"METHOD AND DEVICE FOR NON-INVASIVE PRENATAL DIAGNOSIS"

Technical field

The present invention concerns methods for non-invasive prenatal diagnosis, in particular for the identification of genetic and chromosomic disorders in the foetus.

State of the art

Prenatal diagnosis for chromosomic disorders was introduced with the aim of highlighting abnormalities of the chromosomes in the foetus.

So far, the diagnostic certainty of a foetus being affected by a chromosome disorder can be obtained only by means of invasive diagnostic tests, examining the embryonic cells in order to determine the karyotype by means of amniocentesis, sampling of the chorionic villi or cordocentesis.

All these tests are invasive and involve an increased risk of miscarriage. They are therefore usually recommended for women over the age of 35, or women who in a previous pregnancy have had a child affected by chromosome disorders or when ultrasound scanning identifies a foetus with a malformation.

The discovery of the existence of foetal cells, although rare, in the maternal circulation has led many groups to research and develop methods for the isolation and recovery of said cells which permit non-invasive prenatal diagnosis. In particular, there are three main types of foetal cells able to pass through the placental barrier: lymphocytes, trophoblasts and erythroblasts. Of these, research has been directed above all at studying methods for the isolation of foetal erythroblasts from the peripheral maternal blood and trophoblasts, epithelial cells deriving from the placenta. Isolation of the trophoblasts from the peripheral blood is limited by their multinucleate morphology, whereas it has been

demonstrated [8-13] that these cells are present, between the 6th and 15th week of gestation, in transcervical samples. It should be noted that the trophoblasts which migrate from the placenta often adhere to other trophoblasts or maternal cells forming clumps.

The identification of foetal cells has also recently been made possible by molecular biology methods applied directly to non-cultivated foetal cells. Said methods are for example prenatal FISH (Fluorescent In Situ Hybridization) and Quantitative Fluorescent-Polymerase Chain Reaction (QF-PCR). QF-PCR is a method able to identify and simultaneously quantify chromosome-specific DNA sequences which, being applicable to individual cells, has permitted genetic analysis on the basis of a very low number of foetal cells. In literature there are numerous publications including some reviews [1-6, see References at the end of the description], the content of which is incorporated here for the parts necessary for simple reference, concerning the use of QF-PCR in prenatal analyses.

D.W. Bianchi and colleagues developed [14-27] a system of isolation of the foetal nucleated erythrocytes (NRBCs) from maternal blood based on multiparametric scoring; the parameters include two morphological characteristics (roundness and morphology of the nucleus) and two properties of the foetal haemoglobin marking (fluorescence intensity and peripheral luminosity of the cytoplasm). The protocol provides for separation of the mononucleated cells on density gradient and enrichment by depletion of leukocytes (MACS with antibodies for CD15 and CD45) and isolation on cytofluorimeter with FACS method using a gamma anti-haemoglobin antibody. The cells identified via the multiparametric scoring are recovered using a micromanipulator under microscopic observation.

The scoring system is very laborious and use of the micromanipulator causes the loss of part of the cells. In

general this method has shown a 74% sensitivity in the recovery of foetal cells combined with a frequency of false positives of 5%.

Separation of the mononucleated cells on density gradient and enrichment of the sample by MACS with depletion of CD71+ cells, further marked with specific antibodies for the gamma and epsilon foetal haemoglobin chains, is also known from other works [7]. Said marking can, however, produce aspecifics, as there are cases of production of foetal haemoglobin in adult cells, or due to the cross-link between the antibody for the foetal haemoglobin and the adult haemoglobin, caused by the similarity of these haemoglobin chains.

MonaLiza Medical Ltd. (US patent 2005/0181429 A1) developed a prenatal genetic analysis method using transcervical cells. The method is based on the use of a Pap smear cytobrush for the recovery of transcervical samples, which are processed by means of cytocentrifugation for the preparation of slides. The transcervical cells are marked and analysed under the microscope and their location and coordinates on the slide are memorised. The slide is analysed in FISH and the trophoblastic cells are identified using the coordinates obtained previously. The disadvantage of said method is that during the processing for preparation of the slide, part of the transcervical cells are lost, with a no-call due to lack of trophoblasts.

AVIVA-Biosciences Corporation (see for example EP-A-1439897) developed a system of enrichment based on biochips to isolate foetal cells from maternal blood. This method uses a reagent which permits removal of the majority of the erythrocytes, sorting by means of highly specific magnetic beads and a cocktail of specific antibodies for foetal antigens and enrichment by means of high resolution filtering chambers with

pores having variable diameter according to the type of cells to be isolated.

Said method, however, is confined to the selection of foetal cells by means of enrichment of the sample, which does not limit the possibility of having contaminating maternal cells and therefore the consequent risk of an unreliable genetic analysis.

In short, none of the above-mentioned non-invasive methods has so far demonstrated that it can be used as a routine practice for the diagnosis of foetal aneuploidies and/or other chromosome defects.

The aim of the present invention is therefore to provide a method for non-invasive prenatal diagnosis, based on the sampling of an organic maternal fluid, with high probability of containing circulating foetal nucleated cells and their subsequent isolation, in particular a uterine, endocervical or transcervical fluid, or peripheral maternal blood. A further aim of the present invention is to provide a method for prenatal diagnosis that can be automated, without false negatives, and with a low number of false positives and no-calls.

Summary of the invention

The present invention concerns methods and devices for non-invasive prenatal diagnosis, in particular for the identification of genetic abnormalities in the foetus.

According to the present invention, therefore, the diagnosis is performed on the basis of an organic maternal fluid with high probability of containing foetal nucleated cells and the subsequent isolation thereof, in particular from a uterine, endocervical or transcervical fluid or peripheral maternal blood, identifying and analysing the foetal cells present in

it, proceeding according to the method of claim 1.

According to the present invention, the uterine, endocervical or transcervical fluid or the peripheral maternal blood is firstly processed via one or more enrichment stages of the foetal nucleated cells. The method is characterised by the use of a microfluidic system able to individually select, in an easily automatable and repeatable way, single cells from the enriched sample. By means of a microfluidic system of isolation of single cells, it is possible to obtain a set of foetal cells with purity sufficient to perform a genetic diagnosis.

By microfluidic device we mean a device suitable for managing volumes of liquid with a laminar flow.

By microfluidic device we furthermore mean a device which has at least one dimension smaller than 1 mm.

By device able to individually select single cells we mean a device able to perform the selection of one or more single cells, one at a time or simultaneously, on the basis of parameters assessed individually on each cell.

The genetic analysis can then be performed via techniques such as Quantitative Fluorescent PCR (QF-PCR), if necessary using analysis of the cells of the mother for the comparison, FISH, karyotype or Comparative Genomic Hybridization (CGH).

The use of a microfluidic system offers various advantages including the possibility of having disposable systems, without risks of contamination between the different analyses or the need for thorough washing of the equipment. Furthermore the use of a microfluidic system offers the possibility of having automatic or semiautomatic systems, characterised by a high level of reliability.

Further characteristics and advantages of the invention will appear clear from the following description of some non-limiting implementation examples, with reference to the figures of the accompanying drawings.

Brief description of the figures

Fig. 1 shows a summary diagram of the non-invasive prenatal diagnosis method according to the present invention.

Fig. 2 shows an image of the chip with 10X magnification with filter for fluorescence of the DAPI. Three nuclei can be seen, corresponding to three single cells.

Fig. 3 shows an image of the chip with 10X magnification with filter for fluorescence of the FITC. The area photographed is the same as that of fig. 2 and two Hb-e positive cells and one Hb-e negative cell can be seen.

Fig. 4 shows an electropherogram relative to analysis of the chromosome marker AMXY, D21S11 and HPRT. The upper plot refers to the analysis performed on foetal cells recovered from maternal blood, the lower plot refers to the analysis performed on maternal cells. The graph shows the presence of the sex chromosomes X and Y in the foetus and at D21S11 the foetal cells have a first allele inherited from the mother (M) and a different second allele (of paternal origin (P)).

Fig. 5 shows an electropherogram relative to analysis of the chromosome markers D18S391, D13S631 and D21S1411. The upper plot refers to the analysis performed on the foetal cells recovered from maternal blood, the lower plot refers to the analysis performed on maternal cells. The graph shows for the markers D13S631 and D21S1411 the presence of two alleles (normal heterozygote) and contamination by maternal cells can be excluded.

Fig. 6 shows an electropherogram relative to analysis of the marker D21S1437 and D21S1446. The upper plot refers to the analysis performed on foetal cells recovered from maternal blood, the lower plot refers to the analysis performed on maternal cells. The graph shows the presence of two alleles (normal heterozygote) and contamination by maternal cells can be excluded.

Fig. 7 shows an electropherogram relative to analysis of the marker D18S535. The upper plot refers to the analysis performed on foetal cells recovered from maternal blood, the lower plot refers to the analysis performed on maternal cells. The graph shows the presence of two no-call alleles (normal heterozygote).

Fig. 8 shows a flow chart of a preferred embodiment of the method according to the present invention.

Fig. 9 shows the trend of the number of foetal cells in a single cage (NCISCC) according to the mean density of cells per cage (ACPC).

Fig. 10 shows schematically an example of a device for implementation of the method (or of the substantial and characterising part of it) according to the invention.

Detailed disclosure

The subject of the present invention is a method for performing non-invasive prenatal diagnosis.

Collection of transcervical samples

The transcervical samples (TCC) can be taken from different levels of the uterus (external bone, lower part of cervical canal, lower uterine pole, intrauterine cavity) by means of various techniques: aspiration of the cervical mucus,

cytobrush or swab, endocervical lavage and intrauterine lavage (IUL).

Enrichment starting from peripheral blood

The proportion of foetal cells can be enriched using various methods, for example centrifugation on density gradient, consisting of solutions such as Ficoll or Percoll; mechanical enrichment, based on microfabricated filters which select nRBC and empty the sample of RBC; enrichment via dielectrophoretic separation by means of a specific device, the Dielectrophoretic activated cell sorter (DACS); selective lysis, for example selective lysis of the erythrocytes of no interest; immunomagnetic separation, by means of immunomagnetic beads with positive selection (using beads linked to specific antibodies for the foetal population to be recovered) or negative selection (depletion of cellular populations of no interest), and in which the two types of selection can be coupled to increase the specificity of the method (as, for example, in US2006/0051775 - Bianchi); FACS, on cells marked with specific fluorescent antibody for foetal antigens.

The majority of these methods are also automated and all the separation methods can be preceded by separation of total mononucleated cells by means of centrifugation on density gradient or alternatively they can be applied on blood in toto.

In general the process starts from a dilution, but it is not strictly necessary for all the techniques.

Other enrichment techniques

A further technique well known to persons skilled in the art is called MACS by Miltenyi Biotech, or Easy-sep by Stem-cell technologies.

To summarise, considering the case of organic fluid consisting of peripheral maternal blood, enrichment of the blood sample in the population of cells comprising at least one type of foetal cells can be obtained via a process in successive phases with, for example, a first phase in which total mononucleated cells are separated from the maternal sample, previously diluted in PBS/EDTA by means of Ficoll density gradient centrifugation. Obviously, as an alternative, any one of the other methods summarised in figure 1 could be used, for example enriching of the maternal sample via a selection of cells made on the basis of at least one parameter chosen from the group consisting of:

a. density;
b. morphology;
c. electrical properties;
d. chemical properties;
e. mechanical properties;
f. expression of surface antigens;
g. expression of intra-cytoplasmatic antigens;
h. dielectric properties;
i. magnetic properties;

or combinations of the same.

Subsequently enrichment of the foetal cells is further obtained by means of a second phase in which the positive or negative selection of cells is made, for example expressing the CD71, from the mononucleated cells recovered in the first step. Obviously the second enrichment step can comprise a selection made on the basis of at least one of the following characteristics of the population of cells comprising at least one type of foetal nucleated cells:

a. express the CD71 surface antigen (as already described and which represents the preferred form of the invention);
b. express the CD34 surface antigen;
c. express the GPA surface antigen;
d. not express the CD14 surface antigen;

e. not express the CD15 surface antigen;
f. not express the CD45 surface antigen.

Furthermore said second phase of enriching the sample in the population of cells comprising at least one type of cells can be performed via one of the following techniques:
a. MACS or Magnetic Activated Cell Sorter;
b. DACS or Dielectrophoretic Activated Cell Sorter;
c. FACS or Fluorescence Activated Cell Sorter.

Marking of the foetal cells
Immunostaining of trophoblasts
If the sample is a TCC sample, before marking, the sample is incubated with acetyl-cysteine and agitated vigorously to dissolve the clumps and obtain a suspension of single cells.

For identification of the foetal cells, a marking with a specific antibody for the foetal cells (able to discriminate them from the maternal ones) is used, proceeding as in the known art. The trophoblasts can be marked using a variety of antibodies directed against specific antigens:
HLA-G, NDOG-5, BC1, Factor XIII, FDO202N, JunD, Fra2, HASH2 and PP5 (placental protein), specific for the extravillous trophoblasts;
FT1.41.1, I03, NDOG-1 and AB-154, specific for the syncytium trophoblasts;
CK-7 (cytokeratin-7), CHL1 (CD146), CHL2, H315, HLA-C, aHCG, IGF-II, PAI-1 and p57, expressed on the trophoblasts;
PLAP (placental alkaline phosphatase), AB-340 and D6, expressed on syncytium trophoblasts and cytotrophoblasts;
Tapasin and CAR, specific for invasive or extravillous but not villous trophoblasts;
PLAC1, PLAC4, PLAC8 and PLAC9, placental specifics, in particular of the trophoblast lineage cells;
PAR-1 (protease activated receptor), expressed on placental

cells from the 7th to the 10th week of gestation;
GLUT-12 (glucose transporter protein), expressed on syncytium trophoblasts and extravillous trophoblasts from the 10th to the 12th week of gestation;
NDPK-A (nucleoside diphosphate kinase A), expressed on extravillous trophoblasts during the first three months of pregnancy.

Immunostaining of erythroblasts from samples of peripheral maternal blood
For identification of the foetal cells, a marking with a second specific antibody for the foetal cells (able to discriminate them from the maternal ones) is used, different from the antibody used for the enrichment which, as described above, is not necessarily specific for the foetal cells.

In this case the following can be used:

- antibodies that recognise surface antigens (like i-antigens);
- intra-cellular antigens (for example, the globin chains γ or ε. In these cases the cells are preferably fixed and permeabilised, as in the known art, to permit good marking.

It should be noted that although from previous studies use of the anti-i-antigen antibody to mark foetal cells is known, said antibody was used directly in a device based on density gradient. Marking of the foetal cells by means of i-antigen antibody preceded by enrichment of the CD71+ cells has the advantage of performing a pre-selection of the foetal cells (sample enriched in foetal erythroblasts) and facilitating identification of the cells of interest, furthermore obtaining a very sensitive and specific marking.

Cells already marked
The foetal cells can be already marked if an antibody which is specific for the foetal cells (able to discriminate them from

the maternal ones) and is fluorescent or conjugated with a fluorescent bead or conjugated with a fluorescent secondary or tertiary antibody has been used in the enrichment. In this case the enriched sample can be injected directly into the microfluidic device able to select single cells, since it is already possible to identify the foetal cells.

Isolation of single foetal cells

Subsequently, the sample containing the cells is placed in a microfluidic device able to individually select single cells, of any known type. For said purpose a dielectrophoretic isolation can be used (DEPArray, using for example the techniques described in PCT/IB2007/000963 or in PCT/IB2007/000751, or [31] and [32]), or opto-electronic traps or optophoretic isolation or laser tweezers [28-30]. The content of said documents [28-30] and [31-32] is incorporated here for the parts necessary for simple reference.

Identification of the cells of interest can be performed for example by sensors:

- external
- optical such as a fluorescence microscope,

or also

- internal
- optical, as illustrated in the patents PCT/IB2006/000636 and WO2007010367, which describe an integrated method of identification of the fluorescent cells
- impedentiometric, as illustrated in the patents PCT/IB2006/000636 and WO2007010367 to identify dielectric beads associated with cells.

Genetic analysis

Various types of analysis can be performed on the foetal cells recovered permitting genetic or chromosomic characterisation at different levels of resolution and sensitivity and according to the diagnostic purpose of the study.

In the event of supposed chromosome disorders, analysis of the karyotype with classic or molecular method (FISH) or the study of chromosome markers by means of QF-PCR can be performed. The acquisition or loss of genetic material can also be investigated by means of Comparative Genomic Hybridization.

Examples of preferential embodiments of the invention

By way of non-limiting example of the object of the invention, a preferential embodiment of the method according to the present invention is given, following the flow chart indicated in Fig. 8.

Collection of sample

10 ml of peripheral blood are taken from a pregnant woman.

Enrichment

The preferred embodiment of the invention comprises a process in successive phases having a first phase in which total monoucleated cells are separated from the maternal sample.

Said phase comprises a 1:1 dilution of the maternal blood with PBS pH 7.2. The sample diluted is then stratified on a single Ficoll gradient 1.077 g/ml and centrifuged at a speed of 300 g for 30 min at 22°C. The ring of cells that has accumulated above the Ficoll is collected and transferred to a sterile test tube.

According to a characteristic of the present invention, a part of the blood or mononucleated cells is not further processed and is used for analysis of the maternal DNA.

Enrichment of the foetal nucleated cells is then further obtained via a second phase, in which cells expressing the CD71 are positively selected from the mononucleated cells recovered in the first step. The positive selection is

performed by means of immunomagnetic separation (MACS - Miltenyi Biotec) with the use of anti-CD71 antibodies conjugated with magnetic beads.

For the immunomagnetic separation, the cells are re-suspended in 80 μl of PBS every 10^7 cells and then 20 μl of anti-CD71 micro-beads (Miltenyi Biotec) are added every 10^7 cells. After an incubation of 15 min at 4°C, the cells are passed through a column connected to a magnetic field which retains the positive CD71 cells. The cells retained are then eluted from the column and used for the subsequent phase.

Isolation

The CD71+ cells (positive for CD71) thus obtained are fixed with formaldehyde 3.7% for 15 min at 22°C. The cells fixed are then permeabilised with a solution of NP-40(Sigma Aldrich) 0.1% in PBS. An antibody (1 μg/ml) which recognises the gamma chain of the foetal hemoglobin conjugated with the fluorochrome FITC is added to the permeabilised cells.

According to an important aspect of the invention, the sample is furthermore, in combination, countermarked with DAPI (or other suitable marker) to show the nuclei of all the cells.

Before being loaded in the chip for dielectrophoretic manipulation and isolation of the foetal cells, the sample undergoes a quality control to verify the fluorescence intensity of the marking and the total cellular content. A portion of the marked sample is re-suspended in a minimum specific buffer volume useful for the dielectrophoretic manipulation and is loaded in a device for quality control of the sample and examined under the fluorescence microscope: the fluorescence intensity of the cells is observed in the various channels and a count is performed of the cells marked in DAPI (total nucleated cells). If the cellular concentration is above the optimal concentration for correct operation of the

device for isolation of the single cells, the sample is diluted to obtain the required concentration; if the total number of cells is too low to allow the recovery of a minimum number of foetal cells, the foetal cells are not recovered (no-call result).

For calculation of the optimal concentration of cells we refer to a specific commercial device (DEPArray™, Silicon Biosystems SpA), WO0069565, based on mobile dielectrophoresis cages, in particular the model CONV600k comprising 100,000 cages.

A preferential method of use of said device provides for the following steps:

1. Select the cages containing a foetal cell (positive to marking).
2. If the cell is part of a cluster, i.e. it shares the cage with other non-foetal cells, divide the cells of the cluster into separate cages until the foetal cell is isolated in a cage not shared with other non-foetal cells. If the foetal cell does not separate from the other non-foetal cells, discard it.
3. Recover all the foetal cells in one single cage.

A further alternative preferential method of use of said device provides for the following steps:

1. Select the cages containing a foetal cell (positive to the marking).
2. If the cell is part of a cluster, discard it from the list of cells to be recovered.
3. Recover all the foetal cells in a single cage.

In this second case, the choice of the mean density of cells per cage (ACPC) to be injected in the chip is made taking account of the number of total cells present in the sample (NCELLSTOT) and the expected percentage of foetal cells (PCI).

In fact, when the ACPC increases, the number of foetal cells present in the manipulation chamber of the chip increases. However, the probability of each cell belonging to a cage with single cell decreases, and the number of foetal cells in a single cage (NCISCC) therefore reaches a maximum for ACPC~=1. Said value is independent of PCI, and can be defined a normalised value of NCISCC with respect to the theoretical maximum for ACPC=1, the trend of which is shown in the graph of Fig. 9. Said concentration maximises the number of cells that can be recovered at each flushing of sample into the manipulation microchamber. This is a good choice if the total number of foetal cells that can be recovered is sufficient for the genetic analysis downstream.

As ACPC increases, there is a growing monotonic increase in the percentage of cells to be discarded due to the fact that they share the cage with other non-foetal cells, as illustrated in Fig. 9 in reference to the normalised value with respect to the number of foetal cells present in the manipulation microchamber (normalized cell-waste).

If the number of recoverable cells is below the minimum required for the analysis downstream, the sample can be diluted and a greater number of flushings performed to recover a greater number of foetal cells.

The optimal ACPC value to recover a sufficient number of foetal cells with the lesser number of flushings can be calculated on the basis of the statistical analysis determined above, for example with calculations based on the expected number of foetal cells and the minimum number of cells for the genetic analysis, therefore identifying a minimum recovery efficiency (ratio between cells in single cage/cells in cage with other cells) of the foetal cells present in the sample. From said efficiency a maximum value of ACPC can be deduced, hence a minimum number of flushings to process the entire

sample with said recovery efficiency.

The sample is then loaded in a chip for the isolation of cells by means of mobile dielectrophoresis cages (DEPArray™ WO0069525, Silicon Biosystems SpA, for example as part of a package or overall device like the one schematically illustrated in figure 10) and undergoes scanning, identification and selection, sorting and recovery of the foetal cells. The caged cells are observed (scanning) automatically or manually under a microscope with three different fluorescence channels (or in three different wavelengths): in the non-limiting case described here, the blue channel allows verification of the presence of the nucleus and if necessary its morphology (e.g. marking with DAPI) and the green channel highlights the cells that have been marked with the specific foetal antibody which is conjugated with a fluorophore emitting in the green wavelength (e.g. FITC). According to one aspect of the invention, a third channel is also used, different from the first two (e.g. the channel emitting in the red wavelength, as for the filter used to detect the fluorescence of the TRITC), and for which no fluorescent marker has been used; this permits identification of the autofluorescent cells, for which any signal detected in the DAPI and green channels would not be specific.

Alternatively the third channel can be used to highlight cells conjugated with an antibody linked to a fluorophore, like for example the CD45, to identify the cells to be discarded.

The selection of cells is therefore made by selecting the cages that contain one single nucleated cell (positive to the DAPI, Fig. 2), which have a strong specific foetal antibody signal (positive to the FITC/Alexa, Fig. 3), and which have low or nil autofluorescence, detected on other channels (e.g. red channel).

To further improve the selectivity of the method, the fluorescent markers, in particular the foetal marker, instead of consisting of simple fluorescent molecules, can consist of fluorescent beads conjugated with antibody able to recognise the cells of interest, in this case the foetal cells.

The cells are recovered in a few microlitres (< 40 microlitres) in a 0.2 millilitre PCR tube.

Genetic analysis

From said cells thus obtained the DNA is extracted which is amplified and analysed for the presence of chromosomic aneuploidies, preferably operating in the same microfluidic device as the one used for the selection and, if necessary, already used previously for at least part of the enrichment process (for example in the case of use of the DACS technology); in this case the device can resemble the one illustrated in figure 10.

The equipment contains an array of electrodes as in the known art, but is characterised by a main microchamber (CHM) and a plurality of secondary microchambers (CHJ), all delimited on at least one face by one single chip or by a plurality of separate chips, bearing an array of electrodes that can be activated. The main microchamber can be filled with a sample comprising at least one cell via the relative inlets (IM1) and outlets (OM1). Each secondary microchamber (CHJ) is preferably of substantially greater dimensions but comparable to those of a cell. Preferably each secondary microchamber is connected to the main microchamber via a configuration channel (length and/or form) sufficient to forestall (prevent or at least limit) dispersion of the sample by diffusion and contamination towards other microchambers, in the time necessary for the analysis. According to the example illustrated, there is a plurality of secondary microchambers for the lysis connected to a channel for the capillary electrophoresis on chip, for

example with cross joint. Alternatively a series of channels can be provided for the capillary electrophoresis with double T junction, according to the known art. Optionally, at the end of the channel for the capillary electrophoresis, there is an integrated sensor, of the impedentiometric and/or optical type, able to produce an electropherogram based on the migration time of the compounds analysed from the intersection (cross or double T) to the sensor itself. According to figure 10, in particular, each microchamber of the plurality of microchambers is connected to a capillary for the electrophoresis (CAPJ) via a fluidic outlet (OJ) of each secondary microchamber.

Extraction of the DNA of the recovered foetal cells is performed by alkaline thermolysis.

The determination of chromosome alterations is performed via analysis of the STR (Short Tandem Repeat) or microsatellites by means of QF-PCR. The technology of fluorescence capillary electrophoresis permits the simultaneous analysis of several STRs via the appropriate choice of fragments of DNA marked with different fluorescent molecules.

At least three different STRs, with high heterozygosis frequency in the population, of each of the chromosomes 13, 18 and 21 and three markers of the sex chromosomes are amplified in multiplex-PCR. In the case of a no-call, for example due to the presence of STR in homozygosis, a further STR of the same chromosome is amplified. The STRs analysed are: D21S11, D21S1410, D21S1411, D21S1412, D21S1435 and D21S1446 for analysis of the chromosome 21; D13S631, D13S634, D13S258, D13S305 and D13S742 for the chromosome 13; D18S535, D18S386, D18S391, D18S858 and D18S51 for the chromosome 18; for analysis of the sex chromosomes the markers AMXY and SRY, and the STR X22, DXYS218, DXS6803, DXS6809, DXS8377, HPRT and SBMA are analysed.

Parallel to amplification of the DNA of the foetal cells, the maternal DNA is analysed to recognise, according to one aspect of the invention, the presence of possible maternal contamination of the foetal cells or a possible external contamination of the QF-PCR (Fig. 4-7).

In the electropherograms, obtained from the capillary electrophoresis of the product of PCR with the automatic sequencer (for example with ABI prism 310), the areas and the dimensions of the peaks corresponding to the various alleles of the amplified microsatellites are analysed. Simultaneous analysis of the maternal DNA can help as a control to interpret the result of the genetic analysis, helping to identify possible cases of laboratory contaminations and contamination of the recovered foetal cells with maternal cells.

The genetic analysis phase can also be performed, according to a possible variation of the invention, by means of karyotype, in which case the phase of enrichment of at least one population of cells comprising at least one type of foetal nucleated cells furthermore comprises the phases of:

I. blocking the cells in metaphase.
After stopping the cells in metaphase, fixing and permeabilisation are performed for identification of the foetal cells by means of an intra-cytoplasmatic antibody.

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CLAIMS

1. Method for non-invasive pre-natal diagnosis comprising the following steps of:

a. obtaining a sample of an organic fluid having a high probability of containing foetal nucleated cells from a pregnant woman;

b. enriching said sample of organic fluid in at least one population of cells comprising at least one type of foetal nucleated cells;

c. isolating at least one cell from among said at least one type of foetal nucleated cells;

d. performing a genetic analysis on said at least one cell isolated from among said at least one type of foetal nucleated cells in order to highlight at least one genetic characteristic of said at least one foetal nucleated cell suitable for permitting said diagnosis;

characterised in that said step of isolating at least one cell from among said at least one type of foetal nucleated cells is performed by individually selecting single cells in a microfluidic device designed for said purpose.

2. Method as claimed in claim 1, characterised in that said step of isolating at least one cell from among said at least one type of foetal nucleated cells is performed by capturing single cells, preferably each at a specific site of a plurality of sites of said microfluidic device positioned in the microfluidic device according to an array, and subsequently selecting single cells from among the cells captured, on the basis of at least one parameter which can be detected by means of a sensor internal or external to the microfluidic device.

3. Method as claimed in claim 1 or 2, characterised in that said step of enriching said sample of said organic fluid comprises a selection of cells made on the basis of at least

one parameter chosen from the group consisting of:

a. density;

b. morphology;

c. electrical properties;

d. chemical properties;

e. mechanical properties;

f. expression of surface antigens;

g. expression of intra-cytoplasmatic antigens;

h. dielectric properties;

i. magnetic properties;

or combinations of the same.

4. Method as claimed in claim 3, characterised in that said step of enriching said sample of said organic fluid in at least one population of cells comprising at least one type of foetal nucleated cells comprises the step of treating said sample of said organic fluid in order to separate the nucleated cells and consequently enrich it in nucleated cells.

5. Method as claimed in claim 4, characterised in that said step of treating the sample of said organic fluid in order to separate the nucleated cells and consequently enrich it in nucleated cells comprises the step of centrifuging said sample of said organic fluid in density gradient.

6. Method as claimed in any one of the preceding claims characterised in that said enrichment step comprises a selection made on the basis of at least one of the following characteristics of said population of cells comprising at least one type of foetal nucleated cells:

a. Express the surface antigen CD71;

b. Express the surface antigen CD34;

c. Express the surface antigen GPA;

d. Not express the surface antigen CD14;

e. Not express the surface antigen CD15;

f. Not express the surface antigen CD45

7. Method as claimed in one of the preceding claims, characterised in that it comprises the step of diluting said sample of said organic fluid after said step of enrichment and before said step of isolation of at least one cell from among said at least one type of foetal nucleated cells; said dilution is performed on the basis of a count of the number of nucleated cells present in a portion of pre-established volume of said sample, which is separated from the sample of said organic fluid immediately after said enrichment step.

8. Method as claimed in any of the preceding claims characterised in that at least one or both said steps of enrichment and performance of a genetic analysis are carried out in combination with said step of isolation within said microfluidic device used for performing the isolation step.

9. Method as claimed in claim 8, characterised in that a microfluidic device is used provided with a plurality of different chambers, separated from one another and hydraulically connected, delimited on at least one face by one single chip or by a plurality of separate chips.

10. Method as claimed in any one of the preceding claims, characterised by the phase of comprising a phase of marking of the foetal cells with a specific antibody for the foetal cells suitable for discriminating them from the maternal ones, performed preferably by using a variety of antibodies directed against a specific antigen selected from the group consisting of:

- antibodies that recognise foetal trophoblast antigens such as: HLA-G
- antibodies that recognise foetal surface antigens such as: i antigens
- antibodies that recognise foetal intracellular antigens

such as: haemoglobin chains γ and ε

11. Method as claimed in claim 10, characterised in that said step of isolating at least one foetal cell is performed by individually selecting single cells present in said microfluidic device, previously marked with said at least one marker.

12. Method as claimed in one of the claims 10 or 11, characterised in that said at least one marker is a fluorescent marker.

13. Method as claimed in one of the claims 10 or 11, characterised in that said at least one marker is a fluorescent bead conjugated with said at least one specific antibody for foetal cells.

14. Method as claimed in one of the claims from 11 to 13, characterised in that it comprises the phase of marking with a first marker specific for the cell nucleus, nucleated cells of said blood sample, and marking with a second marker, distinguishable from the first, at least said population of cells comprising at least one type of foetal nucleated cells, in order to select in said step of isolating at least one foetal cell only single cells which highlight the presence of both said first and second marker.

15. Method as claimed in claim 14, characterised in that both said first and second marker are fluorescent markers having emission in a first and a second wavelength respectively, different from each other, preferably in the blue and green wavelength respectively.

16. Method as claimed in claim 15, characterised in that said phase of individually selecting single cells in said step of isolating at least one foetal cell is performed detecting also

the emission of a third wavelength, preferably in the red wavelength, which can be generated by autofluorescence by said cells in said sample of said organic fluid, in order to select only cells that emit in both the first and second said wavelength but which, at the same time, do not emit in said third wavelength.

17. Method as claimed in one of the claims from 10 to 16 characterised in that it comprises, after marking the cells, a phase of fixing and permeabilisation for identification of the foetal cells by means of an intra-cytoplasmatic antibody.

18. Method as claimed in one of the preceding claims, characterised in that said phase of genetic analysis is performed by means of QF-PCR and comprises a phase of comparison between genetic information carried by said at least one foetal nucleated cell and at least one maternal nucleated cell.

19. Method as claimed in any one of the preceding claims, characterised in that said step of enriching said sample of said organic fluid in at least one population of cells comprising at least one type of foetal nucleated cells comprises the phases of placing in a culture said sample of said enriched organic fluid in said at least one population of cells comprising at least one type of foetal nucleated cells and subsequently repeating on said sample an enrichment phase.

20. Method as claimed in any one of the preceding claims, characterised in that said sample of said organic fluid contains cells selected from the group consisting of: uterine, transcervical or endocervical cells or peripheral maternal blood.

21. Method as claimed in any one of the preceding claims, characterised in that said microfluidic device for the

selection of single cells is a disposable device.