detected before and after CS were compared. The FMH (fetal RBC) volume was calculated using the following formulas and by assuming a maternal RBC volume of 1750 ml:[15]

(1) delta (Δ) fetal RBCs = post-CS value – pre-CS value

(2) fetal RBC percentage (%) =
Δ fetal RBCs / number of maternal RBCs x 100

(3) fetal RBC volume (ml) =
fetal RBC % x maternal RBC volume (ml)

### *Alpha-fetoprotein analysis*

Two ml of each sample was centrifuged at 1200 *g* for 10 minutes. Plasma (0.5-1.0 ml) was collected and stored at -80° C until further processing. After sample collection was completed, the AFP measurements were performed in a single run using a Chemiluminescent Microparticle Immuno Assay (Architect I-2000, Abbott, Hoofddorp, The Netherlands). The AFP concentration was expressed in μg/l. The AFP concentrations in the pre- and post-CS samples were compared and the Δ AFP was computed.

### *Statistical analysis*

The student's *t* test and the paired samples *t* test (SPSS 10.0 for windows, SPSS Inc., Chicago, IL) were used to compare means. The Pearson's correlation coefficient ($r^2$) was used for correlation study (SPSS 10.0 for windows). The significance level was set at $p<0.05$. Data are displayed as line charts (Excel2000, Microsoft Inc., Redmond, WA).

## Results

The fetal RBC count and the AFP concentration before and after CS of all women were measured (figure 1 and 2). The mean fetal RBC count detected before and after CS was 6.1 cells / 400 microscopic fields (range 0–130, 95% CI [0.9, 11.34]) and 27.9 cells / 400 microscopic fields (range 0 – 540, 95% CI [7.0, 48.8]), respectively. This increase is significant (p=0.036). The mean Δ fetal RBCs was 21.8 cells / 400 microscopic fields (range -8 – 533, 95% CI [1.5, 42.1]). The mean AFP concentration before and after CS was 144.5 μg/l (range 13 – 542, 95% CI [116.8, 172.2]) and 132.1 μg/l (range 12 – 419, 95% CI [108.1, 156.0]), respectively. A significant decrease of the AFP concentration was observed after CS (p=0.028). The mean Δ AFP concentration was -12.4 μg/l (range -123 – 144, 95% CI [-23.4, -1.4]). The correlation between the Δ fetal RBCs and the Δ AFP was poor ($r^2$=0.244). When comparing a number of subgroups, the Δ fetal RBCs and the Δ AFP concentrations were not significantly different between groups (table 2).

From the Δ fetal RBCs we calculated the transfused fetal RBC volume. In 41 women (71.9%) a FMH volume <0.1 ml was detected. In 15 women (26.3%) the FMH volume ranged from 0.1 to 2.0 ml. In 1 woman (1.8%) the FMH volume was 4.8 ml. Of these 16 women with a FMH >0.1 ml, 2 were carrying a dichorionic twin pregnancy, 1 had a placenta previa and the remaining 13 underwent an elective CS without complicating factors. In 2 women in whom the removal of the placenta was difficult the FMH volume was <0.1 ml.

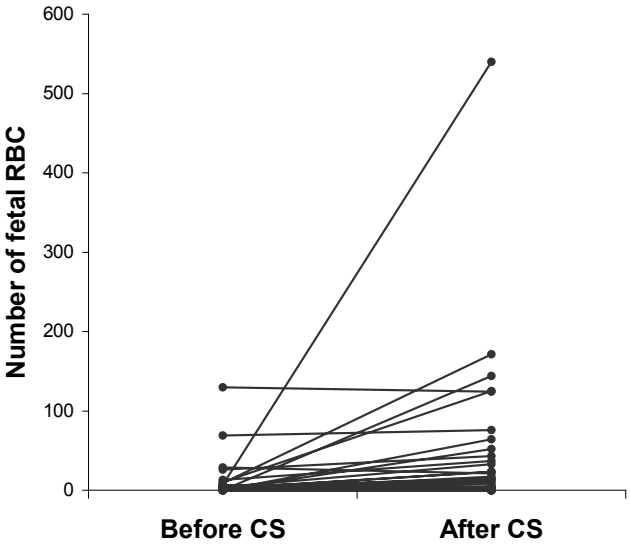


**Figure 1 -** The number of fetal RBCs before and after CS in maternal blood

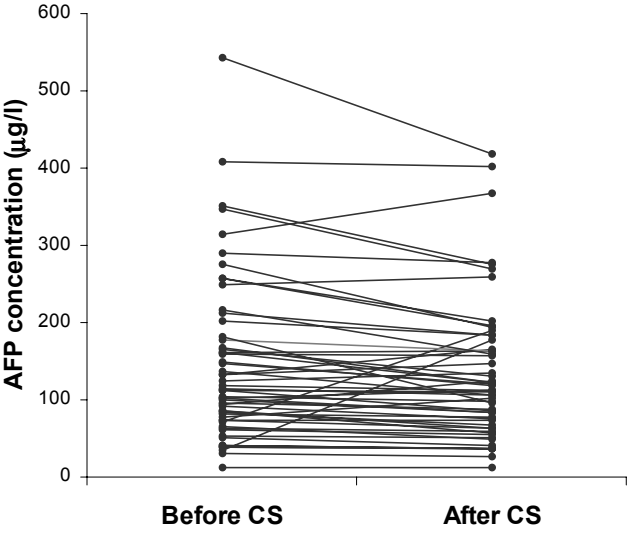


**Figure 2 -** The AFP concentration before and after CS in maternal blood

**Table 2 -** Comparison of the Δ fetal red cells and the Δ AFP concentration for different subgroups by univariate analysis

| clinical characteristics | cases (n) | Δ AFP (μg/l)* | p† | Δ fetal red cells* | p† |
|---|---|---|---|---|---|
| related to pregnancy | | | | | |
| nulliparous | 27 | -2.5 (-87-144) | 0.087 | 34.4 (-8-533) | 0.265 |
| multiparous | 30 | -21.3 (-123-34) | | 10.4 (-4-144) | |
| singleton pregnancy | 53 | -12.0 (-87-118) | 0.921 | 22.7 (-8-533) | 0.753 |
| twin pregnancy‡ | 4 | -18.3 (-123-144) | | 10.0 (1-21) | |
| vertex presentation | 28 | -16.7 (-123-53) | 0.446 | 16.5 (-8-162) | 0.616 |
| breech presentation | 29 | -8.2 (-75-144) | | 26.8 (-6-533) | |
| related to procedure | | | | | |
| planned CS | 50 | -10.4 (-87-144) | 0.733 | 23.3 (-6-533) | 0.587 |
| emergency CS | 7 | -5.5 (-123-32) | | 1.5 (-8-1) | |
| first time CS | 53 | -12.9 (-123-144) | 0.49 | 1.0 (-8-533) | 0.71 |
| repeat CS | 4 | -7.0 (-19-11) | | 0 (0-6) | |
| controlled cord traction | 37 | -13.1 (-123-118) | 0.858 | 26.5 (-8-533) | 0.529 |
| manual removal of placenta | 20 | -11.1 (-82-144) | | 13.0 (-6-162) | |

* expressed as mean (range)
† student's *t* test
‡ twin pregnancies grouped by presentation of the first twin

## Discussion

In the present study FMH was quantified in pregnant women before and after CS by the KBT and the measurement of the AFP concentration. We found a small but detectable FMH volume in the majority of the women after CS. Conversely, the AFP concentration decreased significantly after CS. This is most likely due to the administration of intravenous fluids during and after the operation to all women with spinal, epidural or combined spinal/epidural analgesia resulting in an artificial dilution of maternal plasma, given the fact that the maternal hematocrit decreased significantly after CS (data not shown). The fetal RBC percentage, which was calculated from the proportion of fetal and maternal red cells, is not affected by dilution. Subgroup analysis to identify possible risk factors for the occurrence of FMH, e.g. delivery mode of the placenta or fetal presentation, revealed no significant differences between groups, which is in agreement with previous studies.[13,16]

In addition to these results, we retrospectively reviewed the post-CS KBT results of 113 women after CS in whom FMH assessment postpartum was indicated for Rh incompatibility over the past 5 years. Ninety-three women (82.3%) had no detectable

FMH and in 18 (15.9%) FMH ranged from 0.1 to 6.5 ml. In 2 women (1.8%) the FMH volume was larger than 30 ml.

Feldmann et al. quantified the FMH volume using the KBT in 199 women with various indications for CS with the majority of women (81.4%) having no detectable FMH.[12] In 16.1% FMH ranged from 1-30 ml and 2.5% had a FMH of more than 30 ml, thus requiring additional doses of anti-D immunoglobulin. However, in 3 of these 5 women the indication for CS (e.g. fetal distress, eclampsia and abruptio placentae) rather than the procedure itself may have contributed to the large FMH. In another study on patients at high risk for FMH with 179 patients undergoing CS comparable results were found.[13] The FMH volume was quantified using an enzyme-linked anti-globulin test. In 12.3% FMH was detectable with 3 women (1.7%) having a FMH volume of more than 30 ml. A recent study reported a much higher incidence of FMH following CS compared to previous studies.[17] In 313 women undergoing CS a FMH volume of more than 30 ml in 6.4% was found in this study.

In contrast to these studies the FMH volume in the present study is the transfused fetal RBC volume derived from the difference between the pre- and post-CS values.[12,13,17] The FMH volumes calculated in these cited studies represent the fetal whole blood volumes and are calculated solely from post-CS values. It is well known that during pregnancy the frequency of fetal RBCs in the maternal circulation increases and that in the third trimester even before the onset of labor 45% of the population have circulating fetal cells.[18] Therefore, we calculated the FMH volume present in maternal blood before CS. Fifty-two women had <0.1 ml FMH before CS. Four women had a FMH within the range of 0.1-0.6 ml. In 3 of them external cephalic version had been attempted but failed, and in 1 patient with preeclampsia and intra-uterine growth restriction the pre-CS sample was drawn during labor, which was complicated by fetal distress. The highest pre-CS value detected was 1.2 ml in a woman with myoma, breech presentation and a non-reassuring fetal heart rate pattern.

When comparing FMH volumes from different studies one has to take into account the variables used to calculate the amount of fetal RBCs transfused. It is important to ensure which formula was used to calculate the FMH volume. Dependent on whether a maternal RBC of 1800 ml or whole blood volume of 5000 ml was assumed with adjustment for the difference between the fetal and maternal Hct, the reported FMH volume represents the transfused fetal RBC volume or the fetal whole blood volume. Calculation of the FMH volume is based on a number of assumptions and may therefore slightly differ from the individual FMH volume, but more importantly misinterpretation of the formula sometimes leads to incorrect FMH volumes and therefore may result in an inadequate administration of anti-D immunoglobulin in Rh D negative individuals.

Approximately 50% of the women delivering an ABO-compatible infant have demonstrable circulating fetal RBCs in their circulation.[19] Potential risk factors for the occurrence of FMH have been identified in the past, e.g. forceps delivery, manual removal of the placenta, multiple gestation and CS.[13,20,21] The recognition of certain risk factors is important, but the fact that women having an uncomplicated delivery are at risk for large FMH is even more important. Guidelines on the use of anti-D immunoglobulin recommend assessment of the FMH volume for all Rh negative women who deliver a Rh positive child and not only for those with complicated deliveries.[3,5] In some countries it is common practice to administer a standard dose of 300 μg anti-D immunoglobulin after an uncomplicated delivery without FMH assessment.

In conclusion, we found increased numbers of fetal RBCs in maternal blood after CS with the majority of women undergoing CS under standard clinical conditions and without presumed risk factors for FMH. Our findings indicate that the procedure itself results in a small but detectable FMH volume. As recommended assessment of the FMH volume should be performed in all rhesus negative women undergoing CS.

## Acknowledgments

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# Fetal cell survival in maternal blood after large fetomaternal hemorrhage

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## Abstract

***Objective:*** To investigate the clearance rates of alpha-fetoprotein (AFP), fetal red blood cells (RBCs), and fetal mononuclear cells (MNCs) from maternal blood postpartum following large fetomaternal hemorrhage (FMH).

***Methods:*** Blood samples of a patient diagnosed with a large FMH at 36+2 weeks of gestation were drawn at several points of time during a period of 2 years. From each sample plasma was collected, whole blood smears were prepared and MNCs were isolated and stored. The AFP concentration was measured and fetal RBCs were quantified by the Kleihauer-Betke test. Fetal MNCs were detected by fluorescence microscopy by staining the paternally derived human leukocyte antigen (HLA)-A2, which was absent in the mother.

***Results:*** Based on the Kleihauer-Betke test a red cell FMH of 8.1% was detected, corresponding to a volume of 284 ml fetal whole blood transfused into the mother. The AFP concentration decreased very rapidly, whereas fetal RBCs were undetectable in maternal blood after 2.5 months. Postpartum extremely small numbers of fetal MNCs were detected as opposed to the relatively high number of fetal RBCs. Only 13 fetal per $1x10^6$ maternal MNCs were found in the first postpartum sample. The number of fetal MNCs decreased further in time and disappeared completely from maternal blood after 2 years.

***Conclusions:*** Following large FMH the AFP, fetal RBCs and fetal MNCs showed different clearance rates from maternal blood. The detection of fetal MNCs, based on HLA allelic differences between mother and child was enabled by a fluorochrome conjugated human monoclonal antibody directed against a paternally derived HLA alloantigen (HLA-A2) and is independent of the fetal sex. Our findings underline the low frequency of fetal MNCs circulating in maternal blood postpartum.

## Introduction

It is well established that in normal pregnancy small amounts of fetal cells are present in the maternal circulation and that the frequency of these cells increases as pregnancy progresses.[1,2] Large fetomaternal hemorrhage (FMH) is a serious complication of pregnancy, which occurs in approximately 3 out of 1000 deliveries.[3] FMH may cause severe fetal anaemia, in some cases leading to fetal death due to exsanguination. Other serious consequences that may arise from fetal-maternal cell trafficking are red blood cell (RBC) and platelet immunization in case of blood group incompatibility between mother and fetus, potentially affecting present and future pregnancies.[4,5] Both under normal and pathological conditions, such as placental abruption or abdominal trauma, significant FMH may occur.[3,6-9] In addition, obstetrical interventions during pregnancy and delivery that cause breakdown of the placental barrier place patients at risk for FMH.[3,10-13] The acid elution test described in 1953 by Kleihauer and Betke has been the key method in the study of transplacental passage of fetal RBCs and the cause and prevention of RBC immunization.[14] The routine administration of anti-D immunoglobulin to Rh D negative women during pregnancy and after delivery of a Rh D positive child has decreased the incidence of RBC immunization significantly.[15,16]

Over the last decade the presence of fetal nucleated cells in maternal blood has been described and the possible role of microchimerism in the pathophysiology of autoimmune disease has been suggested.[17-19] It is currently believed that fetal cells in order to be able to persist in maternal blood and other organs must have stem-cell-like properties.[20] Many techniques are available to detect nucleated cells of fetal origin, but one of the limitations is the low frequency of these cells and restricted application of these techniques to women pregnant of a male fetus by the detection Y chromosome sequences. Our aim was to study the clearance rates of fetal RBCs, fetal mononuclear cells (MNCs) and the alpha-fetoprotein (AFP) after delivery in a patient with large FMH using the Kleihauer-Betke test (KBT) for fetal RBC detection and measurement of the AFP concentration. We present a new approach to detect MNCs from fetal origin using a staining with a monoclonal antibody (mAb) directed against a paternally derived human leukocyte antigen (HLA) alloantigen.

## Case report

The patient was a 40-year-old multipara referred to our hospital postpartum. She had a long-term history of infertility due to premature ovarian failure without further underlying cause of disease. The first pregnancy ended in a miscarriage. The second pregnancy, achieved by oocyte donation in combination with in vitro fertilization was uneventful and she delivered a boy. The third pregnancy was also achieved by oocyte donation. At 36+2 weeks of gestation she consulted her midwife because of decreased fetal movements and was referred to an affiliated hospital. Upon admission all vital signs were within normal range. The fundal height was according to the gestational age. The diagnostic work-up included fetal heart rate monitoring which showed a sinusoidal pattern typical for fetal anemia. An emergency Cesarean section was performed and a boy was delivered. His birth weight was 3085 g. The Apgar scores were 5 and 8 after 1 and 5 min, respectively. The arterial blood pH was 6.99 and the hemoglobin level at birth was 2.6 mmol/l. The placenta weighed 520 g without any macroscopic abnormalities. The physical examination showed no abnormalities. He was intubated because of respiratory insufficiency and was transported to the neonatal intensive care unit of our hospital. He was ventilated mechanically for another 12 h and received a blood transfusion. One day after CS the mother was transferred to the maternity ward of our hospital as well.

The diagnosis of a large FMH was confirmed in a postpartum blood sample of the mother. Both the Kleihauer-Betke test (KBT) and flow cytometry using antibodies against the fetal hemoglobin (HbF) were performed to quantify fetal RBCs. A FMH volume of 284 ml fetal whole blood was found. The blood groups of the mother and the child were both A positive. Pathological investigation of the placenta revealed no abnormalities.

The child recovered well and was discharged from the hospital after 2.5 weeks. At the age of two years his neurological development was normal. After informed consent several maternal blood samples and one neonatal blood sample were drawn starting the day after delivery.

## Materials and methods

### *Patient samples*

In the period from 13 May 2003 to 1 June 2005 maternal blood samples were drawn at several points of time postpartum. The samples were collected in vacutainer tubes containing ethylene diamine tetra-acetic acid (EDTA) and sodium heparin by venipuncture and were processed within 3 hours of collection. From each

maternal EDTA sample glass slides were prepared for fetal RBC quantification by the Kleihauer-Betke test (KBT) and plasma was collected for the measurement of the alpha-fetoprotein (AFP) concentration. One maternal and one neonatal sodium heparin blood sample was used for HLA typing. Low resolution molecular typing was performed on DNA obtained from samples by polymerase chain reaction (PCR) / sequence specific oligonucleotide using a reverse dot-blot method.[21] MNCs were isolated by ficoll-amidotrizoate density gradient centrifugation from other sodium heparin samples and cryopreserved for fetal MNC detection and quantification. Maternal sera taken at delivery and on days 1, 2, 15, 30 and 77 postpartum were examined for total HLA class I and II antibody content by ELISA (LAT, One Lambda Inc, Canoga Park, CA) followed by HLA specificity testing in the complement dependent cytotoxicity (CDC) test against a panel of peripheral blood MNCs from molecularly HLA typed individuals.

The patient gave written informed consent to collect blood for research purposes. The Medical Ethical Review Board of the Leiden University Medical Center approved the protocol (P01.016).

***Measurement of the alpha-fetoprotein concentration in maternal blood***

Plasma was collected and stored at -80° C until further processing. The AFP concentration of all samples was measured in a single run using a Chemiluminescent Microparticle Immuno Assay (Architect I-2000, Abbott, Hoofddorp, The Netherlands).

***Quantification of fetal RBCs in maternal blood***

Of each EDTA sample, 100 µl was mixed with 100 µl phosphate-buffered saline (PBS). Conventional blood smears were prepared on glass slides using 2.5 µl of this dilution. Fixation of the cells and elution of HbA were performed as described previously.[9,14] Fetal RBCs were counted in 400 subsequent microscopic fields using a 40x objective. The total number of maternal RBCs in 400 microscopic fields was estimated by counting the number of background cells in 5 different fields. All slides were evaluated in a blinded fashion. Negative and positive control samples consisted of spiked samples of fetal RBCs in adult RBCs. The FMH volume was calculated using the following formulas. The fetal RBC and whole blood volumes were calculated by assuming a maternal whole blood volume 5000 ml at term, and a fetal and maternal hematocrit of 0.50 and 0.35, respectively.[22]

(1) transfused fetal RBC (%) = fetal RBCs / number of maternal RBCs x 100
(2) transfused fetal RBC volume (ml) = fetal RBC % x 0.35 x 5000 ml
(3) transfused fetal whole blood volume (ml) = fetal RBC % x (0.35 / 0.50) x 5000 ml

***Quantification of fetal MNCs***

Preparation of the anti-HLA-A2 mAb SN230G6

A hybridoma was derived by EBV transformation of lymphocytes from an HLA antibody seropositive multiparous woman, followed by electrofusion of an antibody-secreting EBV line and rigorous subcloning. The specificity of the human HLA mAb SN230G6 was determined by CDC and defined as HLA-A2/B17. The mAb SN230G6 (isotype IgG, λ) was purified from hybridoma supernatant by protein A chromatography. A $F(ab')_2$ fragment was prepared by pepsin digestion followed by protein A chromatography to remove Fc fragments and undigested mAb.[23] The isolated F(ab')2 fragment was conjugated to the fluorochrome Alexa Fluor 546 (Molecular Probes Inc, Eugene, OR) according to the manufacturer's instructions. The Alexa Fluor 546-dye labeled mAb has an absorption and fluorescence emission maximum of approximately 558 nm and 573 nm, allowing detection of red fluorescent cells.

Lymphocyte staining protocol

Upon thawing, 1 x $10^6$ MNCs were incubated with 1.25 µg Alexa Fluor conjugated SN230G6 F(ab')2 fragment in a final volume of 20 µl at 4 C° for 30 minutes in the dark. After incubation, cells were washed once and resuspended in 1 ml PBS supplemented with 10% fetal calf serum (FCS).

Microscopic evaluation

Approximately 150,000 mAb-stained cells were spun on glass slides using a Cytofuge centrifuge (Nordic Immunological Laboratories, Tilburg, The Netherlands) and air-dried for 1 hour. Slides were mounted in 0.01 µg/ml 4'-6-Diamidino-2-phenylindole (DAPI) in Vectashield (Vector Laboratories, Burlingame, CA).

Of each blood sample drawn at different points of time postpartum, 10 glass slides (equivalent to approximately 1.5 x $10^6$ MNCs) were evaluated in a blinded fashion. The slides were manually analyzed using a Leica DMRXA fluorescence microscope (Leica Microsystems, Wetzlar, Germany) equipped with a 40, 63 and 100x oil immersion objective and 4 different filters: a DAPI (blue excitation), a HQ-TRITC (red excitation), a HQ-FITC (green excitation) and a triple band pass filter (red, green and blue excitation). All slides were analyzed for their cell content, morphology and staining intensity. Cells with a brightly positive HLA-A2 membrane staining (red) were classified as fetal. Photographs were recorded using the same microscope and a CCD camera (CH250, Photometrics, Tucson, AZ).

### Validation study of mAb SN230G6 anti-HLA-A2

To investigate sensitivity and linearity of detection of mAb SN230G6 stained MNCs, spiked samples of HLA-A2 positive MNCs derived from term umbilical cord blood in HLA-A2 negative, non-pregnant adult MNCs were prepared. Negative control samples stained with mAb SN230G6 consisted of: 1) HLA-A2 negative MNCs of a non-pregnant healthy adult, 2) HLA-A2 negative MNCs of a pregnant woman near term carrying a HLA-A2 negative child and 3) mixtures of HLA-A2 negative MNCs from term umbilical cord blood in HLA-A2 negative MNCs of a pregnant woman near term carrying a HLA-A2 negative child. Additionally, HLA-A2 positive and negative MNCs without staining for mAb SN230G6 were included. Positive controls consisted of HLA-A2 positive MNCs from term umbilical cord blood and blood from non-pregnant adults. Spiked samples consisted of 0.1%, 0.01% and 0.001% concentrations. Of each mixture and control sample 6 glass slides were available. All slides were evaluated in a blinded fashion by two investigators, resulting in 12 replicate measurements per mixture or control sample.

### *Statistical analysis*

Validation study of the mAb SN230G6 was performed by using Pearson's correlation coefficient (Excel2000, Microsoft Inc., Redmond, WA) and calculation of confidence intervals (SPSS 10.0 for windows, SPSS Inc., Chicago, IL). The clearance rates of AFP, fetal RBCs and fetal MNCs are displayed as scatterplots (Excel2000).

## Results

### *AFP and fetal RBC quantification*

The AFP concentration and fetal RBC percentage at different points of time after delivery are shown in table 1. The AFP concentration decreased very rapidly, whereas fetal RBCs were undetectable in maternal blood after 2.5 months (figure 1a and 1b). The $t_{½}$ of AFP and fetal RBCs in maternal blood were 3 and 15 days, respectively.

### *Fetal MNC quantification*

#### HLA class I and II typing of the mother and child

The HLA types of the mother and child were:

| | | |
|---|---|---|
| Mother: | class I: | A*01, A*29, B*37, B*44, Cw*06, Cw*16 |
| | class II: | DRB1*07, DRB1*11, DRB3, DRB4, DQB1*02, DQB1*03 |
| Child: | class I: | A*01, A*02, B*08, B*40, Cw*03, Cw*07 |
| | class II: | DRB1*13, DRB1*14, DRB3, DQB1*05, DQB1*06 |

**Table 1 -** Different clearance rates of AFP, fetal RBCs and fetal MNCs from maternal blood after delivery

| Follow-up postpartum | AFP concentration | Fetal RBCs | | Fetal MNCs | |
|---|---|---|---|---|---|
| | (µg/l) | per $1x10^6$ | (%) | per $1x10^6$ | (%) |
| 1 day | 3953 | 81000 | 8.1 | ND* | ND |
| 2 days | 2753 | 68000 | 6.8 | 13.3 | 0.0013 |
| 4 days | 656 | 54000 | 5.4 | 10.7 | 0.0011 |
| 15 days | 95 | 39000 | 2.9 | 10.7 | 0.0011 |
| 30 days (1 month) | 9 | 23000 | 2.3 | 5.3 | 0.0005 |
| 52 days (1.5 month) | 5 | 2000 | 0.2 | 6.7 | 0.0007 |
| 77 days (2.5 months) | ND | 0 | 0 | 2.0 | 0.0002 |
| 102 days (3.5 months) | ND | 0 | 0 | ND | ND |
| 574 days (1.5 year) | ND | 0 | 0 | 2.6 | 0.0003 |
| 726 days (2.0 years) | ND | 0 | 0 | 0 | 0 |

ND: not determined (sample volume too small)

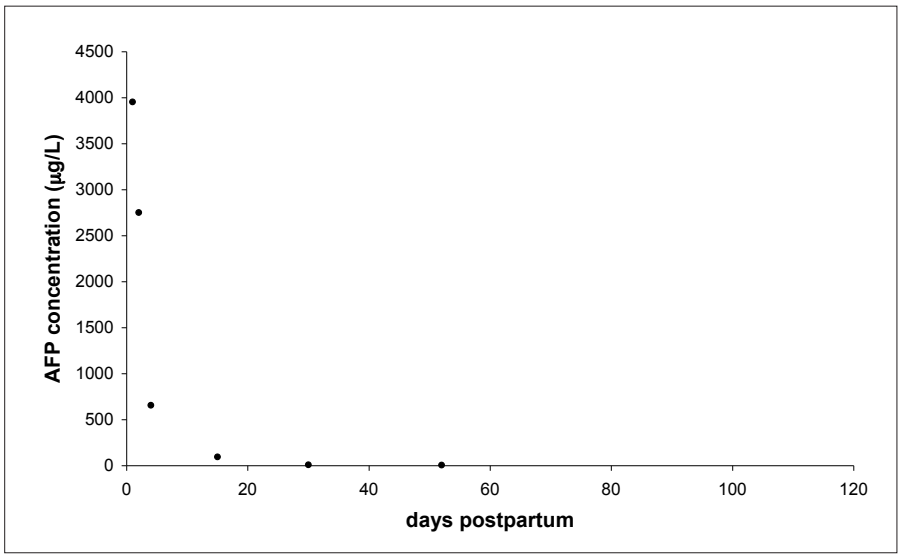


**Figure 1a -** The clearance rate of AFP from maternal blood after delivery

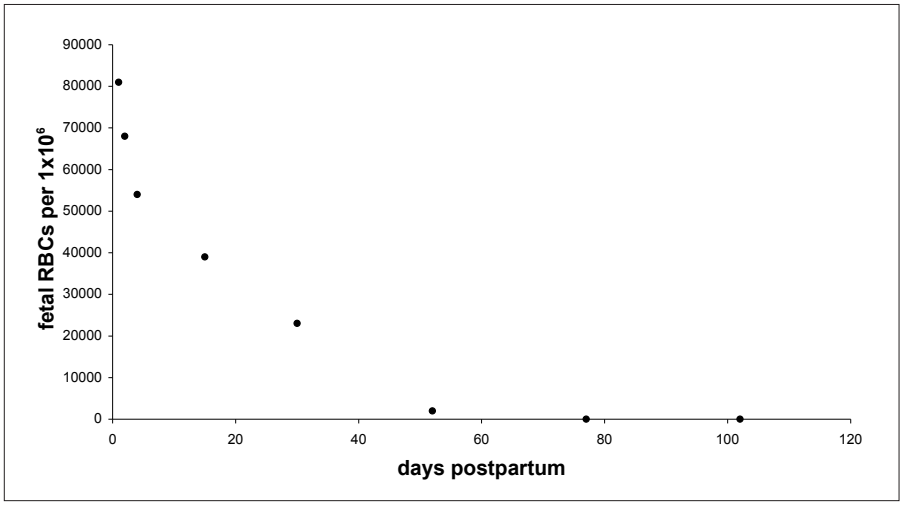


**Figure 1b -** The clearance rate of fetal RBCs from maternal blood after delivery

**Table 2 -** Quantitative assessment of HLA-A2 positive fetal MNCs in spiked samples: expected versus measured cell numbers per 1x10$^6$ adult MNCs

| Theoretical number of HLA-A2 positive MNCs | | Detected mean number of HLA-A2 positive MNCs | | |
|---|---|---|---|---|
| % | per 1x10$^6$ | % | per 1x10$^6$ | 95% CI |
| 0 | 0 | 0 | 0 | [0 – 0] |
| 0.001 | 10 | 0.0013 | 13 | [3 – 23] |
| 0.01 | 100 | 0.0077 | 77 | [50 – 103] |
| 0.1 | 1,000 | 0.0682 | 682 | [620 – 743] |

Based on the HLA type differences between mother and child, it was decided to apply mAb SN230G6, which was reactive with the fetal HLA-A2 antigen.

### Validation study of mAb SN230G6 anti-HLA-A2

No systematic differences between both investigators were found, therefore the data were pooled. No fetal MNCs were found in negative control samples. Samples consisting of HLA-A2 positive MNCs derived from adult and umbilical cord blood were

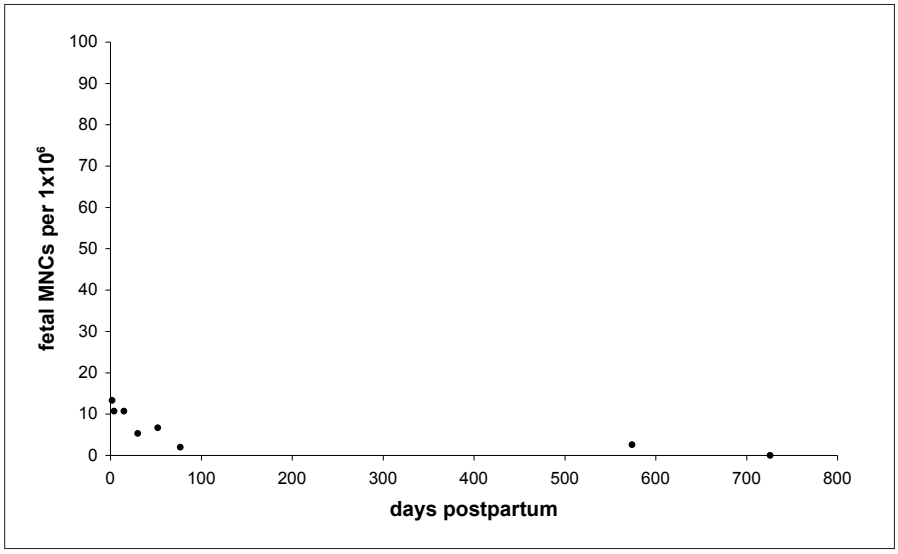


**Figure 1c -** The clearance rate of fetal MNCs from maternal blood after delivery

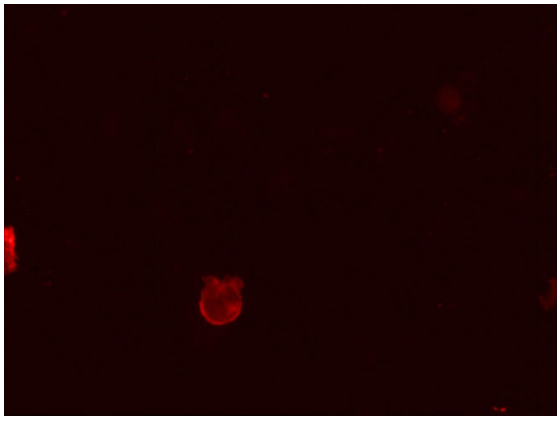

**Figure 2a**
Patient sample stained with mAb SN230G6 anti-HLA-A2: 1 fetal MNC positive for HLA-A2 (red membrane staining) against a background of HLA-A2 negative maternal MNCs.

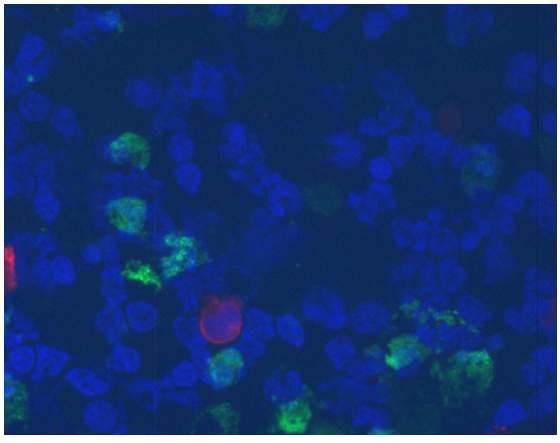

**Figure 2b**
Patient sample stained with a combination of mAb SN230G6 anti-HLA-A2 and DAPI: 1 fetal MNC positive for HLA-A2 (red membrane staining) against a background of HLA-A2 negative maternal MNCs.

100% positive. The correlation between the theoretical and detected concentrations of fetal MNCs was good ($r^2$=0.99), especially in the lower ranges. These results are given in table 2.

### Fetal MNC quantification in patient samples

Postpartum, mAb SN230G6 detected extremely small numbers of fetal MNCs, which decreased further in time (table 1). The clearance rate of fetal MNCs from maternal blood is given in figure 1c. An HLA-A2 positive fetal MNC (red membrane staining) against a background of negative maternal MNCs is shown in figure 2a and 2b. At two years postpartum no fetal MNCs could be identified.

### Antibody formation in the mother

In the maternal serum sample taken on day 4 postpartum, HLA antibodies against HLA-A2 were detected. Samples taken earlier were negative. Fifteen days postpartum antibodies against HLA-B40 were found in addition.

## Discussion

Little is known about the lifespan of fetal blood cells transfused into the maternal circulation. We studied the clearance rates of AFP and fetal RBCs and fetal MNCs in maternal blood samples at several points of time postpartum in a patient diagnosed with large FMH aiming to contribute to the discussion on the persistence of fetal cells. Our results indicate that AFP is cleared very rapidly after large FMH and that ABO and Rh compatible fetal RBCs have a lifespan of approximately 80 days in maternal blood. As compared to fetal RBCs, fetal MNCs are either transferred to maternal blood in extremely small amounts or cleared very rapidly from the maternal circulation.

Our technique to detect fetal MNCs using a staining with a mAb against the paternally derived HLA alloantigen provides a possibility to study microchimerism on a cellular basis in all pregnancies irrespective of the fetal sex. The mAb SN230G6 is specific for the diaminoacid GE 62-63 sequence, a determinant shared between HLA-A2 and HLA-B17. The absence of this determinant on any of the HLA alleles of the mother allowed the unequivocal detection of fetal MNCs on the basis of cell surface expression of HLA-A2. The principle of discrimination between fetal and maternal MNCs based on a paternally inherited HLA alloantigen by fluorescence activated cell sorting has been used previously.[24]

Passage of fetal cells into the maternal circulation does occur at the fetomaternal interface. In the human hemochorial placenta the villi are surrounded by maternal

intervillous blood. Under normal physiological conditions the fetal and maternal circulations are separated by the so-called placental barrier, consisting of the following layers: (1) a continuous layer of syncytiotrophoblast cells, (2) an initially (in the first trimester) complete, but later on (second and third trimester) discontinuous layer of cytotrophoblast cells, (3) a trophoblastic basal lamina, (4) connective tissue derived from the extra-embryonic mesoderm, and (5) the fetal endothelium.[25] Throughout pregnancy the placental barrier becomes progressively thinner while simultaneously fetal blood flow and blood pressure increase as the villous tree enlarges.[26] Due to spontaneous or induced disruption of the placental barrier fetal plasma and blood cells including their precursors will leak into the maternal circulation.

In our patient we found an extremely high AFP concentration in the first postpartum sample underlining the large FMH volume. In the following postpartum samples a relatively rapid decline of the AFP concentration was observed.

AFP is a major serum glycoprotein synthesized during fetal life mainly by the yolk sac and trophoblast early in the first trimester followed shortly thereafter by the fetal liver. In the human fetus the concentration of AFP peaks at 13 weeks of gestation (3-4 mg/ml), falls to about 50 μg/ml at term and disappears after birth.[27] The AFP concentration in adults is approximately 5 μg/l. The primary roles of AFP are indirectly regulation of cell growth by controlling apoptosis, involvement in inflammatory reactions and immunoregulation.[28] At term the placenta does not synthesize AFP and with an intact placental barrier the presence of AFP in the placenta is a reflection of transplacental transport via a receptor-mediated mechanism.[29,30] Elevated levels of AFP in maternal blood during pregnancy are associated with fetal malformations, such as neural tube defects and with placental tissue damage and fetomaternal hemorrhage. The large influx of the AFP into the maternal circulation as observed in our patient is explained by the breakdown of the placental barrier and corresponds with the amount of transfused fetal blood. AFP disappeared from the maternal circulation within 1 month.

The transfused volume of ABO and Rh compatible fetal RBCs was estimated at 142 ml or 284 ml fetal whole blood. Given a fetoplacental volume at term of 125 ml/kg, this fetus has lost 75% of its circulating volume, which corresponds well with the extremely low hemoglobin level at birth.[22] Precise calculation of the FMH volume is important in case of Rh incompatibility between mother and child. The transfused RBCs had a maximum lifespan of approximately 77 days and a $t_{½}$ of 15 days. The clearance rate of fetal RBCs from maternal blood after FMH depends on a number of facts: the ABO and Rh compatibility, administration of anti-D immunoglobulin and the time of entry in the maternal circulation. Results on the lifespan of ABO Rh compatible fetal RBCs described in literature showed different clearance rates. Some

studies report a shorter lifespan compared to adult RBCs corresponding with our results.[31,32] Two other studies on this subject report a fetal RBC lifespan equal to adult RBCs.[3,33] Differences in the observed lifespan of fetal RBCs in maternal blood are most likely due to a varying age distribution of cells entering the maternal circulation.

Many factors may influence the transfer of fetal cells into maternal blood during and after pregnancy. Fetomaternal cell trafficking is increased in conditions such as preeclampsia, intra-uterine growth restriction, fetal abnormalities and termination of pregnancy.[34-37] The amount of fetal to maternal cell transfer may be influenced by histocompatibility. Certain maternal HLA class II alleles, such as HLA-DQ A1*0501 were found to be more frequently associated with fetal cell microchimerism.[38,39] Also, very little is known about the role of maternal antibody formation against fetal specific HLA alloantigens in fetal cell microchimerism.

Different types of fetal nucleated cells can be identified and isolated: trophoblasts, nucleated erythroid cells, and lymphocytes including lymphoid progenitor cells. The biological implications of fetomaternal cell trafficking are currently being explored. Various research groups working on fetal cell isolation for the purpose of non-invasive prenatal genetic diagnosis found evidence for the survival of fetal progenitor cells in the circulation of women many years after delivery.[40-43] This finding led to the hypothesis that fetal cells persisting in maternal blood and tissues are involved in the pathogenesis of auto-immune disease in women after their child-bearing years. So far various techniques and strategies have been used for the detection of rare cells. In studies focussing on the detection of intact fetal cells different cell separation protocols have been applied using fluorescence activated cell sorting (FACS) and magnetic activated cell sorting (MACS) to enrich for specific subpopulations of fetal cells followed by fluorescence *in situ* hybridization (FISH) with chromosome specific probes and PCR to amplify fetal specific gene sequences based on the genotype of the parents.[44,45] One of the limitations of these techniques is the not yet optimal sensitivity and specificity. Therefore, fetal cell microchimerism is still difficult to detect using techniques currently available.

The frequency of fetal cells in the maternal circulation increases with the gestational age.[4] The number of fetal nucleated cells in maternal blood is estimated to be 1-6 cells/ml in uncomplicated second-trimester pregnancies.[36,37] At near-term all women have circulating fetal cells in their blood.[46] After delivery the number of fetal nucleated cells rapidly decreases. Fetal cell microchimerism can be detected in as many as 90% of healthy woman after delivery and thus is a widespread phenomenon, but is difficult to detect.[41] The majority of fetal MNCs are mature and they most probably become apoptotic as a consequence of the maternal T cell response, providing an explanation for the very low frequency at which these cells are found. It is currently believed that fetal cells, which persist in maternal blood and

other organs, must have stem-cell-like properties.[20] The hypothesis is that these cells persist in a maternal stem cell niche, where they are able to engraft. Later in life they may repopulate and home to a damaged organ in case of injury and thus contribute to the maternal repair response.

In conclusion, following large FMH the AFP, fetal RBCs and fetal MNCs show different clearance rates from maternal blood. We demonstrate a new technique to detect fetal MNCs by the use of a mAb directed against the paternally derived alloantigen HLA-A2. This approach is independent of the fetal sex. Our findings underline the low frequency of fetal MNCs circulating in maternal blood postpartum.

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# The incidence of large fetomaternal hemorrhage and the Kleihauer-Betke test

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*(Obstet Gynecol 2005;106:642-643)*
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## Introduction

International guidelines for appropriate management of Rh D negative women at risk for rhesus immunization are based on large observational studies on the incidence of fetomaternal hemorrhage in patients undergoing obstetrical interventions.[1,2] A number of risk factors for fetomaternal hemorrhage have thus been identified.[1-4]

In a recently published study the incidence of fetomaternal hemorrhage after vaginal and Cesarean deliveries was much higher than previously reported in the literature.[5] We commented on this study by the following "Letters To The Editor", both published with a reply from the authors of the original article. We demonstrate that due to an incorrect formula in an obstetrical textbook to calculate the fetomaternal hemorrhage volume, the reported incidence of fetomaternal hemorrhage after vaginal and Cesarean delivery was incorrectly high.

## To the editor *(by Pelikan et al.)*[6]

The study of Salim et al. entitled "The incidence of large fetomaternal hemorrhage and the Kleihauer-Betke test" reports on the frequency of large fetomaternal hemorhage (FMH) in caesarean sections compared with vaginal deliveries.[5] The quantification of fetal red cells was performed by the use of the Kleihauer-Betke test, which is based on resistance of fetal hemoglobin (HbF) to acid elution and widely used to determine the FMH volume.[7] Although this method is clinically useful in the detection of fetal red cells in maternal blood, a high inter-observer and inter-laboratory variability are reported, mainly due to the analysis of an insufficient number of microscopic fields.[8] Since many modifications of this method are known, it would have been meaningful, if the authors had provided information on which modification was applied, how many fields were counted and how the number of background cells was estimated. All these factors influence the fetal cell percentage.

More importantly, the authors' calculation that a volume of 30 ml fetal whole blood corresponds with 0.4% fetal red cells is incorrect. The formula (fetal red cells = (maternal blood volume x maternal hematocrit x % fetal red cells) / newborn hematocrit) calculates the FMH whole blood volume and not only the fetal red cell volume by assuming a maternal blood volume of 5000 ml at term, a maternal hematocrit of 35%, and a newborn hematocrit of 50%. The authors defined large FMH as 30 ml of fetal whole blood, the amount inactivated by 300 μg anti-D immunoglobulin. As the formula already adjusts for the fetal hematocrit, the computed FMH volume is the fetal whole blood volume and not the fetal red cell volume. A 30 ml fetal whole blood volume then corresponds with 0.85% fetal red cells and is

calculated as follows:

5000 ml x 0.35 x 0.0085 ≈ 15 ml (= fetal red cell volume) / 0.50 (= newborn hematocrit) ≈ 30 ml fetal whole blood. Based on an incorrect application of the formula, too many patients were included in the large FMH group, instead of only those with 0.85% fetal red cells. Therefore, the reported incidence of large FMH is too high and the calculated odds ratios are incorrect.

## **In reply** *(by Salim et al.)*[9]

We appreciate Dr. Scherjon and colleagues' interest in our research.[5] Similarly, we are grateful for the opportunity to clarify our views about the concerns they raise. Regarding the method used for detecting fetal red blood cells in maternal blood, which is based on the Kleihauer-Betke test, 3 steps were performed. First, the total number of erythrocytes in 5 fields was counted, and the average number per field was determined. Then the number deeply stained fetal hemoglobin-containing erythrocytes in about 30 fields was counted, and the average number per field was determined. Finally, the percentage of fetal hemoglobin-containing erythrocytes was calculated on the basis of the total number erythrocytes per field. On each day of our testing, blood from a newborn and blood from an adult male (presumed not to have any hemoglobinopathy) were used and inspected on the same slide and served as positive and negative controls, respectively.

The formula used in our research for calculating the amount of fetomaternal hemorrhage is adopted from a well-respected obstetric textbook.[10] Moreover, raising the cut-off to 0.85%, as suggested, leaves nearly a rate of 4% of large fetomaternal hemorrhage in both the vaginal and the cesarean delivery groups. This rate is still higher than the rate reported in the literature. (0.23-1%).[11-13] Our results show a rate of large fetomaternal hemorrhage that is substantially higher than previously reported, with no difference between vaginal and cesarean deliveries. This may reflect inaccuracies with the current method used to estimate the degree of fetomaternal hemorrhage. Further studies, preferably using immunological identification measures, are needed to verify also whether fetomaternal hemorrhage truly occurs before delivery and whether current preventive strategies should be modified.

## To the editor *(by Pelikan et al.)*[14]

The Green Journal recently published our letter to the editor[6] commenting on the article "The incidence of large fetomaternal hemorrhage and the Kleihauer-Betke test"[5] together with a reply by the authors.[9] After reading the authors' reply we now understand the source of the serious error in the study by Salim et al., leading to falsely high incidences of fetomaternal hemorrhage.[9] The error lies in the formula the authors adopted from the 21st edition of Williams Obstetrics[10] to calculate the fetomaternal hemorrhage volume. In the recent 22nd edition of Williams Obstetrics[15] the correct formula to calculate the transfused fetal *whole blood* volume is given: fetal blood volume (ml) = the fetal *whole blood* volume = (maternal blood volume x maternal hematocrit x fetal red cell %) / newborn hematocrit.

In the 21st edition the result of the formula, which represents the fetal *whole blood* volume, is divided wrongly by the fetal hematocrit once again. Based on the correct formula in the 22nd edition, a fetal red cell percentage of 0.85% instead of 0.4% corresponds to a fetal whole blood volume of 30 ml. A maternal blood volume of 5000 ml, a maternal hematocrit of 0.35 and a newborn hematocrit of 0.50 are assumed in this calculation, as recommended.[10,15] By referring to the 21st edition and thus using the wrong formula the authors have not realized that this has led to falsely high incidences and incorrect confidence intervals of fetomaternal hemorrhage in all their study groups. We have our concerns and disagree with the reply by the authors that even when the cut-off level is raised to 0.85% the incidence of large fetomaternal hemorrhage is still higher than reported before in literature. Nevertheless, all the incidences and confidence intervals of fetomaternal hemorrhage in their study are incorrect because of the wrong formula and must be recalculated.

It is important for clinical practice and for investigators who will be referring to this article in the future that the correct incidences and confidence intervals will be available.

## In reply *(by Salim et al.)*[16]

We appreciate Dr. Pelikan and colleagues' interest in our research,[5] and at the same time we are grateful for the opportunity to clarify again our views on the concerns they raise. Even though the formula we used appears in both the 20th and 21st editions of the textbook,[10] we recalculated our results according to the new formula. Raising the cut-off to 0.85%, according to the formula recently reported in the 22nd edition,[15] still leaves, according to our results, an incidence of nearly 4% of large fetomaternal hemorrhage in the vaginal and cesarean delivery groups. This rate is

still higher than reported in the literature (0.23-1%),[11-13] with no significant difference between the groups. The similarity among the groups has no relationship to which formula was used.

We therefore stand behind our ultimate outcome that shows a rate of large fetomaternal hemorrhage, which is substantially higher than previously reported, with no difference between vaginal and cesarean deliveries. More clarifications are provided in the original study.[5]

## Comment

Quantification of fetomaternal hemorrhage is important for routine clinical practice. The amount of anti-D immunoglobulin, which needs to be administered to Rh negative women with a Rh positive child to prevent rhesus immunization is based on the calculation of the fetomaternal hemorrhage volume. Several formulas to calculate the transfused fetal blood volume have been described in literature. An error in the formula adopted from the 21st edition of Williams Obstetrics[10] has led to falsely high incidences of fetomaternal hemorrhage in the cited study.[5] In the recent 22nd edition of Williams Obstetrics the correct formula to calculate the transfused fetal whole blood volume is given.[15] In this comment we aimed to demonstrate the understanding of basic physiological principles is needed to apply the formula, which transforms the fetal to maternal red cell ratio detected in maternal blood into a transfused fetal whole blood volume correctly.

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# Summary and General Discussion

8

Transplacental passage of fetal cells is a common phenomenon in pregnancy and delivery.[1,2] Since the discovery of trophoblast cells in women dying from eclampsia by Schmorl in 1893,[3] many investigators have detected cells of fetal origin in maternal blood and other organs.[4-10] Although the fetal and maternal circulations are separated from each other by the placental barrier, small microscopic disruptions of this barrier allow fetal cells to leak into maternal blood. These cells mainly originate from the fetal hematopoietic system and trophoblast layer. In addition, fetal plasma proteins and DNA, can be detected in the maternal circulation. Fetomaternal cell trafficking may cause complications, such as fetal anemia and alloimmunization. Particularly in cases of Rh D incompatibility between mother and fetus precise quantification of the fetomaternal hemorrhage (FMH) volume is important to ensure the administration of an appropriate dose of anti-D immunoglobulin.

The recent discovery of long-term persistence of fetal hematopoietic progenitor cells in maternal blood leading to microchimerism in multiparous women has opened up new fields of investigation.[11] Different hypothesis regarding the consequences of fetal cell microchimerism in women after their child-bearing years have been discussed extensively in the literature.[11-16]

In this thesis we focused on microscopic and flow cytometric methods suitable to quantify fetal red cells in maternal blood. We compared several methods for FMH quantification both in spiked samples and samples from pregnant women following invasive procedures and complications in pregnancy. We developed a technique for automated read-out of Kleihauer-Betke stained slides to enumerate fetal red cells in maternal blood. We studied the biological behaviour of fetal red and mononuclear cells (MNCs) in the maternal circulation after large FMH and the rate by which these cells are cleared from the maternal circulation. We identified fetal MNCs using a staining with a monoclonal antibody (mAb) directed against the paternally inherited HLA-A2. Furthermore, we discuss the importance of a correct use of the formula to calculate the FMH volume. The results of these studies are summarized and discussed in this chapter.

A general introduction on FMH is presented in **chapter 1**. The biological basis of fetomaternal cell trafficking and the involvement of different fetal cell types, risk factors for the occurrence of FMH and the consequences of fetal cells in maternal blood are reviewed.

The manual KBT is generally used to quantify the transfused fetal red cell volume in patients at risk for FMH. Over the past decades studies on the poor performance of this test were published, reporting both under- and overestimation of the fetal red cell

count and a large inter-observer variability.[17-20]

In **chapter 2** we developed a new strategy for the evaluation of Kleihauer-Betke stained slides using automated microscopy aiming at two goals: 1) to distinguish fetal and maternal red cells in an unbiased way and 2) to analyse a larger area of the slide as compared to the standard manual KBT being able to evaluate many more cells to improve accuracy. A computer-assisted microscope scanned the stained slides on the basis of preset characteristics using bright field analysis. The positive, fetal red cells were distinguished from pale adult cells by the intensity and intracellular distribution of the pink staining and cell size. The automatically detected cells were stored in a database and relocated for direct microscopic inspection by an operator. An important feature contributing to precision and reproducibility of data analysis was the use of a two optical filter-switch to produce two-colour images of each detected cell in order to recognize and exclude white blood cells.

We compared the performance of the standard manual KBT and the automated KBT in spiked sample experiments containing fetal red cell percentages from 0.0001% to 1%. Of each dilution, 5 replicate measurements were performed and stored in a separate selection gallery. Each gallery was reviewed by two independent investigators in a blinded fashion, resulting in 10 paired measurements per dilution. Repeated measurements of each fetal red cell concentration by the automated KBT resulted in a high reproducibility and very small inter-observer and intra-observer variability. Typical coefficients of variation were 3-4% for fetal red cell percentages ranging from 0.001% to 0.1%. The automated KBT showed strong correlation between the theoretical and detected fetal red cell percentage. In the range from 0.0001% to 0.001% the standard KBT underestimated the fetal red cell percentage, whereas the automated KBT was very precise. These findings indicate that an automated readout of Kleihauer-Betke stained slides improves the accuracy of the fetal red cell detection in the range from 0.0001% to 0.1%.

The suitability of the automated KBT to detect small numbers of fetal red cells in maternal blood has led to the study described in **chapter 3**. In this study we compared the manual and automated microscopical KBT and flow cytometry using anti-HbF immunostaining in clinical samples. Indications for FMH quantification were manual removal of the placenta and cesarean section in Rh D negative patients and vaginal bleeding, abdominal trauma, antepartum fetal death, placental abruption and fetal anemia in both Rh D positive and negative patients.

Blood samples from 44 patients between 25 and 42 weeks of gestation, were analyzed by all three methods. To compare the results from manual KBT, automated KBT and flow cytometry, we subdivided FMH into the categories 0%, <0.1% and >0.1%. In 13 patients FMH was undetectable by all three methods. A FMH varying

from 0.0001% to 0.1% was found in 27 patients either by the automated KBT (n=10), the manual KBT (n=3) or both methods (n=14). In these patients, flow cytometry was not capable to distinguish the signal of fetal cells from the background of HbF containing adult cells. In 4 patients flow cytometry and both the manual and automated KBT detected a FMH >0.1%. Risk factors for the occurrence of FMH in these 4 patients were a complicated delivery, cesarean section, choriocarcinoma and placental abruption, respectively. Flow cytometry tended to detect slightly higher fetal red cell percentages than the manual and automated KBT. The correlation between the expected and detected fetal red cell percentage measured in control samples by manual KBT, automated KBT and flow cytometry was good. The coefficient of variation for each method studied in 5 replicate determinations was less than 5% in the majority of the control samples. Moderate agreement was observed between the automated and manual KBT. Agreement between flow cytometry and the manual KBT was fair.

The results presented in this study indicate that the automated KBT is a highly sensitive and reliable method in the quantification of FMH. Flow cytometry is not suitable to detect FMH <0.1%. With the use of an automated microscopic approach the anti-D immunoglobulin dose might be further fine-tuned taking a margin of safety into account in patients with small FMH. These considerations are important in perspective of a possible future shortage of anti-D immunoglobulin, which is obtained from volunteers with high circulating antibody levels, and the fact that anti-D immunoglobulin is a blood product with a small potential risk of viral transmission.

In **chapter 4** we studied FMH in patients undergoing CVS. By the lack of a sensitive method to detect small numbers of fetal red cells early in pregnancy, FMH after first trimester CVS has been studied predominantly by measurement of the AFP concentration. To investigate whether CVS results in a transplacental passage of proportional amounts of AFP and fetal red cells, we applied the automated KBT to blood samples from 59 patients before and after CVS and compared the results with the AFP concentration in the same samples. All pregnancies were cytogenetically normal. The biopsy forceps or the canula were used for transcervical CVS in 56 patients. An aspiration needle was used for abdominal sampling in 3 patients.

The median AFP concentration increased significantly after CVS, but no significant increase of the median fetal red cell number was observed. The AFP increase tended to correlate with the amount of villi aspirated, but not with an increase of fetal red cells. No relation was found between the number of passes and an increase of AFP or fetal red cells. We hypothesize that the AFP increase is caused by release of this protein into maternal blood due to destruction of cytotrophoblast cells. An alternative explanation for the discrepancy between the AFP concentration

and fetal red cells in maternal blood would be that when villi are damaged by CVS, and red cells presumably spill into the maternal lacunae bathing the villi in vivo, most of the fetal red cells do not find their way into the maternal circulation. They are perhaps caught up in a clot formation local to the site of spillage. Both explanations regarding a significant increase of AFP without fetal red cell increase after CVS place the use of AFP as a marker of FMH in doubt.

From the results in this study it can be concluded that the AFP rise is discordant with red cell FMH. In view of our findings, the risk of maternal immunization to a fetal red cell antigen, following CVS is extremely small, but not absent.

It is well known that the risk of FMH during pregnancy increases due to the fact that the placental barrier becomes progressively thinner while simultaneously fetal blood flow and blood pressure increase. Small microscopic disruptions of the placental barrier allow fetal cells to enter the maternal circulation. Although large FMH occasionally occurs after normal vaginal deliveries, traumatic deliveries, such as CS, are more likely to be associated with an increased risk of FMH. We quantified FMH in a prospective cohort of 57 patients undergoing CS in **chapter 5**.

Fifty women had a planned CS for various indications and 7 underwent an emergency CS because of fetal distress. We compared the fetal red cell numbers measured by manual KBT and the AFP concentrations before and after CS. We aimed to assess whether CS contributes to the transplacental passage of fetal red cells and AFP and to identify possible risk factors for FMH. A significant increase of fetal red cells was found after CS, while the AFP concentration decreased significantly. A FMH <0.1 ml was found in 71.9% of the patients. In 28.1% the FMH volume ranged from 0.1 to 4.8 ml. No specific risk factors for FMH were found. The increased fetal red cell numbers detected in maternal blood following CS, indicate that the procedure itself results in a small but detectable FMH volume in an unselected population undergoing CS. The AFP decrease is most likely explained by intravenous fluid supply in combination with per-operative blood loss resulting in dilution of maternal plasma.

Many different cells from fetal origin can be detected in maternal blood as a consequence of fetomaternal cell trafficking. It is possible to identify trophoblast cells and fetal hematopoietic cells, including their progenitors. In the study presented in **chapter 6**, we investigated the clearance rates of fetal red cells, fetal MNCs and AFP in a patient diagnosed with large FMH near term.

Based on the KBT a red cell FMH of 8.1% was detected, corresponding to a volume of 284 ml fetal whole blood transfused into the mother. We hypothesized that within the transfused population of fetal MNCs into maternal blood, the cells with

proliferating potential may be detected in maternal blood long after delivery. Maternal blood samples were drawn at several points of time postpartum. The last sample was drawn 2 years after delivery. As expected, the AFP concentration decreased very rapidly, whereas fetal red cells could not be demonstrated anymore by the KBT after 2.5 months. To identify fetal MNCs we used a staining with a mAb directed against the paternally derived HLA-A2 alloantigen, which was absent in the mother. In contrast to the relatively high number of fetal red cells detected in maternal blood postpartum, extremely small numbers of fetal MNCs were detected. Only 13 fetal per $1x10^6$ maternal MNCs were found in the first postpartum sample. The number of fetal MNCs decreased further in time and could not be demonstrated anymore after 2 years.

The use of human mAbs against HLA offers the advantage that whole fetal cells can be detected microscopically and irrespective of the fetal sex. The method described in this chapter was designed as a proof of principle study to identify fetal cells in maternal blood on the basis of HLA polymorphism. With further improvement of enrichment strategies using MACS or FACS, staining with specific anti-HLA mAbs may be implemented in a protocol for fetal cell detection.

The identification of risk factors for the occurrence of FMH is important for appropriate management of Rh D negative women to prevent red cell immunization. International guidelines are based on large observational studies in obstetric patients.

In **chapter 7** we comment on a recently published study on the incidence of large FMH after vaginal delivery and CS.[21] In this study the incidence of FMH after vaginal and cesarean deliveries was much higher than previously reported in the literature. In a letter to the editor we demonstrate that due to an incorrect formula to calculate the FMH volume, derived from an obstetrical textbook, the reported incidence of FMH after vaginal delivery and CS was incorrectly high. The importance of the use of a correct formula to quantify the FMH is pointed out in this chapter. Failure to understand basic physiological principles, which are needed to transform the fetal to maternal red cell ratio into a transfused fetal whole blood volume correctly, may lead to errors and subsequently to inappropriate administration of anti-D immunoglobulin.

## Fetal cells in maternal blood: clinical applications and future perspectives

***Assessment of FMH in clinical practice and for research purposes***

In this thesis we have studied several methods available for the quantification of FMH. Traditionally the manual KBT is performed in most laboratories, but a large inter-observer variability is one of the limitations of this method.[17-20] Since studies on the use of flow cytometry for FMH quantification have reported improved accuracy in the range of 0.1% to 10% FMH, some laboratories have abandoned the KBT and now use flow cytometry.[17-20,22,23]

In our experiments, which consisted of both spiked and patient samples, we compared the manual and automated KBT with flow cytometric analysis using staining with monoclonal anti-HbF, dual staining with anti-HbF/anti-i and anti-HbF/anti-CA. We obtained accurate and consistent results from the manual KBT within the range of 0.001% to 1%. If recommendations are followed and a sufficient number of microscopic fields is counted using a 40x objective, then, in our opinion the manual KBT is a reliable method to quantify FMH. The automated KBT was even more sensitive than the manual KBT and provided accurate quantification in the range of 0.0001% to 1%. With the automated analysis of large numbers of cells, we could detect 1 fetal red cell per 1,000,000 maternal red cells. Flow cytometry using staining with anti-HbF was accurate in quantifying FMH within the range of 0.1% to 10%, but insensitive to detect FMH smaller than 0.1%.

Both in microscopic and flow cytometric analysis it may be difficult to distinguish the true fetal red cell population from maternal F cells. In a pilot study we tested diagnostic assays for flow cytometry using dual staining with anti-HbF/anti-i and anti-HbF/anti-CA. Unfortunately, due to problems with the specificity of the anti-i and anti-CA antibodies, no improvement of accuracy could be obtained.

Considering the fact that the cut-off level for additional anti-D immunoglobulin administration is a fetal red cell percentage of 0.85%, corresponding to 15 ml of fetal red cells, a method capable to quantify FMH above this level should be applied. We agree that flow cytometry using staining with anti-HbF is an objective method to quantify large FMH and therefore useful in clinical practice to identify patients, who need additional anti-D immunoglobulin. However, particularly in clinical settings and interventions, in which small FMH is likely to occur, the application of an automated KBT can be helpful in quantifying the FMH volume. With the use of an automated microscopic approach the anti-D immunoglobulin dose might be further fine-tuned taking a margin of safety into account in patients with small FMH. These considerations are important in perspective of a possible future shortage of anti-D

immunoglobulin, which is obtained from volunteers with high circulating antibody levels, and the fact that anti-D immunoglobulin is a blood product with a small potential risk of viral transmission.

***Non-invasive prenatal diagnosis***

The detection of fetal cells in maternal blood early in pregnancy is a very elegant and promising approach to perform non-invasive prenatal diagnosis. As opposed to invasive techniques, such as CVS and amniocentesis, it would provide prenatal diagnosis without the risk of pregnancy loss. If reliable detection of fetal genetic disorders could be achieved with respect to sensitivity and specificity, such test would be superior to current non-invasive screening techniques.

Technical advances in the field of cell sorting (e.g. FACS and MACS), in immunology (increasing availability of monoclonal antibodies), and in molecular genetics (FISH and PCR) have helped to establish the presence of fetal cells in maternal blood and to improve the detection rate. However, despite numerous efforts by research groups worldwide, non-invasive prenatal diagnosis has not been optimized yet for implementation in clinical practice.[24-27] Problems encountered are the low number of fetal nucleated cells in maternal blood, the lack of a 100% specific fetal cell marker, and the possible persistence of fetal progenitor cells from previous pregnancies. Currently, the fetal NRBC seems to be the optimal target cell for non-invasive prenatal diagnosis of trisomy 21, trisomy 18, trisomy 13 and sex chromosomal aneuploidies. This cell type is unlikely to persist from previous pregnancies. A frequently used strategy to detect and confirm fetal nucleated cells from maternal blood is based on FISH analysis with X and Y specific probes. One of the restrictions of FISH analysis is the fact that it is only applicable in women carrying a male fetus.

In this thesis we studied the possibility of staining with a mAb directed against a paternally inherited HLA antigen present on fetal cells and absent on maternal cells, an approach very similar to the one originally described by Herzenberg et al. using FACS.[28] Such an approach could be useful for the purpose of non-invasive prenatal diagnosis as it is independent of the fetal sex.

The recent discovery of fetal DNA in maternal plasma has opened up new possibilities.[29,30] With the application of PCR analysis for the detection of fetal DNA in maternal plasma, reliable non-invasive prenatal diagnosis of the fetal Rh D status, sex-linked disorders and other paternally inherited genetic disorders can be performed.[31-33] The detection of fetal DNA in maternal plasma is easier than detecting fetal nucleated cells in maternal blood and does not require prior enrichment. It is known that fetal cells circulating in maternal blood are constantly destroyed and digested, producing a pool of fetal DNA, that is many times higher than the equivalent

number of intact circulating fetal cells. Given the fact that fetal DNA is cleared rapidly from maternal plasma after delivery, persistence from previous pregnancies is impossible. Obviously, quality and relative intactness (e.g. fragment length) of the fetal DNA in the maternal circulation is an issue that needs to be considered when designing PCR primers to detect disease related to DNA abnormalities.

In conclusion, further refinements in the techniques used for the detection and isolation of cells and DNA from fetal origin are needed for the implementation of non-invasive prenatal diagnosis in clinical practice.

***Fetal cell microchimerism***

The study of fetomaternal cell trafficking and the implications of fetal cell microchimerism for parous women, is an emerging field of research. Whether fetal cell microchimerism has beneficial or adverse effects on the health of parous women after their child-bearing years has not been cleared yet.

Persisting fetal cells are present in higher numbers in women with autoimmune disease, such as systemic sclerosis, than in control groups.[34,35] Also, in nonautoimmune disorders, such as hepatitis C[36] and cervical cancer,[37] fetal cell microchimerism has been observed. In recent studies male fetal cells were detected in 30-50% of healthy women who had prior male pregnancies.[38] The detection of microchimeric male cells, bearing epithelial, leukocyte, or hepatocyte markers, in a variety of maternal tissue specimens suggest the presence of fetal cells with multilineage potential.[39]

The recently proposed theory of “good microchimerism” suggests that persistent fetal cells, instead of inducing a maternal immune response, survive in a maternal stem cell niche and provide a rejuvenating source of fetal progenitor cells in case of maternal tissue injury. They may home to the damaged organ and differentiate as a part of the maternal repair response.[40,41]

The detection of fetal hematopoietic cells based on HLA polymorphism, as described in this thesis, combined with laser capture microdissection and single cell PCR in the future, may provide further insight in the interesting field of fetal cell microchimerism.

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# Samenvatting

9

Transplacentaire passage van foetale cellen naar de maternale circulatie tijdens de zwangerschap of de bevalling komt bij vrijwel alle zwangeren in meer of mindere mate voor. Hoewel de foetale en de maternale circulatie van elkaar gescheiden zijn door de zogenaamde placentaire barrière, kunnen foetale cellen, als gevolg van microscopisch kleine onderbrekingen in deze barrière, terechtkomen in de maternale circulatie. Deze cellen zijn voornamelijk afkomstig van het foetale hematopoietische systeem en van de trofoblastcellaag. Tevens kunnen plasma-eiwitten en DNA van foetale origine worden aangetoond in maternaal bloed. Wanneer sprake is van een kleine hoeveelheid cellen wordt gesproken van foetomaternaal celverkeer. In geval van een groter volume wordt meestal de term foetomaternale transfusie gebruikt.

In 1893 beschreef Schmorl voor het eerst trofoblastcellen in longcapillairen van vrouwen die waren overleden ten gevolge van eclampsie. Hierna hebben vele onderzoeksgroepen foetale cellen aangetoond in maternaal bloed en andere organen, zoals beenmerg, lever en huid.

Het is bekend dat foetomaternaal celverkeer complicaties kan veroorzaken in de vorm van foetale anemie of alloimmunisatie. Het bepalen van het exacte volume foetaal bloed dat naar de moeder is getransfundeerd, is met name van belang voor het toedienen van de juiste hoeveelheid anti-D immunoglobuline aan rhesus D negatieve vrouwen die zwanger zijn van een rhesus D positieve foetus.

In dit proefschrift worden microscopische en flowcytometrische methoden beschreven om foetale erythrocyten en lymfocyten in maternaal bloed aan te tonen. Om de verschillende methoden met elkaar te kunnen vergelijken, zijn artificiële verdunningen alsook bloedsamples van zwangere vrouwen na een invasieve procedure of een zwangerschapscomplicatie geanalyseerd. Automatische microscopische analyse van bloeduitstrijken, gekleurd volgens de Kleihauer-Betke methode als nieuwe techniek voor detectie van foetale erythrocyten in maternaal bloed, is ontwikkeld. Vervolgens zijn de verdwijningscurves van foetale erythrocyten en lymfocyten bestudeerd. Voor het aantonen van foetale lymfocyten is een humaan monoclonaal antilichaam gericht tegen het human leukocyte antigen (HLA) gebruikt. Voorts werd het belang van het toepassen van de juiste formule voor het berekenen van het foetomaternale transfusievolume uiteengezet. De resultaten van de afzonderlijke studies die verband houden met foetomaternale transfusie, worden beschreven in deze samenvatting.

In **hoofdstuk 1** worden verschillende aspecten van foetomaternale transfusie uiteengezet. In dit inleidende hoofdstuk wordt een literatuuroverzicht gegeven waarop de hypothesen van dit proefschrift zijn gebaseerd.

Het is bekend dat bij meer dan de helft van de zwangeren al tijdens de

zwangerschap foetale cellen aantoonbaar zijn in de circulatie. Postpartum worden bij vrijwel alle zwangeren foetale cellen in het bloed gevonden. In de meeste gevallen gaat het om kleine hoeveelheden. Echter, van een aantal obstetrische ingrepen en complicaties in de zwangerschap is bekend dat deze een verhoogd risico geven op een grote foetomaternale transfusie. Voorbeelden van ingrepen met een verhoogd risico zijn chorion villus biopsie (vlokkentest), amniocentese (vruchtwaterpunctie), cordocentese (navelstrengpunctie), abortus provocatus, versiepoging bij stuitligging, sectio caesarea (keizersnede) en manuele verwijdering van de placenta. Het risico is ook verhoogd bij zwangerschapscomplicaties, zoals een solutio placentae (loslating van de placenta), abdominaal trauma, een intra-uteriene vruchtdood en choriocarcinoma.

Door de samenstelling van foetaal bloed worden voornamelijk foetale erythrocyten aangetroffen in maternaal bloed. Het rhesussysteem is één van de vele bloedgroepantigenen die voorkomen op erythocyten. Wanneer de foetus rhesus D positief is, kan immunisatie van de moeder optreden, hetgeen tot complicaties kan leiden bij volgende zwangerschappen. Derhalve wordt bij rhesus D negatieve zwangeren die ingrepen ondergaan of complicaties doormaken in de zwangerschap anti-D immunoglobuline toegediend ter voorkoming van rhesusimmunisatie.

Op basis van bestaande literatuurgegevens zijn in het verleden richtlijnen geformuleerd, die voor elke ingreep of complicatie voorgeschrijven welke hoeveelheid anti-D immunoglobuline gegeven moet worden. Na een ingreep in de eerste helft van de zwangerschap, wanneer het getransfundeerde foetale bloedvolume minimaal is, wordt in Nederland een standaarddosering van 375 IE anti-D immunoglobuline aanbevolen. Met het vorderen van de zwangerschap en daarmee ook het stijgen van het foetoplacentaire volume, neemt ook de kans op een foetomaternale transfusie toe. Daarom wordt bij een zwangerschapsduur van 30-32 weken een profylactische dosis van 1000 IE anti-D immunoglobuline aanbevolen. Na een ongecompliceerde vaginale partus krijgt een rhesus negatieve zwangere die bevalt van een rhesus positief kind 1000 IE anti-D immunoglobuline toegediend volgens de Nederlandse richtlijn. Deze hoeveelheid is voldoende om rhesusimmunisatie te voorkomen tot een getransfundeerd foetaal volbloedvolume van 20 ml. In geval van een verhoogd risico moet nadere diagnostiek worden verricht om het foetomaternale transfusievolume te kwantificeren vóór de toediening van de standaarddosering anti-D immunoglobuline. Wanneer sprake is van een volume groter dan 20 ml, dient een aanvullende dosis anti-D immunoglobuline te worden gegeven.

Het onderscheid tussen foetale en maternale erythrocyten wordt bemoeilijkt door het ontbreken van een uniforme foetale marker. Een veelgebruikte methode om het getransfundeerde foetale bloedvolume te bepalen bij patiënten met risicofactoren

voor een foetomaternale transfusie is de Kleihauer-Betke test. Deze test werd voor het eerst beschreven in 1957 door Kleihauer, Betke en Braun en maakt gebruik van het verschil in zuurresistentie tussen foetale en volwassen erythrocyten. Door bloeduitstrijken te behandelen met een citroenzuurfosfaatbuffer, denatureert het hemoglobine in de minder zuurresistente volwassen erythrocyten, terwijl het zuurresistente foetale hemoglobine in de foetale erythrocyten intact blijft. De foetale erythrocyten kunnen dan vervolgens onder de microscoop zichtbaar gemaakt worden na aankleuring van het foetale hemoglobine met erythrosine. De afgelopen jaren zijn veel publicaties verschenen die de betrouwbaarheid en de objectiviteit van de Kleihauer-Betke test bekritiseren. Met name bij het kwantificeren van grotere hoeveelheden foetaal bloed in de maternale circulatie werd een grote inter- en intra-observer variabiliteit beschreven. Een nieuwere techniek om het foetomaternale transfusievolume te bepalen is flowcytometrie. Foetale erythrocyten in een maternaal bloedsample kunnen met deze objectieve en snelle techniek worden aangetoond na aankleuring met fluorescerende monoclonale antilichamen gericht tegen het foetale hemoglobine. Een relatief nadeel van deze methode is de ongevoeligheid om kleinere hoeveelheden foetale erythrocyten aan te tonen. Zowel bij de Kleihauer-Betke test als bij flowcytometrie kunnen zogenaamde maternale F cellen een probleem vormen. Deze cellen bevatten een kleine hoeveelheid foetaal hemoglobine, waardoor ze moeilijk te onderscheiden zijn van de echte foetale erythrocyten.

Foetale hematopoietische cellen van lymphoide en myeloide origine komen slechts in zeer lage frequenties voor in maternaal bloed en zijn derhalve moeilijk aantoonbaar. In de afgelopen jaren is wereldwijd veel onderzoek gedaan naar het detecteren van zeldzame kernhoudende foetale cellen in maternaal bloed. Een dergelijke strategie zou in de toekomst non-invasieve prenatale diagnostiek mogelijk kunnen maken. Het grote voordeel ten opzichte van invasieve methoden, zoals de chorion villus biopsie en de amniocentese, is het afwezige risico op een miskraam. Foetale kerhoudende cellen die hiervoor in aanmerking komen zijn trofoblastcellen en erythroblasten, omdat deze celtypen niet kunnen persisteren uit een voorgaande zwangerschap. Klinische implementatie is echter nog niet binnen bereik wegens problemen met betrekking tot de sensitiviteit en specificiteit van de non-invasieve prenatale diagnostiek. Recent werd door Lo et al. circulerend foetaal DNA in maternaal serum aangetoond met behulp van een polymerase chain reaction (PCR). Op dit moment is het mogelijk om betrouwbaar de foetale rhesusfactor en het foetale geslacht (van belang bij geslachtsgebonden aandoeningen) antenataal te bepalen in het maternale serum.

Door het onderzoek naar foetale cellen in de maternale circulatie voor non-invasieve prenatale diagnostiek ontstonden nieuwe inzichten in de gevolgen van foetomaternaal celverkeer. Het aantonen van foetale hematopoietische

voorlopercellen in maternaal bloed tot 27 jaar postpartum door Bianchi et al., heeft geleid tot vele publicaties met betrekking tot microchimerisme van foetale origine. Tot op heden is echter nog onbekend welke gevolgen microchimerisme kan hebben voor vrouwen op langere termijn.

In **hoofdstuk 2** wordt een nieuwe techniek beschreven om bloeduitstrijken die werden gekleurd volgens de Kleihauer-Betke methode te beoordelen. Volgens op voorhand vastgestelde criteria werd een microscopische analyse verricht met behulp van een computer-gestuurde microscoop. De foetale erythrocyten werden onderscheiden van de maternale erythrocyten op grond van de intensiteit van de roze kleuring en de celgrootte. De automatisch gedetecteerde cellen werden opgeslagen in een database en konden vervolgens nog worden beoordeeld door een onderzoeker. Geautomatiseerde microscopie biedt voordelen ten opzichte van een handmatige beoordeling omdat een groter oppervlak van het objectglas in korte tijd kan worden geanalyseerd aan de hand van objectieve criteria.

De resultaten van de geautomatiseerde en handmatige Kleihauer-Betke test werden met elkaar vergeleken in artificiële verdunningen die varieerden van 0.0001 tot 1% foetale erythrocyten in maternaal bloed. De twee methoden correleerden goed met elkaar. Herhaalde analyse van dezelfde verdunningen toonde aan, dat met een geautomatiseerde microscopische analyse een hoge reproduceerbaarheid kan worden bereikt met een kleine inter- en intra-observer variabiliteit. Met behulp van geautomatiseerde microscopie bleek het zelfs mogelijk om een 1 foetale erythrocyt per 1.000.000 maternale erythrocyten te detecteren.

Om de toepasbaarheid van geautomatiseerde microscopische analyse van Kleihauer-Betke gekleurde preparaten te toetsen, werden in **hoofdstuk 3** bloedsamples van zwangeren beoordeeld. De resultaten van de handmatige en automatische Kleihauer-Betke test werden vergeleken met een flowcytometrische analyse van dezelfde samples, die werden aangekleurd met monoclonale antilichamen tegen foetaal hemoglobine. De patiëntenpopulatie bestond uit zwangeren opgenomen op de afdeling verloskunde, bij wie op grond van risicofactoren een indicatie bestond om een Kleihauer-Betke test te verrichten.

De indicaties waren sectio caesarea, manuele placentaverwijdering, vaginaal bloedverlies, abdominaal trauma, intra-uteriene vruchtdood, solutio placentae en onverklaarde foetale en neonatale anemie. De zwangerschapsduur varieerde van 25 tot 42 weken. Bij 13 patiënten werd met geen enkele techniek een foetomaternale transfusie aangetoond. Een zeer geringe foetomaternale transfusie tot 0.1% werd alleen gedetecteerd met de handmatige en/of de geautomatiseerde Kleihauer-Betke test. De zeer geringe foetomaternale transfusie bij deze patiënten, die wel

microscopisch kon worden aangetoond, werd niet gevonden met flowcytometrie. Bij 4 patiënten met obstetrische complicaties werd een foetomaternale transfusie van meer dan 0.1% gevonden met behulp van alle drie technieken. Het ging hierbij om patiënten met een gecompliceerde vaginale partus, een sectio caesarea, solutio placentae, en een choriocarcinoma. Uit een statistische analyse kwam naar voren dat er een redelijk goede overeenstemming was tussen de handmatige en geautomatiseerde Kleihauer-Betke test. Een vergelijking tussen de handmatige Kleihauer-Betke test en de flowcytometrie liet een matige overeenstemming zien.

Door implementatie van een zeer gevoelige methode om foetale erythrocyten in maternaal bloed te detecteren, zoals bijvoorbeeld de geautomatiseerde Kleihauer-Betke test, zou de anti-D immunoglobuline dosering kunnen worden aangepast, rekening houdend met een bepaalde veiligheidsmarge.

Het foetoplacentaire volume in het eerste trimester van de zwangerschap is zeer gering. Door het ontbreken van een gevoelige test om zeer kleine aantallen foetale erythrocyten te detecteren, wordt derhalve in de literatuur een stijging van het alpha-foetoproteine gerelateerd aan het optreden van een foetomaternale transfusie.

Het alpha-foetoproteine komt in zeer hogere concentraties voor in het foetale plasma, maar is nauwelijks aantoonbaar in gezonde volwassenen. Wanneer een foetomaternale transfusie optreedt, dan zal door een influx van het foetale plasma de maternale plasmaconcentratie van alpha-foetoproteine aanzienlijk stijgen. In voorgaande studies werd bij diagnostische ingrepen zoals een chorion villus biopsie of een amniocentese een stijging van het alpha-foetoproteine aangetoond na de ingreep.

In **hoofdstuk 4** werd bij zwangeren die merendeels op leeftijdsindicatie een chorion villus biopsie ondergingen, de alpha-foetoproteineconcentratie en het aantal foetale erythrocyten voor en na de ingreep gemeten. De geautomatiseerde Kleihauer-Betke test werd gebruikt om het aantal foetale erythrocyten in maternaal bloed te kwantificeren. Hoewel een significante stijging van de alpha-foetoproteineconcentratie optrad, werd verrassend genoeg geen stijging van het aantal foetale erythrocyten gevonden. Uit eerder onderzoek is gebleken dat trofoblastcellen vroeg in de zwangerschap alpha-foetoproteine synthetiseren en dat de concentratie van dit eiwit intracellulair hoog is. Als gevolg van trofoblastdestructie tijdens de chorion villus biopsie komt het intracellulaire alpha-foetoproteine vrij en kan in de maternale circulatie terechtkomen. Een concentratiestijging van alpha-foetoproteine in maternaal bloed is dan geen goede maat voor het foetomaternale transfusievolume. Een andere verklaring is dat op de plaats van biopsie een stolsel ontstaat waardoor de foetale erythrocyten worden weggevangen en niet in de maternale circulatie terecht kunnen komen. Bij één patiënt werd echter een geringe

stijging van het aantal foetale erythrocyten gevonden. Derhalve blijft het aanbevolen om na een chorion villus biopsie anti-D immunoglobuline toe te dienen aan rhesus negatieve zwangeren.
In de loop van de zwangerschap wordt de placentabarrière dunner en neemt daarmee de kans op foetomaternaal celverkeer toe. Ingrepen in het laatste trimester van de zwangerschap en tijdens de bevalling zouden het risico op een foetomaternale transfusie verhogen. Om te onderzoeken of een sectio caesarea een foetomaternale transfusie induceert, werd bij 57 patiënten voor en na de ingreep maternaal bloed afgenomen.

De resultaten van deze studie worden beschreven in **hoofdstuk 5**. Er werd een geringe foetomaternale transfusie gedetecteerd na een sectio caesarea, zonder dat hiervoor risicofactoren werden gevonden. De concentratie alpha-foetoproteine in maternaal bloed daalde, hetgeen kan worden verklaard door het feit dat bij alle patiënten die een sectio caesarea ondergingen spinaal analgesie werd toegepast. Bij deze vorm van analgesie is het gebruikelijk intraveneus vocht toe te dienen, waardoor verdunning van het maternale bloed optreedt.

In tegenstelling tot foetale erythrocyten, is het technisch gezien veel moeilijker om foetale lymfocyten in maternaal bloed aan te tonen vanwege hun zeer lage frequentie. In **hoofdstuk 6** werd bij een zwangere met een grote foetomaternale transfusie van 284 ml onderzocht in welke verhouding en hoe lang foetale erythrocyten en lymfocyten aantoonbaar zijn in maternaal bloed. In dit geval was sprake van het optreden van een spontane foetomaternale transfusie bij een zwangerschapsduur van 36 weken. Vanwege foetale nood op basis van anemie werd een spoedsectio verricht.

Tot twee jaar postpartum werden maternale bloedsamples afgenomen en onderzocht op de aanwezigheid van foetale cellen. Voor het kwantificeren van foetale erythrocyten werd gebruik gemaakt van de Kleihauer-Betke test. Voor het detecteren van foetale lymphocyten werden specifieke humane antilichamen gebruikt, gericht tegen het HLA-A2 dat wel aanwezig was op de foetale maar niet op de maternale cellen. Uit de analyse bleek dat de ABO en rhesus D compatibele, foetale erythrocyten een relatief korte overlevingsduur van ongeveer 80 dagen hebben in maternaal bloed. In tegenstelling tot het aantal foetale erythrocyten direct postpartum aangetroffen in het maternale bloed, werd slechts een zeer gering aantal foetale lymfocyten gevonden. Dit aantal nam nog verder af in de tijd.

De resulaten van deze studie tonen aan dat foetale lymfocyten in maternaal bloed kunnen worden gedetecteerd door gebruik te maken van HLA specifieke antilichamen en dat deze cellen zeer waarschijnlijk slechts in hele kleine aantallen circuleren. Dit zeer lage aantal van getransfundeerde foetale lymfocyten was echter

voldoende om een maternale immuunrespons op te wekken.

Een recent gepubliceerde studie met betrekking tot de incidentie van een grote foetomaternale transfusie bij patiënten na een sectio caesarea of een vaginale partus, was de aanleiding voor het schrijven van **hoofdstuk 7**. In dit hoofdstuk wordt aan de hand van een “letter to the editor” gewezen op een fout in de door de auteurs van deze studie gehanteerde oorspronkelijke formule voor het omrekenen van het percentage foetale erythrocyten in maternaal bloed naar het getransfundeerde volume foetaal volbloed. In deze “letter to the editor” wordt uiteengezet dat de fout in de gerefereerde studie heeft geleid tot een te hoge incidentie van een grote foetomaternale transfusie zowel na een sectio caesarea als na een vaginale partus.

De studies beschreven in dit proefschrift naar methoden om foetale erythroide en lymphoide cellen in maternaal bloed aan te tonen, kunnen bijdragen tot de kennis van foetomaternaal celverkeer gedurende de zwangerschap en specifiek met betrekking tot een aantal complicaties en interventies.

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## Curriculum vitae

Denise Pelikan werd geboren op 1 januari 1972 te Groningen. Zij behaalde het VWO diploma aan het Mencia de Mendoza Lyceum te Breda in 1990, waarna zij in datzelfde jaar begon aan de studie Geneeskunde aan de Rijksuniversiteit Leiden. In de daarop volgende tijd deed zij naast haar studie onderzoek naar de behandelingsresultaten van schildkliercarcinoom op de afdeling Endocrinologie van het LUMC. Haar afstudeerproject betrof een studie naar de behandeling van goed gedifferentieerd schildkliercarcinoom met radio-actief jodium, eveneens op de afdeling Endocrinologie van het LUMC onder begeleiding van Dr. B.M. Goslings.

Na het behalen van het doctoraal examen in 1994 ging zij naar de Mayo Clinic in Rochester, Minnesota voor een klinische stage op de Neonatal Intensive Care Unit en de afdeling Otorhinolaryngology. In januari 1995 begon zij met haar co-schappen. Als keuze co-schap deed zij een wetenschappelijk onderzoek naar de rol van Interleukine-8 in meconium bij het ontstaan van het meconiumaspiratie-syndroom bij de afdeling Neonatologie van het LUMC onder begeleiding van Dr. A.J. de Beaufort en Prof. Dr. H.M. Berger.

In januari 1997 behaalde zij haar artsexamen. Vanaf april 1997 was zij een jaar werkzaam als agnio op de afdeling Neonatologie van het Wilhelmina Kinderziekenhuis te Utrecht (Prof. Dr. F. van Bel) en daarna een half jaar als agnio op de afdeling Gynaecologie en Verloskunde van het Reinier de Graaf Gasthuis te Delft (Dr. J.C. Kuijpers).

In oktober 1998 begon zij aan haar opleiding tot gynaecoloog in het LUMC (opleiders: Prof. Dr. H.H.H. Kanhai en Prof. Dr. G.G. Kenter) en het Groene Hart Ziekenhuis te Gouda (opleider: Dr. M. Helfferich, Dr. J.C. van Huisseling), waarna in 2000 een agiko stipendium werd toegekend door ZonMw voor het in dit proefschrift beschreven onderzoek. Dit onderzoek naar foetomaternale transfusie was een samenwerkingsverband tussen de afdelingen Verloskunde (Prof. Dr. H.H.H. Kanhai en Dr. S.A. Scherjon) en Moleculaire Celbiologie (Prof. Dr. H.J. Tanke) van het LUMC. Het laatste jaar van haar opleiding tot gynaecoloog zal zij werkzaam zijn in het LUMC en het Groene Hart Ziekenhuis.

## Abbreviations

| | |
|---|---|
| **AGM** | aorta/gonad/mesonephros |
| **AFP** | alpha-fetoprotein |
| **BSA** | bovine serum albumin |
| **CA** | carbonic anhydrase |
| **CD** | cluster of differentiation |
| **CDC** | complement dependent cytotoxicity |
| **CS** | Cesarean section |
| **CV** | coefficient of variation |
| **CVS** | chorionic villus sampling |
| **DAPI** | 4'-6-diamidino-2-phenylindole |
| **DNA** | deoxyribonucleic acid |
| **EDTA** | ethylene diamine tetra-acetic acid |
| **FACS** | fluorescence activated cell sorting |
| **FCM** | flow cytometry |
| **FCS** | fetal calf serum |
| **FISH** | fluorescence in situ hybridization |
| **FITC** | fluorescein isothiocyanate |
| **FSC** | forward scatter |
| **Hb** | hemoglobin |
| **HbA** | adult hemoglobin |
| **HbE** | embryonic hemoglobin |
| **HbF** | fetal hemoglobin |
| **Hct** | hematocrit |
| **HLA** | human leukocyte antigen |
| **HPA** | human platelet antigen |
| **HSC** | hematopoietic stem cell |
| **KBT** | Kleihauer-Betke test |
| **mAb** | monoclonal antibody |
| **MACS** | magnetic activated cell sorting |
| **MHC** | major histocompatibility complex |
| **MNC** | mononuclear cell |
| **NAITP** | neonatal alloimmune thrombocytopenia |
| **NRBC** | nucleated red blood cell |
| **PBS** | phosphate-buffered saline |
| **PCR** | polymerase chain reaction |
| **RBC** | red blood cell |
| **Rh** | rhesus |
| **SD** | standard deviation |
| **SSC** | sideward scatter |
| **UA** | unidentified alarm |

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