

Anna Slunga-Tallberg
Sakari Knuutila

Department of Medical Genetics,
University of Helsinki, Finland

Can Nucleated Erythrocytes Found in Maternal Venous Blood Be Used in the Noninvasive Prenatal Diagnosis of Fetal Chromosome Abnormalities?

Key Words

Fetal cells
MAC (morphology antibody
chromosomes) technique
Maternal blood
Nucleated erythrocytes
Prenatal diagnosis

Abstract

Nucleated erythrocytes found in the venous blood of pregnant women have been seen as the most promising type of cell for prenatal diagnosis of fetal abnormalities because of their short life span and their scarcity in the venous blood of healthy non-pregnant women. However, this review demonstrates that a great proportion of the nucleated erythrocytes found in venous blood during pregnancy are of maternal origin and that pregnancy per se induces nucleated erythrocytes of maternal origin to appear in the peripheral blood. More efficient and specific enrichment techniques are therefore needed to obtain purified nucleated erythrocytes of fetal origin in such quantities that reliable prenatal diagnoses can be performed.

Fetal Cells in Maternal Venous Blood

Investigations in preeclampsia patients, interphase cytogenetics using Y body scoring, karyotype analyses and more recent molecular technologies all indicate that maternal blood contains cells of fetal origin [1-4]. Fetal gene sequences amplified from cells in maternal blood now include the Y chromosome [5, 6], hemoglobin Lepore^{Boston} [7], rhesus D [8] and HLA DQ α [9]. Fetal chromosome abnor-

malities, such as trisomies 21 and 18 [10-12], sex chromosome aneuploidies 47,XXY and 47,XYY [13, 14] have been identified in interphase cells from maternal blood by fluorescence in situ hybridization (FISH). The proportions of fetal cells in maternal venous blood reported in different studies vary greatly: from 1/27 to 1/100,000,000 [reviewed in ref. 3]. More studies will be required to establish whether all pregnant women have fetal cells in their venous blood. Fetal cells have

been detected in maternal blood from the 4th week of gestation by amplification of Y chromosome DNA sequences [15] and with in situ hybridization using Y-chromosome-specific probes from the 6th week of gestation [16]. The number of fetal cells appears to increase with advancing gestation [3, 17]. Fetal cells encountered in the maternal circulation include nucleated erythrocytes [17], lymphocytes [3], granulocytes [16] and trophoblasts [18]. The number of fetal cells of each cell type in the maternal blood is so low that without enrichment, their presence is not useful in diagnosis [3, 4]. Attempts to enrich trophoblasts have not yet been successful, because maternal cells tend to absorb trophoblast antigens [19, 20]. Fetal lymphocytes can be detected by enriching cells with paternal HLA alleles in maternal blood [21–23]. The need to study both parents and the long life span of lymphocytes are, however, drawbacks to using this method. Nucleated erythrocytes have emerged as the most promising target cells for use in prenatal diagnosis in maternal blood because of their short life span and because they are present in the blood of pregnant women but are rare in the blood of healthy adults [24, 25]. Furthermore, the maturation of red blood cells in the fetus starts between the 5th and 6th week of gestation and a large number remain nucleated in the fetal circulation [26]. The main question is: are the nucleated erythrocytes in maternal venous blood fetal in origin?

Nucleated Erythrocytes

Nucleated erythrocytes can be identified on the basis of morphology, but as the morphological classification of erythroblasts is not very reliable, monoclonal antibodies should be used [27]. Glycophorin A (GPA) is a sialoglycoprotein found only on cells of the ery-

throid lineage, and is present from the erythroblast stage [28]. The transferrin receptor (CD71) is another antigen found on erythroid cells. It is expressed from the burst-forming-unit erythroid stage and disappears in the late reticulocyte stage [29]. Unfortunately, the corresponding antibody (anti-CD71) also recognizes other activated cells, such as activated lymphocytes [30]. Antibodies against hemoglobin chains are specific indicators of red cells, and fetal hemoglobin (HbF, $\alpha_2\gamma_2$) can be used as a marker for fetal cells, even though about 1% of adult hemoglobin is of this type [31, 32]. Antibodies to the leukocyte common antigen, CD45 can be used for indirect enrichment of cells of the erythroid cell lineage. CD45 is an antigen found on all other hematopoietic cells but erythroid cells. Whether CD45 also recognizes early erythroid precursors is an open question [29, 30].

The Enrichment of Nucleated Erythrocytes

To enrich nucleated erythrocytes in maternal blood, methods such as density gradient centrifugation, fluorescence-activated cell sorting (FACS) [33], Dynal magnetic beads [25], or micromagnetic beads (MACS) [34] are combined. Both positive and negative selection can be employed. Positive selection uses antibodies which recognize cells of the erythroid lineage (e.g. anti-GPA, anti-CD71 or anti-HbF), whereas negative selection is based on antibodies (e.g. anti-CD45) which remove leukocytes. Although enrichment by MACS and FACS increases the proportion of nucleated erythrocytes, the separation does not yield a pure cell fraction [25, 35]. As the presence of cells other than nucleated erythrocytes decreases the specificity and sensitivity of molecular cytogenetic methods, analysis which targets fetal erythroid cells is needed.

Culturing nucleated erythrocytes from maternal blood in the presence of erythropoietin (Ep) and stem cell factor (SCF) has given very promising results in the molecular cytogenetic analysis of mitotic cells [36]. This indicates that it is possible to immunophenotype nucleated erythrocytes even during mitosis.

The MAC Method for Targeted Analysis of Nucleated Erythrocytes

The MAC (morphology antibody chromosomes) method and related techniques allow analysis of the three parameters (morphology, immunophenotype and cytogenetics) required for cell classification of interphase as well as mitotic cells [37–41]. The MAC technique has been restricted until now to cancer and mutation research [41]. The following is a brief description of MAC as applied to prenatal diagnosis of fetal cells in maternal blood.

For classification, interphase cells are first enriched using any of the available techniques, then cytopsin preparations are made and the cells are left to dry. The cells are fixed according to the chosen cytochemical or immunocytochemical technique. The classified cells can be studied and photographed before in situ hybridization (consecutive system), or the process can proceed directly to in situ hybridization (simultaneous system). For the classification of mitotic cells (e.g. nucleated erythrocytes in culture), the cells are treated with mild cell-membrane-stabilizing solutions to obtain chromosome spreading inside the intact cell membrane on the cytocentrifuge preparations.

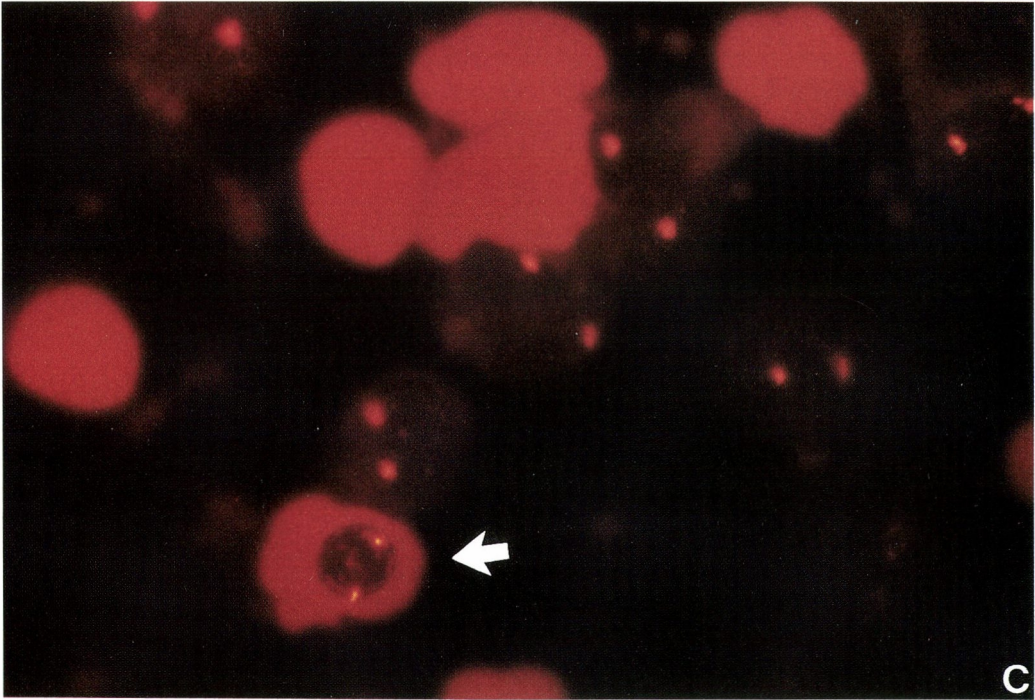
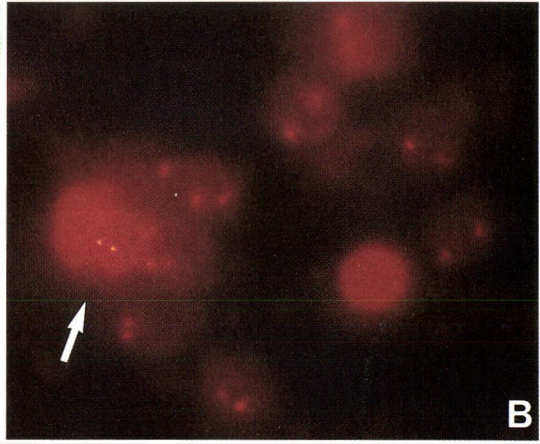
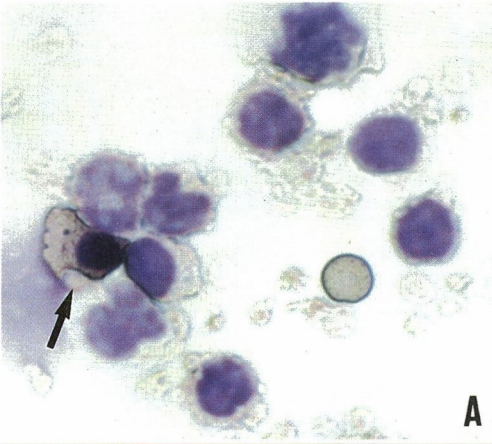
Immunofluorescence, immunoperoxidase or alkaline phosphatase anti-alkaline phosphatase (APAAP) techniques can be used for immunophenotyping. Hybridization signals can be detected either by immunofluorescence or immunoenzymatic methods. All the

procedures, including hypotonic treatment, fixation, cytochemical and immunocytochemical stainings, in situ hybridization techniques, signal detection systems (either fluorescent or enzymatic) and scoring of the signals are described in detail in our recent MAC manual [41].

Figure 1 shows two X-chromosome-specific fluorescence in situ hybridization signals in a GPA-positive nucleated erythrocyte detected in maternal venous blood. Figure 2 shows an enzymatically detected Y-specific signal in a GPA-positive cell from maternal

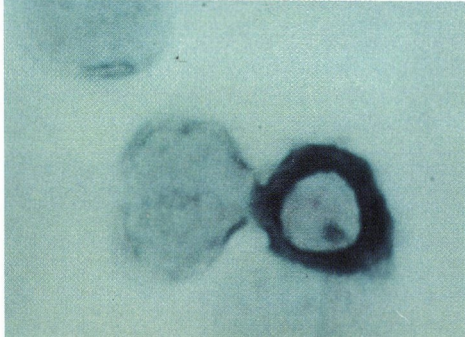
Fig. 1. A A GPA-positive nucleated red cell (arrow) and to the right GPA-negative cells in venous blood from a nonpregnant woman. Procedure: Ficoll-Paque density gradient centrifugation; enrichment by immunomagnetic beads with anti-CD45 monoclonal antibody; cytopsin preparation; APAAP immunostaining with anti-GPA monoclonal antibody. **B** The same cells after in situ hybridization with an XY contig probe (provided by Integrated Genetics, Framingham, Mass., USA). All nucleated cells including the GPA-positive nucleated red cell (arrow) have two red X-chromosome specific hybridization signals indicating their maternal origin. Procedure: APAAP reaction removed with acidic fixation; in situ hybridization; detection of the XY cocktail probe. The X chromosome was detected with the fluorochrome Cy3 (yellowish/orange signal) and the Y chromosome with fluorochrome fluorescein isothiocyanate (FITC; green signal). Note that after in situ hybridization, GPA-positive cells fluoresce a brilliant red in a fluorescence microscope with a dual-band pass filter, FITC/rhodamine. The picture is taken on Ektachrome 400 HC film for color slides which makes the focused red signals in the GPA-positive cell look yellowish/orange. **C** A GPA-positive nucleated red cell (arrow) in venous blood from a pregnant woman. The two yellowish/orange X signals indicate its maternal origin. Procedure: see panels **A** and **B**.

Fig. 2. A fetal cell in maternal blood. Shown is a GPA-positive nucleated erythrocyte with an enzymatically detected signal of the Y-specific probe pY431 (provided by Dr. K. Smith, Baltimore, Md., USA). For methodological steps see figure 1.



1

C



2

blood. Note that the positive cell resembles a nucleated erythrocyte in morphology. The photographs illustrate the usefulness of the MAC method when targeted analysis of in situ hybridization is needed. MAC used with in situ hybridization on nucleated erythrocytes can therefore be a powerful tool in the prenatal diagnosis of fetal aneuploidies in maternal blood.

Presence of Nucleated Erythrocytes in Maternal Blood

Biological variation in the number of CD71-positive cells in the blood of pregnant women has been demonstrated by Bianchi et al. [24] and the number of nucleated erythrocytes has been shown to increase with increasing gestational age [17, 42]. On average, 100 nucleated erythrocytes in 40 ml maternal venous blood have been reported by Gänshirt et al. [42]. They also showed that previous invasive procedures or pregnancies did not cause any increase in the number of nucleated erythrocytes circulating in the maternal blood. In the same study, the number of nucleated erythrocytes in the maternal blood was shown to be significantly higher in pre-eclamptic than in normal pregnancies. The proportions of fetal and maternal nucleated erythrocytes in the venous blood of pregnant women have yet to be defined.

Our MAC studies on 40 women carrying a male fetus (at 6–17 weeks of gestation) showed a low frequency of nucleated erythrocytes in the blood of pregnant women, ranging from 1 to 230 per 20-ml sample (50–10,000 per 1,000 ml) [43] and the majority of these cells were of maternal origin. In 6 of the 10 nulligravid controls, 1 to 3 nucleated erythrocytes were found per 20 ml venous blood. Enrichment of the cells was performed by negative magnetic activated cell sorting (mini-

MACS) using an anti-CD45 monoclonal antibody, and they were detected by APAAP using anti-GPA monoclonal antibody and Giemsa counterstaining. The genotype of the detected cells was studied by FISH using X- and Y-specific probes. These results show that pregnancy induces nucleated erythrocytes of maternal origin to appear per se in the maternal venous blood.

Although the two other groups [44, 45] who have used direct targeted analysis on nucleated erythrocytes enriched from maternal venous blood have found nucleated erythrocytes of fetal origin, the number of cells has still been too low to allow reliable prenatal diagnosis using FISH analysis. Our finding that a great proportion of nucleated erythrocytes are of maternal origin is confirmed by the recent results of Bianchi et al. [35] and Büsch et al. [46] obtained by indirect techniques using PCR on fractions of enriched nucleated erythrocytes from maternal blood.

Can Nucleated Erythrocytes Be Used for Noninvasive Prenatal Diagnosis?

As a high proportion of nucleated erythrocytes in the blood of pregnant women seems to be of maternal origin, more efficient and specific enrichment techniques will be needed to obtain purified nucleated erythrocytes of fetal origin in sufficient quantities to allow reliable prenatal diagnoses.

It will no doubt be established whether or not all pregnant women have fetal nucleated erythrocytes in their peripheral blood, and consequently, what proportion of women could benefit from noninvasive prenatal diagnosis using maternal venous blood.

References

- 1 Walknowska J, Conte FA, Grumbach MM: Practical and theoretical implications of fetal/maternal lymphocyte transfer. *Lancet* 1969;i:1119-1122.
- 2 Schröder J, de la Chapelle A: Fetal lymphocytes in the maternal blood. *Blood* 1972;39:153-162.
- 3 Hamada H, Arinami T, Kubo T, Hamaguchi H, Iwasaki H: Fetal nucleated cells in maternal peripheral blood: Frequency and relationship to gestational age. *Hum Genet* 1993; 91:427-432.
- 4 Björkqvist AM, Slunga-Tallberg A, Wessman M, Ylinen K, Knuutila S: Prenatal sex determination by in situ hybridization on fetal nucleated cells in maternal whole venous blood. *Clin Genet* 1994;46:352-356.
- 5 Lo YMD, Wainscoat JS, Gillmer MDG, Patel P, Sampietro M, Fleming KA: Prenatal sex determination by DNA amplification from maternal peripheral blood. *Lancet* 1989;ii:1363-1365.
- 6 Bianchi DW, Flint AF, Pizzimenti MF, Knoll JHM, Latt SA: Isolation of fetal DNA from nucleated erythrocytes in maternal blood. *Proc Natl Acad Sci* 1990;87:3279-3283.
- 7 Camaschella C, Alfarano A, Gottardi E, Travi M, Primignani P, Cappio FC, Saglio G: Prenatal diagnosis of fetal hemoglobin Lepore-Boston disease on maternal peripheral blood. *Blood* 1990;75:2102-2106.
- 8 Lo YMD, Howell PJ, Selinger M, Mackenzie IZ, Chamberlain P, Gillmer MDG, Littlewood TJ, Fleming KA, Wainscoat JS: Prenatal determination of fetal RhD status by analysis of peripheral blood of rhesus negative mothers. *Lancet* 1993;341:1147-1148.
- 9 Geifman OH, Vadnais TJ, DeMaria MA, Weil GJ, Capeless E, Bianchi DW: Detection of fetal HLA-DQ alpha sequences in maternal blood. *Am J Hum Genet* 1993;53(suppl):A250.
- 10 Price JO, Elias S, Wachtel SS, Klinger K, Dockter M, Tharapel A, Shulman LP, Phillips OP, Meyers CM, Shook D, Simpson JL: Prenatal diagnosis with fetal cells isolated from maternal blood by multiparameter flow cytometry. *Am J Obstet Gynecol* 1991;165:1731-1737.
- 11 Bianchi DW, Mahr A, Zickwolf GK, Houseal TW, Flint AF, Klinger KW: Detection of fetal cells with 47,XY,+21 karyotype in maternal peripheral blood. *Hum Genet* 1992; 90:368-370.
- 12 Gänshirt-Ahlert D, Börjesson-Stoll R, Burschky M, Dohr A, Garritsen HSP, Helmer E, Miny P, Velasco M, Walde C, Patterson D, Teng N, Bhat NM, Bieber MM, Holzgreve W: Detection of fetal trisomies 21 and 18 from maternal blood using triple gradient and magnetic cell sorting. *Am J Reprod Immunol* 1993;30:194-201.
- 13 Simpson JL, Elias S: Isolating fetal cells from maternal blood: Advances in prenatal diagnosis through molecular technology. *J Am Med Assoc* 1993;270:2357-2361.
- 14 Cacheux V, Milesi-Fluet C, Tachdjian G, Druart L, Bruch JF, Hsi BL, Uzan S, Nessmann C: Detection of 47,XXX trophoblast fetal cells in maternal blood by fluorescence in situ hybridization after using immunomagnetic lymphocyte depletion and flow cytometry sorting. *Fetal Diagn Ther* 1992;7:190-194.
- 15 Thomas MR, Williamson R, Craft I, Yazdani N, Rodeck CH: Y chromosome sequence DNA amplified from peripheral blood of women in early pregnancy. *Lancet* 1994;343:413-414.
- 16 Wessman M, Ylinen K, Knuutila S: Fetal granulocytes in maternal venous blood detected by in situ hybridization. *Prenat Diagn* 1992;12:993-1000.
- 17 Bianchi DW, Stewart JE, Garber MF, Lucotte G, Flint AF: Possible effect of gestational age on the detection of fetal nucleated erythrocytes in maternal blood. *Prenat Diagn* 1991;11:523-528.
- 18 Mueller UW, Hawes CS, Wright AE, Petropoulos A, DeBoni E, Firgaira FA, Morley AA, Turner DR, Jones WR: Isolation of fetal trophoblast cells from peripheral blood of pregnant women. *Lancet* 1990;336:197-200.
- 19 Covone AE, Kozma R, Johnson PM, Latt SA, Adinolfi M: Analysis of peripheral maternal blood samples for the presence of placenta-derived cells using Y-specific probes and McAb H315. *Prenat Diagn* 1988;8:591-607.
- 20 Bertero MT, Camaschella C, Serra A, Bergui L, Caligaris-Cappio F: Circulating trophoblast cells in pregnancy have maternal genetic markers. *Prenat Diagn* 1988;8:585-590.
- 21 Herzenberg LA, Bianchi DW, Schröder J, Cann HM, Iverson GM: Fetal cells in the blood of pregnant women: Detection and enrichment by fluorescence-activated cell sorting. *Proc Natl Acad Sci USA* 1979; 76:1453-1455.
- 22 Iverson GM, Bianchi DW, Cann HM, Herzenberg LA: Detection and isolation of fetal cells from maternal blood using the fluorescent activated cell sorter (FACS). *Prenat Diagn* 1981;1:61-73.
- 23 Yeoh SC, Sargent IL, Redman CWG, Wordsworth BP, Thein SL: Detection of fetal cells in maternal blood. *Prenat Diagn* 1991;11:117-123.
- 24 Bianchi DW, Yih MC, Zickwolf GK, Flint AF: Transferrin receptor (CD71) expression on circulating mononuclear cells during pregnancy. *Am J Obstet Gynecol* 1994;170:202-206.
- 25 Slunga-Tallberg A, Wessman M, Ylinen K, von Koskull H, Knuutila S: Nucleated erythrocytes in enriched and unenriched peripheral venous blood samples from pregnant and nonpregnant women. *Fetal Diagn Ther* 1994;9:291-295.
- 26 Oski FA, Naiman JL: *Hematologic Problems in the Newborn*. Philadelphia, Saunders, 1982, vol 4, pp 1-31.

- 27 Bianchi DW, Zickwolf GK, Yih MC, Flint AF, Geifman OH, Erikson MS, Williams JM: Erythroid-specific antibodies enhance detection of fetal nucleated erythrocytes in maternal blood. *Prenat Diagn* 1993;13:293-300.
- 28 Loken MR, Civin CI, Bigbee WL, Langlois RG, Jensen RH: Coordinate glycosylation and cell surface expression of glycophorin A during normal human erythropoiesis. *Blood* 1987;70:1959-1961.
- 29 Loken MR, Shah VO, Dattilio KL, Civin CI: Flow cytometric analysis of human bone marrow. I: Normal erythroid development. *Blood* 1987;69:255-263.
- 30 Barclay NA, Birkeland ML, Brown MH, Beyers AD, Davis SJ, Somoza C, Williams AF: *The Leucocyte Antigen FactsBook*. London, Academic Press, 1993.
- 31 Park VM, Bravo RR, Price JO, Elias S: A model system using fetal hemoglobin to distinguish fetal cells enriched from maternal blood. *Am J Hum Genet* 1993;53(suppl):A252.
- 32 Turpeinen U, Stenman UH: Determination of fetal hemoglobin by time-resolved immunofluorometric assay. *Clin Chem* 1992;38:2013-2018.
- 33 Holzgreve W, Garritsen HSP, Gänshirt-Ahlert D: Fetal cells in the maternal circulation. *J Reprod Med* 1992;37:410-418.
- 34 Gänshirt-Ahlert D, Burschky M, Garritsen HSP, Helmer L, Miny P, Horst J, Schneider HPG, Holzgreve W: Magnetic cell sorting and the transferrin receptor as potential means of prenatal diagnosis from maternal blood. *Am J Obstet Gynecol* 1992;166:1350-1355.
- 35 Bianchi DW, Shuber AP, DeMaria MA, Fougner AC, Klinger KW: Fetal cells in maternal blood: Determination of purity and yield by quantitative polymerase chain reaction. *Am J Obstet Gynecol* 1994;171:922-926.
- 36 Alter BP: Biology of erythropoiesis. Fetal cells in maternal blood: Prospects for non-invasive prenatal diagnosis. *Ann NY Acad Sci* 1994;731:36-47.
- 37 Wessman M, Knuutila S: A method for the determination of cell morphology, immunologic phenotype and numerical chromosomal abnormalities on the same mitotic or interphase cell. *Genet (Life Sci Adv)* 1988;7:127-130.
- 38 Weber-Matthiesen K, Deerberg J, Müller-Hermelink A, Schlegelberger B, Grote W: Rapid immunophenotypic characterization of chromosomally aberrant cells by the FICTION method. *Cytogenet Cell Genet* 1993;63:123-125.
- 39 Weber-Matthiesen K, Pressl S, Schlegelberger B, Grote W: Combined immunophenotyping and interphase cytogenetics on cryostat sections by the new FICTION method. *Leukemia* 1993;7:646-649.
- 40 Knuutila S, Larramendy M, Ruutu T, Paetau A, Heinonen K, Mahlamäki E: Analysis of phenotype and genotype of individual cells in neoplasms. *Cancer Genet Cytogenet* 1993;68:104-113.
- 41 Knuutila S, Nylund SJ, Wessman M, Larramendy ML: Analysis of genotype and phenotype on the same interphase or mitotic cell: A manual of MAC (morphology antibody chromosomes) methodology. *Cancer Genet Cytogenet* 1994;72:1-15.
- 42 Gänshirt D, Börjesson-Stoll R, Burschky M, Garritsen HSP, Neusser M, Smeets FWM, Velasco M, Walde C, Holzgreve W: Successful prenatal diagnosis from maternal blood with magnetic-activated cell sorting. *Ann NY Acad Sci* 1994;731:103-114.
- 43 Slunga-Tallberg A, El-Rifai W, Keinänen M, Ylinen K, Kurki T, Klinger K, Ylikorkala O, Knuutila S: Maternal origin of nucleated erythrocytes in peripheral venous blood of pregnant women. *Hum Genet* 1995;96:53-57.
- 44 Zheng YL, Carter NP, Price CM, Colman SM, Milton PJ, Hackett GA, Greaves MF, Ferguson-Smith MA: Prenatal diagnosis from maternal blood: Simultaneous immunophenotyping and FISH of fetal nucleated erythrocytes isolated by negative magnetic cell sorting. *J Med Genet* 1993;30:1051-1056.
- 45 von Koskull H, Gahmberg N: Fetal erythroblasts from maternal blood identified with 2,3-bisphosphoglycerate (BPG) and in situ hybridization (ISH) using Y-specific probes. *Prenat Diagn* 1995;15:149-154.
- 46 Büsch J, Huber P, Pflüger E, Miltenyi ST, Holtz J, Radbruch A: Enrichment of fetal cells from maternal blood by high gradient magnetic cell sorting (double MACS) for PCR-based genetic analysis. *Prenat Diagn* 1994;14:1129-1140.