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A cell-based NIPD (Non-invasive prenatal diagnosis) procedure to select fetal cells from pregnant women maternal blood

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ABBREVIATIONS

AFS: amniotic fluid sampling
BMI: body mass index
cfDNA: cell-free fetal DNA
CMA: chromosomal microarray analysis
CNA: copy-number aberration
CNV: copy-number variation
CPM: confined placenta mosaicism
CVS: chorionic villous sampling
DAPI: 4′,6-diamidino-2-phenylindole
DLRS: Derivative Log Ratio Spread
cEVT; extravillous trophoblast
FACS: flow fluorescence-activated cell sorting
FBS: fetal blood sampling
FISH: fluorescent in situ hybridization
FN: False Negatives
fnRBCs: Fetal nucleated red blood cells
FP: False Positives
FTC: trimester combined test
gDNA: genomic DNA
LCM: laser capture microdissection
LTC: long-term culture
MACS: Magnetic Activated Cell Sorter
Mb: Megabases
MX: monosomy X
NGS: next-generation sequencing
NIPD: Non-invasive Prenatal Diagnosis
NIPT: Non-invasive Prenatal Testing
NT: nuchal translucency
PAPP-A: Pregnancy-associated plasma protein A
PCR: polymerase chain reaction
RATs: rare autosomal trisomies
RBCs: red blood cells
RMSE: root-mean-square error
STR: Short Tandem Repeats
T13: trisomy 13
T16: trisomy 16
T18: trisomy 18

T21: trisomy 21
TN: True Negatives
TP: True Positives
US: ultrasound scan
WBCs: white blood cells
WGA: whole genome amplification

CHAPTER 1

General introduction

1.1 Prenatal diagnosis

Prenatal diagnosis represents a path aimed at obtaining information about the embryo or foetus during the course of pregnancy, using a set of instrumental, laboratory and multidisciplinary professional investigations.

Prenatal diagnosis can be achieved both through invasive and non-invasive methods. Fetal pathological conditions, leading to a diagnostic suspicion or a diagnosis of certainty, can be of genetic or non-genetic origin. It is estimated that genetic disorders or birth defects affect about 3% to 5% of the pregnancies[1]. Diseases of non-genetic origin include all those pathologies for which there is no established link with a chromosomal or genetic alteration. Such diseases include congenital malformations, developmental abnormalities of an organ, infections, etc. and are mainly investigated through non-invasive methods: ultrasound, nuchal translucency and biochemical screening. Although these methods are extremely useful in the monitoring of pregnancy, they are inadequate for the diagnosis of many diseases of genetic origin (both chromosomal or monogenic), for which the only possibility is represented by invasive techniques such as fetal blood sampling

(FBS), chorionic villous sampling (CVS) and amniocentesis. Invasive prenatal diagnosis through amniocentesis and CVS began in the late 1960 and 1980 respectively. The establishment of the correct number of human chromosomes by Tijo and Levan in 1956[2] opened the way to define the various chromosome aneuploidies and allowed the association between clinical phenotypes and chromosome abnormalities. Subsequently, Steele and Breg published the first study where they demonstrated the possibility to culture amniotic fluid for chromosome analysis: it was the first evidence in the foundation of fetal diagnostic[3]. Over the past four decades, these invasive techniques have been enormously improved, minimizing the risk of abortion following fetal material withdrawal; however, the risk of miscarriage has not been completely eliminated, and is currently rated around 0.5-1% [4,5]. For this reason, prenatal diagnosis through invasive methods is generally proposed to a limited group of women. In particular, for several years it was addressed to women aged 35 years or more, since after this threshold the risk of having a child affected by chromosomal abnormalities dramatically increases. Indeed, the possibility of having a newborn affected by Down syndrome is approximately 1 in 1,250 for women aged 25 or less, increasing to about 1 in 100 for women above 40

years old[6]. This age-based approach allows detecting only 30-40% of Down syndromes because most of fetuses with trisomy 21 are born of women under the age of 35 and younger women generally have more children than older ones. However, women less then 35 years old and with no particular medical indications (anomalies detected by ultrasound, positive screening test or known family history of genetic disorders) are usually not offered the possibility of having an amniocentesis or CVS, since the risk of carrying a fetus with chromosomal abnormalities is lower than that of losing it because of the procedure itself. As such, numerous efforts have lately been spent to develop non-invasive approaches for genetic disorders analysis, to be offered to all pregnant women population, regardless their inherent risk of having an affected child. These approaches are called screening tests, because they identify cases with a higher probability for chromosomal abnormalities among the general population, thus distinguishing pregnancies at low and high risk for these conditions. There are currently several types of screening tests and the most frequent will be described later.

1.2 Classes of genetic disorders

Genetic disorders derive from an alteration of the genetic heritage and cytogenetics and genetics methods are used for their diagnosis. Generally, genetic disorders can be caused by:

- Chromosomal abnormalities (changes in the number or structure of entire chromosomes);
- Microduplication or microdeletion syndromes (sub-microscopic deletions or duplications of contiguous genes on particular parts of chromosomes);
- Monogenic diseases (mutation in one single gene).

1.2.1 Chromosomal abnormalities

Chromosomal abnormalities include aneuploidy (classified with one or more extra or missing chromosomes), translocations, duplications, and deletions; they represent the most frequent genetic disorder, affecting nearly 1% of newborns. Diagnosis is possible performing fetal karyotype on chorionic villi (obtained by chorionic villus sampling) or amniocytes (obtained by amniocentesis). The severity of chromosomal abnormalities is variable and depends on the chromosome involved and the type of alteration. Some chromosomal anomalies are prematurely lethal because they are

associated with the presence of serious malformations, while other anomalies are related to a less unfavorable expectation and quality of life, resulting in minor physical and mental handicaps; still, some can be completely asymptomatic or leading to infertility.

Trisomy 13, 18 and 21 are the most recurring chromosomal abnormalities and represent approximately 50-70% of karyotype anomalies, with incidence varying according to maternal age[7]. Trisomy 21, or Down syndrome, is characterized by the presence of an extra chromosome 21. It is the most frequent genetic cause of mental retardation and is associated with an increased risk of congenital malformations (cardiac, intestinal, etc.). Affecting about one newborn out of 700[8], it can occur in two ways:

- maternal non-disjunction during the meiotic division (96% of cases). In this case there are 47 chromosomes with 3 separate chromosomes 21 and this condition is not inherited from the parents[9];
- unbalanced translocation (4% of cases): occurs when one chromosome breaks off and attaches to another chromosome. In this case, there are three 21 chromosomes, but one of the 21 chromosomes is attached to another chromosome (translocation Down's syndrome). In about half of cases, the

condition is inherited from one of the two parents presenting a balanced translocation, meaning without loss or gain of the chromosome 21 material. There are no big differences between the patients who have translocation Down's syndrome compared with those who have three separate copies of chromosome 21.

Trisomy 18 (or Edwards's syndrome) and trisomy 13 (or Patau's syndrome) present a supernumerary chromosome 18 and 13 respectively. Both conditions are most of the times lethal, characterized, by severe mental retardation and multiple malformations. They can also result in live births, though with a significantly lower incidence (1/5,000 births).

Sex chromosome aneuploidies are less common than autosomal aneuploidies. In case of an extra chromosome they include Klinefelter's syndrome (47, XXY; it occurs in 1/600 male livebirths) and XYY syndrome (47, XYY; 1/1000 male livebirths). These type of chromosomal abnormalities are generally associated with mild or no symptoms. Indeed, the only known viable monosomy is monosomy X (Turner's syndrome) with a prevalence estimated at 1 out of 2000 female livebirths[10].

1.2.2 Microduplication or microdeletion syndromes

Microdeletion and microduplication syndromes are genetic anomalies characterized by submicroscopic loss or duplication of contiguous genes localized in particulars region of chromosomes. To date, microduplication syndromes are not well characterized, and for many of them the significance is still unclear. Both microdeletions and/or microduplications are quite rare in the general population, with a prevalence varying from 1 case out of 2,000 (like the DiGeorge syndromes) to 1 case out of 50,000 live births. However, unlike chromosomal abnormalities, such syndromes are not related to the increase in maternal age. Hence, microdeletions involve smaller segments of the chromosomes (typically 1 to 3 Megabases) compared to the chromosomal abnormalities, they are not detectable by standard karyotypes. Indeed, only fluorescent in situ hybridization (FISH) and chromosomal microarray analysis (CMA) are used for detection due to the higher resolution of these methodologies and it is likely that in the future, next-generation sequencing (NGS) will replace CMA.

1.2.3 Monogenic diseases

Monogenic diseases are due to an alteration of one or more genes.

They are responsible for several clinical syndromes such as cystic fibrosis, thalassemia, muscular dystrophy, etc. which are individually rare in the general population, however due to the hereditary transmission, the recurrence risks is high. Additionally, genetic disorders can also arise ex novo. In this case the mutation is not present in the parents, but arises in the fetus which, in turn, can pass it on to children. Currently, there is not a laboratory procedure which allows the identification of all monogenic diseases and, in addition, not all of them are diagnosable in the prenatal period. Therefore, prenatal diagnosis of monogenic diseases is performed in a "targeted" way, under specific indication of geneticist and it is usually performed through the polymerase chain reaction (PCR) for amplification of specific sequences of the gene related to the disease.

1.3 Current methods for prenatal diagnosis of genetic disorders

Diagnosis of genetic disorders directly identifies or excludes the presence of the genetic disorders. The diagnostic methods are invasive as they involve the removal of fetal or placental material for genetic analysis (karyotype, chromosomal microarray or other).

1.3.1 Chorionic Villous Sampling

Chorionic Villous Sampling consists in the aspiration of a small quantity of chorionic villi (placental tissue). The procedure is usually performed from 10^{th} to 13^{th} week of pregnancy, although it is possible to execute it in more advanced gestational periods. It is performed on an outpatient basis and is preceded by an ultrasound analysis to evaluate the fetal heartbeat, date of pregnancy and nuchal translucency measurement. The sampling is done under constant ultrasound guidance and can be transcervical or transabdominal. Chorionic villus consists of the outer layer of cytotrophoblast cells and the inner mesenchymal core, both of fetal origin. Cytogenetic analysis can be carried out using either one or both methods. The first is called direct method and analyzes the cytotrophoblast cells in spontaneous division, providing results within 48 hours, allowing to

minimize maternal cells contamination from the decidua, since they have lower mitotic index compared to cytotrophoblasts. The culture method (or long term method) analyzes the mesenchymal cells and requires 7 to 10 days for results. This method usually provides better karyotype preparations compared to the direct one, although maternal contamination could be higher. The complete exam result is provided on average after 21 days. If the analysis is carried out with the direct method only, the possibility of a false negative is considered very rare (1/1,000 to 1/3,000). Using direct analysis instead, as it happens in most of the cases, together with long term analysis, false negative results are even more rare, estimated in one case out of 20,000. Conversely, the possibility of a false positive result cannot be excluded in about 1-2% of cases, almost always due to the presence of placental mosaicisms, a condition characterized by the presence of cells with normal chromosome associated with cells with chromosomal abnormalities and, rarely, for a non-mosaic chromosomal abnormality. Usually, a large amount of fetal DNA (5-30 mg) is obtained from the villi for the analysis of a wide range of genetic conditions, but in some cases (1-2%) the amount of chorionic villus sampling is not sufficient and, therefore, it becomes necessary to perform a second withdrawal. The reasons could be related to an

insufficient quantity of chorionic villi collected, a cell growth failure in culture, or other more sporadic causes. In addition, in about 1-2% of cases, cytogenetic analysis could show the presence of cellular mosaicism (not allowing a precise diagnostic result), thus it may be appropriate to proceed with amniocentesis for clarification. Risks associated to the CVS procedure include miscarriage as already mentioned, uterine infections (very rarely) and Rh sensitization.

1.3.2 Amniocentesis

Amniocentesis consists in the withdrawal of amniotic liquid form the uterus, in which desquamation cells coming from the amniotic sac and the fetus (skin, digestive tube system, respiratory and urogenital tract) are suspended. Such cells can be used for several genetic analyses. Normally, the sampling is carried out starting from the 15th week of pregnancy until the 21st week. The procedure is performed on an outpatient basis. The sampling is performed transabdominally under constant ultrasound guidance. Usually 16-20 ml of amniotic fluid are aspirated and sent to the laboratory for analysis. In very rare cases (0.2%), failure can occur in the cytogenetic analysis and it is necessary to repeat the sampling. The results provided by amniocentesis are highly reliable. The risk of

false negative, mostly due to maternal cells contamination, is estimated in 1 case out of 5,000 (2015 SIEOG Guidelines). Risks associated with this procedure are similar to those of the CVS, but also include leaking of the amniotic fluid through the vagina, needle injury to the fetus (very rare) and the possibility to transmit an infection (such as hepatitis C, toxoplasmosis or HIV) from mother to fetus.

1.3.3 Fetal blood sampling

Fetal blood sampling is done transabdominally like the amniocentesis. Also in this case, a preliminary ultrasound examination is performed to confirm the gestational period, the number of fetuses, the vitality and morphology of these, the quantity of amniotic fluid and the placental location. A needle similar to that of the amniocentesis is inserted though the abdomen and uterine wall, pushed into one of the vessels of the umbilical cord (in the site where the cord inserts in the placenta) for fetal blood aspiration, which is sent to the laboratory for the analysis. The whole procedure is performed under ultrasound control. The procedure, also called Percutaneous Umbilical Blood Sampling (PUBS), cordocentesis or Umbilical Vein sampling, it is normally carried on from the 18th -

20th week of pregnancy onwards. Of course, the later the procedure is, the easier the collection of the material, as the size of the umbilical cord vessels grow with the pregnancy progress. Cordocentesis is mainly indicated for rapid karyotype, inconclusive results of CVS and amniocentesis, evaluation of fetal hematological condition or infection. In the event that the pregnancy is in an advanced period and there is the need for a rapid study of fetal chromosomes, cordocentesis is certainly indicated, given that the outcome of the examination can be obtained in 4-5 days. The procedure can also be used to administer drugs or carry out blood transfusions to the fetus via the umbilical cord. Among the risks that it involves, the most serious are fetal death or spontaneous abortion: its frequency is estimated in about 1-2% of cases[11–13].

1.4 Current methods for prenatal screening of genetic disorders

Screening tests for genetic disorders are aimed to detect, in the general pregnant women population, cases with a higher probability to have a chromosome abnormalities, thus classifying in low and high-risk for these conditions. The tests are based on statistical evaluations and do not provide a diagnosis. The main advantage of a screening test is to be harmless to the mother and fetus, while the disadvantages are the possibility to originated false positive and false negative results. Proportions depend on the type of used test. Currently, the existing screening tests are limited to the evaluation of the most frequents chromosomal abnormalities, such as trisomy 21, 18 and 13. These anomalies represent about 50-70% of all investigable chromosomal anomalies with the fetal karyotype.

1.4.1 First trimester combined test

The combined test is based on the combination of maternal age with ultrasound (nuchal translucency) and biochemicals data (human Chorionic Gonadotropin, β-hCG and pregnancy-associated plasma protein A, PAPP-A), obtained from a maternal blood sampling. The nuchal translucency consists in the measurement of the thickness of

soft tissues at the nape of the fetus. In fetuses suffering from more frequent chromosomal abnormalities, nuchal translucency appears more thickened, from 2-4 mm up to 10 mm. Measurement needs to be performed between the 11th and the 13th week of gestation, while maternal blood sampling can be performed before, at the same time or after the measurement of the nuchal translucency. Pregnancy-associated plasma protein A (PAPP-A) is a high molecular weight (720-850 kD) placental-derived glycoprotein, mainly produced in the syncytiotrophoblast (necessary for implantation of the embryo in the uterus) and it is released into the maternal circulation. Usually, PAPP-A concentration in maternal serum increases rapidly from the beginning of pregnancy but in the presence of a fetus with Down Syndrome, the levels of PAPP-A tend to be almost always lower than expected[14]. Free β-hCG is a glycoprotein with hormonal activity associated with pregnancy: its main function is to provide for the maintenance of gestation, favoring a hormonal and tissue environment suitable for the development of the embryo. Normally, β-hCG values progressively increase in the maternal circulation in the first 8-10 weeks of pregnancy, according to an increase of placental activity, and then decrease and stabilize for the rest of gestation. In cases of Down syndrome, during the first trimester, the

serum concentration of hCG is higher than in pregnancies with a healthy fetus. Combined test results are interpreted together to mathematically calculate the risk that the fetus is a carrier of chromosomal defects. The obtained value is compared with an established cut-off; if the risk is greater, then the screening is considered positive and the woman is directed to further tests, such as CVS or amniocentesis. Detection rate of this screening test allows to identify about 85-90% of fetuses affected by trisomy 21 with 5% of false positives[15,16].

1.4.2 Non-invasive Prenatal Testing (NIPT)

Over the past decade, non-invasive tests based on the analysis of cell-free fetal DNA (cfDNA – cell-free DNA of fetal origin) in the maternal blood, are gaining relevant interest. These tests are based on the discovery in 1997, that an amount of fetal DNA can pass into the maternal circulation[17]. Circulating-cell free DNA derives from the placenta trophoblast which releases fetal DNA , following its apoptosis[18]. The NIPT consists of identification and subsequent quantification of cfDNA circulating in the maternal blood. Because risk assessment through this approach, is based on the presence in excess or shortage of cfDNA with respect to reference values and

not on direct analysis of this DNA, it has a screening value rather than diagnostic. Furthermore, cfDNA comes from the outermost layer of the placenta, and discrepancies between the chromosomes of the placenta and fetus may occurs. The test can be performed starting from the week 10th of pregnancy, but most of the Scientific Guidelines recommend that it is preceded by an ultrasound, possibly with the measurement of the nuchal translucency (therefore between 11-13th weeks of gestation), and following medical consultant (Ministerial Guidelines on the use of NIPT, May 2015). The result is provided after 7-10 working days from blood collection. To be reliable, the test must include the determination of the fetal fraction (meaning the quantity of free fetal DNA detected in the plasma sample), analyzed over the total DNA. In about 2% of cases, the fetal fraction results below the established limit and therefore cannot provide risk assessment. In Italy, the test is currently paid by the patients, although some Regions (such as Emilia Romagna and Lombardia) have introduced or are considering to introduce it in the reimbursement system. It is worth mentioning that the test has been validated on a large number of samples for trisomy 21, 18 and 13 only, hence the results obtained for other chromosomal or genetic abnormalities are of doubtful reliability, due to the scarcity of cases

tested. However, some Companies propose the test for other chromosomal abnormalities, clinically relevant microdeletions or monogenic diseases as well, even if not yet validated. NIPT currently represents the most effective screening test in term of sensitivity and specificity for trisomy 21 (98.6-100%)[19], with a false positive rate lower then 0.1%. Slightly lower results are reported for the trisomy 18 and 13, while performances are lower for sex chromosome abnormalities.

1.5 Circulating fetal cells as alternative source of fetal DNA

Prenatal field is constantly evolving both for the progress of scientific discoveries and for the advancement of technologies. The interest in the development of a non-invasive method that allows achieving the same level of information and performance comparable to that of invasive methods is still high. A part from the cell-free fetal DNA, there is another source of fetal material circulating in the maternal blood that can be used for non-invasive prenatal screening or diagnosis: fetal cells. Great potential of fetal cell is related to the possibility to obtain the whole genome of the fetus, rather than only DNA fragments, free of any maternal contamination. The first evidence of the presence of fetal cells in the maternal circulation, was in 1983 when the German pathologist Schmorl described trophoblast cells in the lung of 14 pregnant women who died of eclampsia[20,21]. In 1969 Walknowska, cultured lymphocytes from the peripheral blood of pregnant women carrying a male fetus[22] identifying cells bearing chromosome-Y-specific DNA sequence, therefore of fetal origin. Years later, in 1970 fetal lymphocytes were successfully isolated using flow fluorescence-

activated cell sorting (FACS) by Herzenberg and his collaborators[23]. In 1990 Diana Bianchi was able to isolated fetal cells from maternal blood and to use the DNA content to diagnose fetal sex[24]. Up to now, it has been reported the presence of three main types of circulating fetal cells in maternal blood and they include: fetal nucleated red blood cells (fnRBCs), trophoblasts and lymphocytes.

1.5.1 Fetal lymphocytes

Fetal lymphocytes have the great potential to proliferate in vitro, allowing to overcome the limit related to their rarity. However, their use for genetic investigations is not recommended since it has been demonstrated that they can persist in the maternal circulation up to 27 years after childbirth[25]. Indeed this condition of microchimerism could cause a diagnostic error, as there is a risk that selected lymphocytes are not belong to the current pregnancy[26].

1.5.2 Trophoblasts

Trophoblast cells are released in maternal blood during the first trimester of pregnancy, so they can be found early and they are rapidly eliminated from the circulation after the delivery[27]. During placenta development, different type of trophoblasts are originated [28], but the most predominant who enter the maternal circulation is

the circulating extravillous trophoblast (cEVT)[29]. HLA-G antigen was used for many years as target for trophoblast positive enrichment or identification with limited success. More recently, the interest in trophoblasts has grown enormously, especially after the discovery of additional specific markers that allowed to improve their enrichment and identification or isolation[30–32]. Predominant antigens currently used include CD105 (endoglin), CD141 (thrombomodulin), EpCAM, anti-Trop2 and cytokeratin (for staining). Other groups are focusing on microfluidic platforms or size-based system, instead[33–35]. The main disadvantages of trophoblasts is related to their placental origin which lead to a risk equal to 1-2% to obtain a non-representative karyotype of the fetus, due to the phenomenon of placental mosaicism[36].

1.5.3 Fetal nucleated red blood cells

Fetal nucleated red blood cells (fnRBCs) also referred as erythroblasts, represent the ideal candidates for prenatal diagnosis, because of their fetal origin, not leading to confined placenta mosaicism (CPM), and short lifespan that eliminated microchimerism risk. Their isolation and identification is much more complicated than the other fetal cell type circulating in maternal

blood. The reason is intrinsic in this cell type. First fnRBCs derived from the erythroid lineage are cells which are differentiating in red blood cells. Therefore, they share most of surface antigens with red blood cells (RBCs) which are the most abundant cell type in the bloodstream. Besides this, it has been reported that during pregnancy nucleated red blood cells of maternal origin are also present in the blood[37]. This adds a degree of complexity since a part from the isolation, it is also necessary to distinguish between fetal and adult nucleated red blood cells. Predominant positive markers explored for fnRBCs enrichment include: CD71 (transferrin reception), CD36, CD47, GPA (glycophorin A) and CD147. Selective direct enrichment from whole blood it is very inefficient because does not allow to remove RBCs and therefore, it is performed prior density gradient centrifugation[38–40] or selective red blood cell lysis[41,42]. Importantly, as previously mentioned, the similarity of the fnRBCs to the RBCs methods that will inevitably lead also to a target cells loss[43]. Other approaches are based on RBCs lysis followed by white blood cells (WBCs) depletion using CD45, CD66, CD15 and CD14, markers in combination with Magnetic Activated Cell Sorter (MACS) technology. Hemoglobin, both embryonic (epsilon and zeta), and fetal (gamma) have been used from many groups for the

identification of fnRBCs from an adult nRBCs. On the other hand, in some conditions, such as beta-thalassemia, gamma subunit it is upregulated, hemoglobin does not represent an adequate marker for discrimination.[44] In 1992 and 1993 several studies were performed on fnRBCs to prove the possibility to detect chromosomal abnormalities after their enrichment using either FACS or MACS[45,46] and therefore a large clinical trials, the NICHD (National Institute of Child Health and Human Development) Fetal Cell Isolation Study (NIFTY) was initiate in 1995. The results were not reproducible due to the low fetal cell isolation yield, demonstrating that clinical implementation of fnRBCs isolation for prenatal diagnosis, was not yet possible.

1.5.4 Technical difficulties in fetal cell isolation

Isolation and subsequent genetic analysis of fetal cells from maternal blood is very challenging mainly because of their rarity and the scarcity of unique elements (such as morphological properties or cellular markers) exclusive to the fetal cells and absent on the predominant maternal blood cell. Incongruences about the real number of circulating fetal cells still exist among several studies, where different factors have been used for the enumeration,

including the type of fetal cell targeted, enrichment and identification methods, genetic confirmation and week of gestation explored. Some groups have tried to quantify the absolute number of fetal cell using quantitative PCR assay without prior enrichment, and data concur on 1 fetal cell out of 1 ml of maternal blood analyzed[47–49]. Due to their rarity, all attempts to isolate and analyze fetal cells made so far were aimed at separating them from maternal cells and were mainly based on multistep approaches, with concomitant or subsequent phases: enrichment, identification, isolation and/or confirmation of their real fetal origin. The most critical step has always been the enrichment from maternal blood, which contains billion of maternal cells, different in size and type, that have to be eliminated, without losing the target of the few fetal cells. Numerous approaches have been used for this aim, including red blood cell lysis, density gradient centrifugation, immunomagnetic spheres, filtration, microfluidic, FACS and MACS. Identification is usually performed by imaging (microscopically or again by FACS) and can be concomitant or prior to isolation. Isolation methods include cell picking, laser capture microdissection (LCM), or FACS again. Fetal cells can be recovered as single cells or in a pool, but usually, due to their low number,

whole genome amplification (WGA) is required to guarantee a sufficient amount of DNA for downstream genetic analysis. All methods both singularly or altogether united in one workflow result in a laborious, operator dependent, time-consuming approach, which until now does not allow a high and consistent purification of fetal cells.

1.6 Scope of the thesis

The scope of my PhD project was to develop a method to enrich, identify and isolate single fetal cells circulating in maternal blood for subsequent genetic analysis. The project was divided in two parts. The first part was dedicated to the research and testing of different specific markers for fetal cells enrichment and identification. Once the enrichment step was optimized and subsequently automatized it was adapted to the Autoprep machine (from Menarini Silicon Biosystem). The throughput has been increased from one to eight samples per time, with minimal hands-on. The enrichment step was then implemented and optimized in a full workflow consisting of the following steps:

1. Maternal blood collection
2. Fetal cell enrichment and staining
3. Identification and single cell sorting
4. Whole genome amplification and fetal origin confirmation
5. Sequencing for chromosomal assessment

Once the workflow was optimized, the second part, a clinical performance evaluation study was performed on pregnant women

enrolled at San Gerardo Hospital (Monza) and Policlinico Mangiagalli Hospital (Milan).

Chapter 2 describes the main results of the clinical evaluation study performed on 372 pregnant women.

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CHAPTER 2

An automated method for isolation of circulating trophoblasts for non-invasive prenatal testing

Running title: cEVTs isolation for prenatal testing

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ABSTRACT

The isolation of intact fetal cells from maternal circulation is a promising approach for non-invasive prenatal testing, representing a relevant but still challenging medical need.

Here we present an automated workflow for the enrichment and genetic characterization of single circulating extravillous trophoblasts (cEVTs). The workflow's performances were evaluated through success rate assessment, the ability of identifying common trisomies by copy-number profiling and the determination of copy-number variation resolution. Recovery rate of at least one fetal cell varies depending on gestation age being 90.7% between 10-11 gestational weeks, 81.0% between 12-14 weeks and 70.6% at 15-20 weeks. The mean number of cEVTs was 3.5 cells, with a peak of 4.2 cells at 10-11 weeks. Copy-number profiling of cEVTs was performed for 131 pregnancies who underwent invasive prenatal diagnosis. Fifteen cases with common aneuploidies were identified on isolated cEVTs showing full concordance with standard karyotyping. Moreover, a reproducible resolution as low as 1.5Mb for micro-imbalances detection was estimated. Overall, these results support the feasibility of an automated and consistent isolation of single cEVTs for non-invasive prenatal testing.

INTRODUCTION

Currently, fetal chromosomal abnormalities are diagnosed prenatally either by chorionic villus sampling (CVS) or amniocentesis (amniotic fluid sampling; AFS) by conventional karyotyping or microarray analysis. Although the procedure-related risk of fetal loss has recently been re-evaluated by different groups and consistently found to be lower than previously thought, CVS and AFS are not risk free[1–4]. Therefore, considerable efforts have been devoted to the development of non-invasive methods for fetal genetic testing[5,6].

The discovery in 1997 of cell-free fetal DNA (cfDNA) in the blood of pregnant women demonstrated the potential for non-invasive prenatal testing/screening (NIPT/S; also called cell-free DNA test, cfDNA test) for common aneuploidies (trisomies 21, 18 and 13 and sex-chromosome aneuploidies). Clinical performances of NIPT has improved over the years and for T21 it reached a sensitivity and a specificity of >99%[7,8] in both singleton and twin pregnancies. Nevertheless, because of confounding maternal and placental factors, cfDNA-based NIPT includes false-positive and false-negative results[9,10]. Monogenic disorders and syndromes involving

microdeletions or microduplications can also be screened by cfDNA, albeit with many limitations mainly related to the low resolution for imbalances of smaller size[11–13].

The isolation of intact fetal cells from maternal circulation is a promising approach for non-invasive prenatal testing[5], but it is technically challenging mainly because of their rare occurrence and individual variability. Circulating extravillous trophoblasts (cEVTs) have been the first fetal cell type found in the maternal blood and, since they are extensively released during the first trimester of pregnancy, they became an attractive target for non-invasive prenatal testing[14,15].

Previous investigations of methods for isolation of cEVTs resulted in laborious protocols with inconsistent and insufficient cell recovery[16] as the identification of cEVTs by manual needle-based cell picking or laser capture microdissection under the microscope is highly operator-dependent[17,18]. In the context of routine clinical application, manual protocols may not be suitable[19], and reproducible, automated and high-throughput cell-recovery procedures are still needed.

Here we present a multistep, automated workflow for the isolation of cEVTs by describing i) the success rate and the mean number of

isolated cEVTs , ii) the feasibility of the copy-number profiling, iii) the concordance rate for common trisomies with fetal karyotype results and iv) the copy-number variation (CNV) resolution of the current downstream genetic analysis.

RESULTS

Automated enrichment and identification of cEVTs for downstream analysis

Fetal trophoblasts were successfully enriched from 20 ml maternal blood through the developed multi step workflow illustrated in Fig. 1A and detailed in the Materials and Methods. Each step and related processing time is shown in Table S1. The entire workflow took approximately 6 h. Whole blood samples were processed with CellTracks AutoPrep System (Menarini Silicon Biosystems, Bologna, Italy) using ferrofluid-conjugated specific antibodies for cEVT enrichment. This process took approximately 3 h and resulted in a sample containing less than 10,000 cells (including maternal contaminants and cEVTs) that can be loaded and inspected using the image-based DEPArray system (Menarini Silicon Biosystems) in 1.5 h. Coarse selection of putative cEVTs was provided by CellBrowser image-analysis software, applying the criteria of pan-cytokeratin positivity and CD45 negativity, followed by a fine confirmation from the user (10 min), based on morphological features (Fig. 1, B and C). Notably, identified cEVTs exhibited heterogeneous cytokeratin staining patterns (Fig. 1 C). In the majority of cEVTs, cytokeratin was condensed in numerous small,

high-density spots, consistent with a previous description of 'bubble' cells[20]. In the remaining cEVTs, cytokeratin displayed a uniform cytoplasmic distribution. Some cEVTs displayed an elongated morphology with one or more cytoplasmic processes. Shape, roundness, condensation levels and size of nuclei were variable among all cEVTs. Occasionally, the dielectrophoretic (DEP) field that was generated in the DEPArray cartridge resulted in the trapping of two or more cells of different types, one cEVT and one maternal white blood cell, hereafter referred to as 'mixed' cells or recoveries, in the same DEP cage (Fig. 1 C, orange dot)[21]. In this circumstance, the ability to visualize each cell individually provided a powerful tool for cell discrimination. Selected target cells were automatically moved from the system and recovered individually. Depending on the number of recovered cells, this step required about 1 h.

Validation of the method on enrolled pregnancies

To validate the method on a clinical cohort, a total of 372 patients were enrolled during the study period (Fig. 2). The overall study design is provided in Figure 2. A summary of the demographic information and indications is reported in Table 1.

Indications for invasive procedures were one or more of the following reasons for increased risk of fetal aneuploidy: a) advanced maternal age (≥35 years, *n* = 42); b) abnormal ultrasonography findings (*n* = 10); c) positive First trimester Combined Test (FCT; patients with ≥1:250 risk of trisomy, *n* = 102); d) positive cell-free DNA (cfDNA) test results (*n* = 8); e) positive family history, parent carrier of a chromosome abnormality and risk for monogenic disorders (*n* = 28).

Success rate and cEVT yields

A total of 1084 cEVTs (mean ± SD = 3.5 ± 3.4 cEVTs per patient with positive recoveries) were isolated from the DEParray and genetically confirmed through Short Tandem Repeat (STR) analysis (Fig. 2; Fig. 1D). In 311 of the 372 enrolled pregnancies (83.6%) at least one cEVT was recovered and genetically confirmed by Short Tandem Repeat (STR) analysis (complete data are provided in Supplementary Data S1).

Overall, the mean volume of maternal blood processed was 17.6 ml (range: 9.5 – 20.5mL). For patients where no cEVTs were recovered (*n* = 61, 16.4%) the mean blood volume was significantly lower compared to patients with cEVTs ≥ 1 (16.8 ml vs 17.7 ml,

nonparametric Mann–Whitney U-test, p <0.01, Fig. S1). The success rate for recovery of at least one cEVT was 90.7% at 10–11 weeks (*n* = 118), 81.0% at 12–14 weeks (*n* = 237) and 70.6% at 15–20 weeks (*n* = 17) (Fig. 3, A), showing a significant linear trend between gestational-age and success rate (Cochran–Armitage test for trend, *p* <0.01). Among women with cEVT recovery (*n* = 311, 1084 cEVTs in total), the mean number of recovered cEVTs was 3.5 cells (mean ± SD = 3.5 ± 3.4), 4.2 cells per patient at 10–11 weeks, 3.1 cells per patient at 12–14 weeks and 2.8 cells per patient at 15–20 weeks. The number of recovered cEVTs was significantly different comparing 10–11 weeks and 12–14 weeks (Kruskal–Wallis nonparametric One-Way ANOVA test followed by Dunn's multiple-comparison test, *p* <0.01). No significant difference was observed in comparisons with the 15–20-weeks group (Fig. 3, B).

Success rate was higher in the CVS/AFS group compared to the FCT/NIPT group (CVS or AFS versus FCT or NIPT; Fisher's exact test, *p* <0.05) (Fig. 3, C and D). The number of isolated cEVTs was not different between CVS or AFS versus FCT or NIPT. This is likely to be due to lower gestational week for FCT compared to that of CVS and AFS. Maternal age, body mass index (BMI), fetal gender and chromosomal abnormalities were not significantly

associated with success rate or the number of isolated cEVTs (Fig. S2).

In total 11 twin pregnancies were enrolled in the study period (Table 2). For these pregnancies a significant increase of recovered cells number compared to singletons (Fig. 3, E and F, nonparametric Mann–Whitney U-test, $p < 0.01$) was observed. As expected, for the 3 Monochorionic pregnancies, only one profile was detected by STR analysis (6 to 25 cells analyzed). STR analysis detected dizygotic twins in 4 out of 7 confirmed or suspected dizygotic pregnancies (Table 2; M031, M040, M184 and M326). In two dizygotic pregnancies only one profile was detected (M012 and M082), while in one case no cells were recovered (M144). In 1 case no information about zygosity/chorionicity was available and 2 different STRs profiles were detected, indicating a dizygotic pregnancy (Table 2).

Comparison of genetic profiling by next generation sequencing (NGS) and karyotyping

Of the 311 pregnancies with successful cEVT recovery, 150 women underwent an invasive procedure (Fig. 2).

In 136 of the 150 samples, 432 cEVTs (298 pure fetal cells and 134 mixed recoveries) underwent WGA and low pass NGS. Fourteen

patients were used for NGS experimental protocol setup and, therefore, excluded from copy-number analysis. Comparison with karyotyping results was possible for 131 women, as interpretable profiles could not be obtained for five women for which only one cEVT was recovered (n = 5, 1 cEVT per each woman) because of the low-quality sequencing libraries, apoptotic-like cells or S-phase mixed recoveries (Fig. S3)[21,22]. In two out of these five missed cases, the invasive procedure indicated the presence of a fetal T21. Our data, although not conclusive, did not support an association between aneuploidies and NGS test failure (Fisher's exact test, p >0.05). Overall, low-quality libraries resulting in noisy and uninterpretable NGS profiles involved 1.6% of cells (7/432); apoptotic cells, characterized by chaotic profiles, were 3.0% (13/432); S-phase cells, indicated by partially replicated genomes with short and evenly distributed losses, represented 7.9% of cEVTs (34/432), however the general profile of S-phase pure single cells was not affected and was anyway interpretable; therefore, the S-phase pure fetal cells (n = 25) were included for aneuploidy detection (Supplementary Table S2). A total of 29 cells (29/432) were not informative and the remaining 403 provided suitable cEVT profiles (93.3% of sequenced cells) for aneuploidy detection enabling a

result for 131 women. The vast majority of them (~85%) had at least one high-quality single fetal cell (13.2% with only mixed recoveries and 1.5% with only S-phase cells). On average, approximately 2.8 cells for each singleton pregnancy were used for evaluation, prevalently consisting of single fetal cells (Table S3, S4 and Fig. S4). Copy-number profiling identified 15 samples with common aneuploidies (Table 3 and Supplementary Data S2): 12 cases with T21, one with T18, one with monosomy X and one with two cell lines, one with T21 and the other with a T21 and a T18 (M115). There was full concordance with fetal karyotype for common trisomies. In all cases with multiple recovered cEVTs from a pregnancy, all cells showed the same abnormality, suggesting an apparently non-mosaic pattern in trophoblasts (Fig.4, A and B and Supplementary Data S2). In the case of mosaicism, among three isolated cEVTs, one showed T21 and two showed the co-existence of a T21 and a T18, suggesting the presence a non-mosaic T21 with mosaic cell line with T18 (Fig. 4C). In this case, CVS analysis identified a 47,XY,+21 karyotype in cytotrophoblasts and a 48,XY,+18,+21 karyotype in mesenchymal cells. These data suggested the presence of a 48,XY,+18,+21 cell line in the

cytotrophoblasts that was undetected by the CVS direct-preparation cytogenetic analysis.

An additional case of trisomy 16 was detected by copy-number profiling, but not confirmed by AFS karyotyping. Finally, although this is not always possible with the current approach, a case with 69,XXY karyotype was identified. Copy-number profiles of single cEVTs not consistent with a diploid asset were detected. Profiles were therefore recomputed with an input ploidy=3, as suggested by the implemented ploidy auto-detection algorithm, correctly identifying three copies for autosomes and the XXY configuration for sex chromosomes.

A normal copy-number profile was identified in all cases with normal karyotype (n = 114).

CNV resolution

A CNV panel from the Coriell Institute was used to assess the ability of our sequencing approach to identify microdeletions and/or microduplications. Copy-number profiles (n = 127) were obtained from triplicate analysis of gDNA from 43 Coriell cell lines (two failed libraries), demonstrating a resolution as low as ~1.5 Mb (Fig. 5, A and B) with 100% sensitivity. Alterations that were smaller than

this threshold could also be detected, but with lower confidence and with the occurrence of false positives. The same observations were obtained by copy-number profiling on single cells[23–25], where even an 800kb-sized target was reproducibly detected on all cells (Fig. 5C). However, the presence of false positives and negatives concentrated in specific targets above 800Kb suggests the existence of a positional bias which, together with the low size, made the identification of some alterations in peculiar genomic regions (low complexity, pericentromeric, etc.) difficult.

DISCUSSION

Our results have demonstrated the feasibility and a strong reliability of an automated method for isolation of cEVTs from maternal blood for prenatal non-invasive genetic testing. The success rate for recovering at least one cEVT decreased with gestational age, being 90.7% at 10–11 weeks, 81.0% at 12–14 weeks and 70.6% at 15–20 weeks. Notably, levels of cfDNA in maternal plasma show an opposite trend, increasing with gestational age by 0.1% per week between 10 and 21 weeks of gestation and by 1% per week beyond 21 weeks of gestation[26]. These opposite trends may reflect the release of trophoblasts into the maternal circulation as a result of their active proliferation during the initiation of invasion of the uterine arteries, followed by a progressive increase in apoptosis after the conclusion of trophoblast invasion[27–30].

Accordingly, the number of cEVTs recovered per patient was higher in the 10–11-week group than in the 12–14-week group. This finding is consistent with a previous observation resulting from microscope identified cells, of relatively high numbers of cEVTs at early gestational age[21].The wide range of values for numbers of isolated cEVTs per patient (0–28) indicates that there is considerable variability between individuals.

We did not observe any significant association between either the number of cEVTs or the success rate and factors such as maternal age or BMI, fetal sex or karyotype. We did, however, observe a significant difference in the number of isolated cEVTs between women with twin and singleton pregnancies, as described by others[21]. We also identified an association between success rate and different clinical indications, although gestational age might have been a confounding factor in this relationship, which should therefore be considered carefully.

In 131 women who underwent both non-invasive cEVT isolation and invasive prenatal diagnosis, molecular characterization of individual cEVTs showed full agreement with standard karyotype for common trisomies and normal results. However, a common biological phenomenon that has shown affecting the analysis of trophoblast cells via cfDNA and CVS cytogenetic analyses is feto-placental mosaicism[31], mainly involving whole-chromosome aneuploidies. Therefore, potential discordant results for aneuploidies with fetal karyotype on amniocytes are possible, even when a fully abnormal or normal karyotype is detected on cytotrophoblasts via analysis of cEVTs. This is expected to be more likely with monosomy X (MX) and rare autosomal trisomies (RATs) than with T21, 18, 13[10,32–34].

Due to the possible discordances involving these aneuploidies in feto-placental unit, our study was mainly aimed at evaluating the concordance between cEVTs and karyotype for common trisomies. Indeed, we found a case where a T16 was identified in all cEVTs, despite the karyotype resulting from AFS was normal, suggesting the presence of a confined placental mosaicism for T16[35]. Notably, when T16 is identified in the cytotrophoblast by CVS cytogenetic analysis, the likelihood of its confirmation on AFS is only ~16%[36]. In addition, when a RAT is detected in all cytotrophoblasts by direct preparation alone (without analysis of long-term cultures), the associated false-discovery rate is of 56.52%[34].

We acknowledge that, similar to cfDNA test, the analysis of cEVTs for aneuploidies, may have limitations due to feto-placental mosaicisms. However, the isolation of individual cEVTs has the potential to detect mosaic in Chorionic Villi (CV) when multiple cells are recovered. Single-cell approach has also the potential for discrimination between mono- and dizygotic twins in multiple pregnancies. Additional studies are required to determine the minimum number of cells required to reliably detect/exclude a mosaic in CV to minimize discordant results with fetal karyotype

and assess the zygosity when chorionicity by US scan and fetal sex are not informative.

Moreover, compared with cfDNA testing, the non-invasive analysis of pure genomic unfragmented DNA from the conceptus is highly advantageous, as it can achieve higher resolution for detection of micro-imbalances[12] and avoid biological confounding factors related to the maternal genetic make-up. Preliminary data obtained with this automated pipeline applied on contrived samples of cell-lines with known pathogenic microdeletions/duplications showed reliable detection of imbalance of about 1.5Mb in size, allowing to non-invasively identify a large proportion of clinically relevant CNVs[37]. Our methodology resulted in 16.4% of samples with no cEVTs recovered and 3.7% with uninterpretable results due to low-quality NGS profiles, apoptotic-like cells or S-phase mixed recoveries. The no-recovery rate can be reduced by increasing the volume at first blood draw (from 20 ml to 40 ml); drawing a second sample in the case of no recovery/uninterpretable results might be helpful, as it is routinely performed with cfDNA testing in cases of no-call results. Our results on recovery rate, number of recovered cells and blood volume support the first strategy.

As a whole, our data fully support the feasibility of an automated workflow potentially well applicable to routine clinical practice: blood samples could be collected before or at the time of routine first-trimester ultrasonographic scan, and stabilized for up 4 days at room temperature, allowing sample transportation to decentralized laboratories. The automated platform enables high throughput and a turnaround time comparable to those of current prenatal genetic methods, requiring minimal user interaction.

These data will be further supported by a larger validation study which is already ongoing and involves the recruitment of 1,500 high-risk pregnancies. The automated isolation of single intact trophoblasts represents a paradigm shift in prenatal diagnostics, enabling non-invasive genetic testing of high quality, pure genomic DNA from the conceptus.

MATERIALS AND METHODS

Study cohort enrollment

Pregnant women aged ≥18 years, with singleton or twin pregnancies, presenting between October 2019 and December 2020 for routine prenatal care (invasive procedures, e.g., CVS and AFS; First trimester Combined Test, FCT; cell-free DNA test, cfDNA) in two public maternity health hospitals located in Lombardy (Milan and Monza), were invited to participate to the study. The study protocol was approved by the local Institutional Review Boards at both study sites (IRB #1227 and #2648).

The study project was described to eligible women, and a signed informed consent was obtained from each participant. Blood sampling was performed at the time of the planned prenatal procedure as per standard care (FCT, cfDNA, ultrasonography) and prior to the invasive procedure, when required.

The success rate according to gestational week and the number of cEVTs isolated per woman were determined for all the enrolled pregnant women, while the genomic analysis of single cEVTs was conducted in a subgroup of women for which CVS or AFS was performed.

Trophoblast enrichment and staining

Twenty ml of maternal blood were collected into two separated CellSave tubes (Menarini Silicon Biosystems) and processed at San Gerardo Hospital or shipped at room temperature to Menarini laboratory in Bologna. Samples were analyzed within 96 hours of blood sampling.

Enrichment was performed with the CellTracks Autoprep (Menarini Silicon Biosystems) automated sample-preparation instrument for immunomagnetic capture and fluorescent labeling of rare cell populations. A customized version of CellSearch Circulating Tumor Cell Kit (Menarini Silicon Biosystems) was used, combining anti-CD105 and anti-EpCAM ferrofluid-conjugated nanoparticles to target trophoblast cell-surface antigens. The 20 ml of maternal blood were aliquoted into three tubes of 6.5 ml each, diluted with 7.5 ml dilution buffer, centrifuged at 800 × *g* for 10 min no brake at room temperature and transferred into the CellTracks AutoPrep system using the CTC-X9 protocol. In this workflow, the enrichment steps, including reagent addition, mixing, incubation, washing and aspiration, were performed automatically by the instrument. Briefly, each tube is moved across nine different stations inside the machine, where plasma is aspirated and ferrofluid particles are added. A

magnetic field is applied, enabling retention of ferrofluid-labeled cEVTs and discarding of unbound maternal cells (mainly red blood cells and leukocytes). Cell suspensions are then permeabilized and stained with phycoerythrin-conjugated anti-pan-cytokeratin and allophycocyanin-conjugated anti-CD45 antibodies, with nuclear staining by 4′,6-diamidino-2-phenylindole (DAPI), washed to remove excess ferrofluid and staining reagents, and finally resuspended in 300 µl cell fixative.

Trophoblast detection and single-cell sorting

The three 300 µl labeled cell suspensions were combined, washed twice with DEPArray Buffer for Fixed Cells buffer (Menarini Silicon Biosystems) and resuspended in 12 µl of the same buffer, prior to loading on a DEPArray cartridge (Menarini Silicon Biosystems) for single-cell sorting, as previously described[38]. Briefly, the DEPArray system automatically injects the sample into the cartridge main chamber, where the cells are randomly distributed, subjected to a dielectrophoresis potential and trapped into cages. The system scans the whole main chamber for the selected fluorescence channels, automatically identifies all events, using DAPI as a common marker, assigns ID numbers, and performs

qualitative and quantitative marker evaluation with the CellBrowser image-analysis integrated software.

Putative fetal trophoblasts were selected based on positivity for expression of cytokeratin and negativity for expression of CD45 (Fig. 1, B and C), and were automatically recovered individually in 0.2 ml PCR tubes. Maternal white blood cells were recovered as single cells or in pools of 10 cells as controls for the downstream analysis.

Fetal-origin confirmation

Any isolated cell was tested for fetal origin through the comparison of Short Tandem Repeats (STR) with maternal DNA (Fig. 1D). Specifically, the isolated single cells and corresponding maternal white-blood-cell pools were lysed with the DEPArray LysePrep kit (Menarini Silicon Biosystems). Cells obtained from women undergoing CVS/AFS were further whole-genome amplified for STR and NGS downstream analyses using the *Ampli*1 WGA kit (Menarini Silicon Biosystems)[39–41]. STR genotyping was performed using the 27 locus PowerPlex Fusion 6C System (Promega Corporation, Madison, WI, USA), including Amelogenin and three Y-STR loci (DYS391, DYS570, DYS576) for gender determination.

Microsatellite amplicons were separated by capillary electrophoresis (ABI 3500 Genetic Analyzer; Thermo Fisher Scientific), with data analysis in GeneMapper ID-X v1.6. Profiles were compared to the corresponding maternal ones. Informative fetal alleles were counted for each locus.

Fetal origin was also subsequently further confirmed by SNP comparison between maternal and single-cell DNAs obtained by low-pass NGS on WGA samples[42].

Detection of whole-chromosome aneuploidies on isolated single cells

Genomic DNA, amplified with the *Ampli*1 WGA kit, was processed using the *Ampli*1 LowPass kit (Menarini Silicon Biosystems)[43] for Illumina library preparation, with a fully automated workflow on a STARLET Hamilton Liquid-Handling Robot (Hamilton Bonaduz AG, Bonaduz, Switzerland). *Ampli*1 LowPass library concentrations were normalized, and sequenced on an Illumina NovaSeq 6000 platform (Illumina, San Diego, CA, USA), with 100 cycles single-read run mode, obtaining ~5M reads per sample on average.

Copy-number profiles were obtained using a pipeline developed in-house based on the common approach for copy-number aberration

(CNA) detection in single-cell sequencing[44]. It includes a first step of alignment of DNA sequences to the reference genome (hg19) using BWA mem[45] with default parameters. Then, alignments were sorted and filtered for a mapping quality >5 with samtools[46]. Reads mapping to non-overlapping 500 kb-sized genomic bins were counted. Only counts on WGA fragments whose mappability was >0.85 were retained (on the basis of alignability of 100mers by GEM from ENCODE/CRG downloaded from http://hgdownload.cse.ucsc.edu). Counts were normalized by fitting a lowess regression obtained using the *Ampli*1 WGA fragment length distribution and GC content[47] of the bin. The ratio per bin was computed using the per bin normalized signal over the median signal across all bins. Segmentation of copy-number profiles was performed with DNAcopy[48], and segment values were rounded to the nearest integer. The copy-number ratio was then converted to the absolute copy number by multiplying it by the multiplication factor between 1.5 and 2.5 (under ploidy=2 assumption) that minimized the root-mean-square error (RMSE) of genomic bins' signal with respect to segmented copy-number profiles[49]. In case of unknown ploidy, the range of multiplication factor can be extended accordingly. Significance of each copy-number call was assessed by

performing Wilcoxon rank-sum test of bins in altered segments versus all bins in non-altered segments. Then, *p*-values were adjusted using Benjamini–Hochberg correction for multiple tests, and copy-number calls were filtered using a 99% significance level. Sample sex was automatically determined by setting a hard cut-off on the fraction of total reads mapped on chromosome Y.

Aneuploidy detection was carried out both on single cEVTs and in mixed recoveries, where two or more joined cells of different type were captured (Fig. 1 B and C). The analysis involved only cells with a suitable copy-number profile (high-quality and S-phase cells), characterized by low background noise and the absence of variable aberrations across the genome (chaotic profiles). The evaluation of profile quality was done using hard cut-offs for noise metrics, such as the Derivative Log Ratio Spread (DLRS), where values <0.35 defined high-quality profiles. Moreover, each single copy-number profile was manually inspected and reviewed to get a final classification.

In case of mixed samples the developed pipeline implements an optional module for the signal correction accounting for maternal contamination (Fig. S5), realized by adjusting the normalized bin counts according to a factor manually set or alternatively

automatically determined based on the median difference between the observed and expected signal[50]. Three different degrees of contamination (0.33, 0.50 and 0.66) were tested, respectively mimicking the 2:1, 1:1 and 1:2 pattern of fetal and maternal cells. Then, the correction factor that produced the profile that best fitted integer copy-number levels was selected by visual inspection. With high maternal cell prevalence, such correction becomes not possible and mixed recoveries resemble maternal profiles, with possible false-positives/false-negatives. Moreover, with mixed recoveries, S-phase cells were not considered (S-phase represents an additional confounding factor to mixed status) and the copy-number analysis was restricted to chromosomes 13, 18, 21 and X only.

Determination of size resolution for submicroscopic pathogenic CNVs

CNV resolution evaluation was initially investigated using a Coriell panel (Catalog ID: CNVPANEL01) of genomic DNA (gDNA) samples extracted from 43 cell lines hosting several partial imbalances of different size (range 0.1–155 Mb). For each cell line, three aliquots of 1 ng human genomic DNA were amplified using the *Ampli*1 WGA kit and sequenced with the *Ampli*1 LowPass for Illumina Kit (Menarini Silicon Biosystems).

After CNV resolution evaluation, 21 cells from 5 Coriell cell lines harbouring microdeletions/microduplications with a size close to the expected limit of detection (~2Mb) were analysed with the same pipeline used for the cEVTs isolated from patients to evaluate reproducibility.

Specifically, a bin size of 100 kb was applied on FASTQ files downsampled to 3M reads. Profile quality parameters, such as the Large-Scale Transition value (>4) and DLRS (>0.35) together with visual inspection, were used to exclude low-quality or apoptotic samples and S-phase cells. CNV target coordinates and expected copy-number levels were downloaded from the UCSC genome browser (https://genome.ucsc.edu/cgi-bin/hgTrackUi?g=coriellDelDup, provided in the Supplementary Data S3). True Positives (TP), True Negatives (TN), False Positives (FP) and False Negatives (FN) were identified by averaging the segmented absolute copy-number signal in all the alteration targets and measuring their deviations from the expected value, using a 0.45 threshold. Moreover, targets were labelled as "Nocall" if the underlying copy-number signal covers <25% of the target, and as "Noeval" if the expected target of the evaluated cell-line overlaps the tested one.

Cytogenetic analysis on CVS and AFS

Fetal karyotyping following CVS and AFS was performed by the Hospital's internal laboratories, according to the Italian guidelines. Q-banding karyotype results were interpreted in accordance with the International System for Human Cytogenetic Nomenclature guidelines 2016[51]. Cytogenetic analysis was conducted following the Italian Cytogenetic guidelines. Briefly, for CVS samples, both direct preparation (dir; analysis of cytotrophoblasts) and long-term culture (LTC; analysis of mesenchyme) were performed and at least a total of 16 metaphases were scored and analyzed (at least six metaphases from dir and 10 from LTC). For AFS samples 10 metaphases from at least 10 colonies of at least two independent cultures were analyzed.

Statistical analysis

All variables were analyzed according to their characteristics and distribution. Categorical variables were presented as percentages. Continuous variables were presented as means, medians, standard deviations, minimum and maximum values. In case of classification, Fisher's exact test with two groups and Cochran–Armitage test for trend with more than two groups were used to verify significant

differences for categorical. Mann–Whitney U-test and Kruskal–Wallis One-Way ANOVA non-parametric tests with Dunn's correction for multiple comparison were used for continuous variables with two or more than two groups, respectively. For all tests, p-values ≤ 0.05 were considered as statistically significant. Statistical analyses were performed with GraphPad Prism version 9.1.1.

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Author contribution

AD, CF, GSHY, TWY, MS, AB, PV, EF, FRG, PRC designed research; AD, CF, CMan, DL, RA, CMar, EDG, GB, CB, RM, MD, LJ, RBA, SS, SP performed research; CF, DL, GSHY, TWY, MS, AB, PV, EF, FRG contributed new reagents/analytic tools; AD, CF, DL, CMan, RA, MD analyzed data; and AD, CF, CMan, DL, RA, LJ, RBA, EF, FRG, PRC wrote the paper.

Conflict of interest: AD, CF, CMan, RA, CMar, EDG, GB, CB, LJ, RBA, are employees of Menarini Biomarkers Singapore Pte Ltd, a Menarini Company Group. RM, EF, FRG and PRC are consultants for A. Menarini Biomarkers Singapore Pte Ltd. All other authors report no conflict of interest.

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Partial results of this manuscript were presented in Abstract #113 Poster Session at the International Society for Prenatal Diagnosis, 25th International Conference on Prenatal Diagnosis and Therapy, virtual event, June 6-8, 2021 and as Oral communication at the ISUOG's 31st World Congress, virtual event, October 15-17, 2021. Partial results will also be presented in Abstract #23 Poster Session at the CoGEN's 8th World Congress, virtual event, November 6, 2021.

THE PAPER EXPLAINED

Problem

Currently, prenatal diagnosis is based on invasive procedures for the identification of fetal aneuploidies or micro-imbalances, which are associated with a low, but present, risk of pregnancy loss. The analysis of single circulating trophoblasts isolated from maternal blood could provide non-invasive alternative of prenatal testing without the risk of fetal loss. Given the rarity of these fetal cells in the maternal circulation and technical challenges, an automated workflow for trophoblasts isolation is still an unmet clinical need, and standardization and throughput pledged by automation are crucial for its successful adoption into clinical prenatal care.

Results

Here we present an automated platform for identification, isolation and genetic characterization of single circulating fetal extravillous trophoblasts from maternal blood. Our data, obtained from the analysis of 372 pregnant women, demonstrated that reproducible and sufficient yields of fetal cells are possible, showing that gestational age and blood volume influence the recovery rate. Furthermore, blinded genetic copy-number characterization of isolated single fetal cells was performed on 131 pregnancies who also underwent

invasive prenatal diagnosis. Full concordance was obtained for normal karyotype and common trisomies.

Impact

This robust, high-throughput and automated methodology for isolation of circulating trophoblasts may open the way to a novel era in prenatal testing. As a whole, our data support the feasibility of an automated workflow well suited to routine clinical practice: blood samples could be collected before or at the time of routine first-trimester ultrasonographic scan, and stabilized for up 4 days at room temperature, allowing sample transportation to decentralized laboratories. The automated platform enables high throughput and a turnaround time comparable to those of current prenatal genetic methods. This workflow is also minimally operator dependent therefore easily standardizable in different laboratories.

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FIGURE LEGENDS

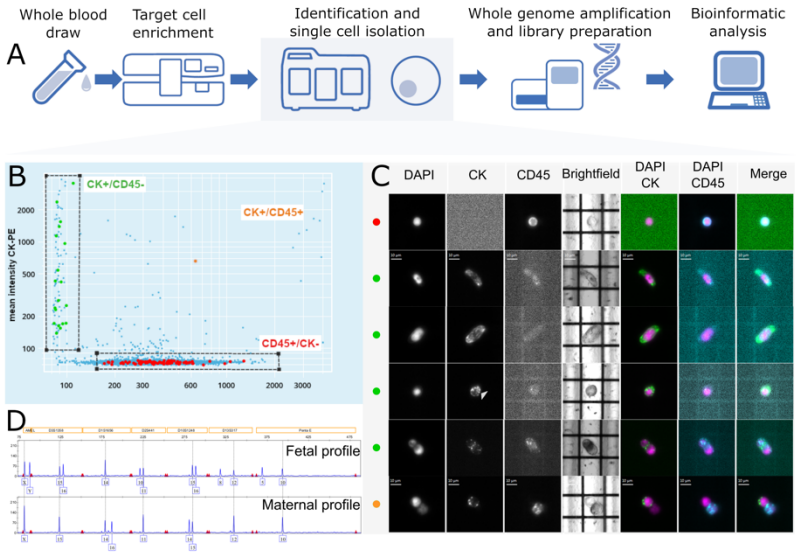


Figure 1. Experimental multistep workflow for single-trophoblast selection and identification.

A) Whole blood was enriched and stained using CellTracks AutoPrep System. After sample preparation, DEPArray system was used for isolation of single fetal extravillous trophoblasts (cEVTs) and maternal white blood cells (WBCs) for downstream analysis. B) Scatter plots of pan-cytokeratin–phycoerythrin (CK-PE) mean intensity (y-axis) versus CD45–allophycocyanin (APC) mean intensity (x-axis) of putative cEVTs ($CK^+/CD45^-$ cells), WBCs ($CK^-/CD45^+$ cells) and a mixed cell ($CK^+/CD45^+$). Selected WBCs, cEVTs and mixed cell are highlighted in red, green and orange,

respectively. C) Image gallery of one representative WBC (side red dot), four representative cEVTs (side green dots) and a representative mixed cell (side orange dot); DAPI, CK, CD45 and brightfield single channels are visualized in grayscale; DAPI/CK, DAPI/CD45 and Merge combined channels are visualized as follows: DAPI signal in purple, CK signal in green and CD45 signal in cyan. The four representative cEVTs exhibit different typical morphological features, from top to bottom: elongated cells with cytoplasmic processes, cells with an even distribution of cytoplasmic cytokeratin, so-called "bubble" cells with cytokeratin high-density spots (arrowhead) and CK+/CK+ doublets. Scale bars: 10 μm. D) Representative microsatellite genotyping results comparing a fetal and a maternal profile. Seven informative short tandem repeat loci (blue channel) are shown for a single cEVT and a pool of 10 maternal WBCs. Dashed bars identify the obligate maternal alleles at each locus.

Abbreviations: cEVTs, circulating Extravillous Trophoblasts; WBCs, white blood cells.

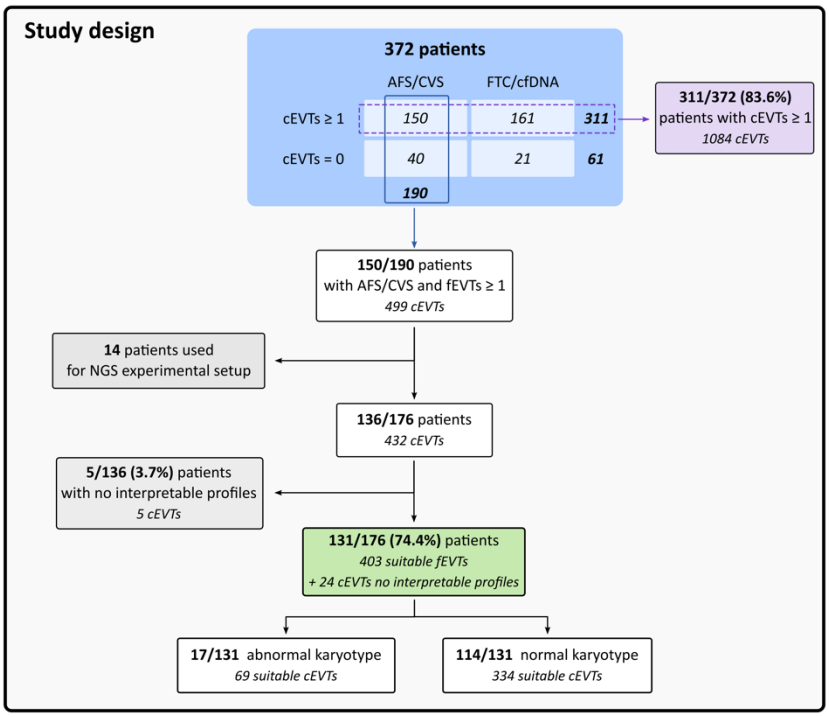


Figure 2. Study structure and enrollment.

A total of 372 women ≥18 years old with singleton or twin pregnancies were enrolled in the study period. Of these, 190 underwent invasive procedure as CVS (n = 173) or AFS (n = 17). The recovery of at least one cEVT was possible for 311 women, achieving an 83.6% success rate (violet box), which include 150 pregnancies with AFS/CVS. From these, 136 were suitable for NGS copy-number profiling with the aim to compare the results obtained by our workflow and those by invasive procedures, while the residual 14 were used for NGS the experimental protocol setup and

were excluded from the evaluation. The final comparison was performed using 131 patients with at least a suitable copy-number profile (403 cEVTs) usable for aneuploidy detection. Additional 5 patients were lost due to apoptotic status of cEVTs or low quality of sequencing libraries (1 cEVT per each case).

Abbreviations: CVS, Chorionic Villus Sampling; cEVTs, circulating Extravillous Trophoblasts; AFS, Amniocentesis; NGS, Next-Generation Sequencing.

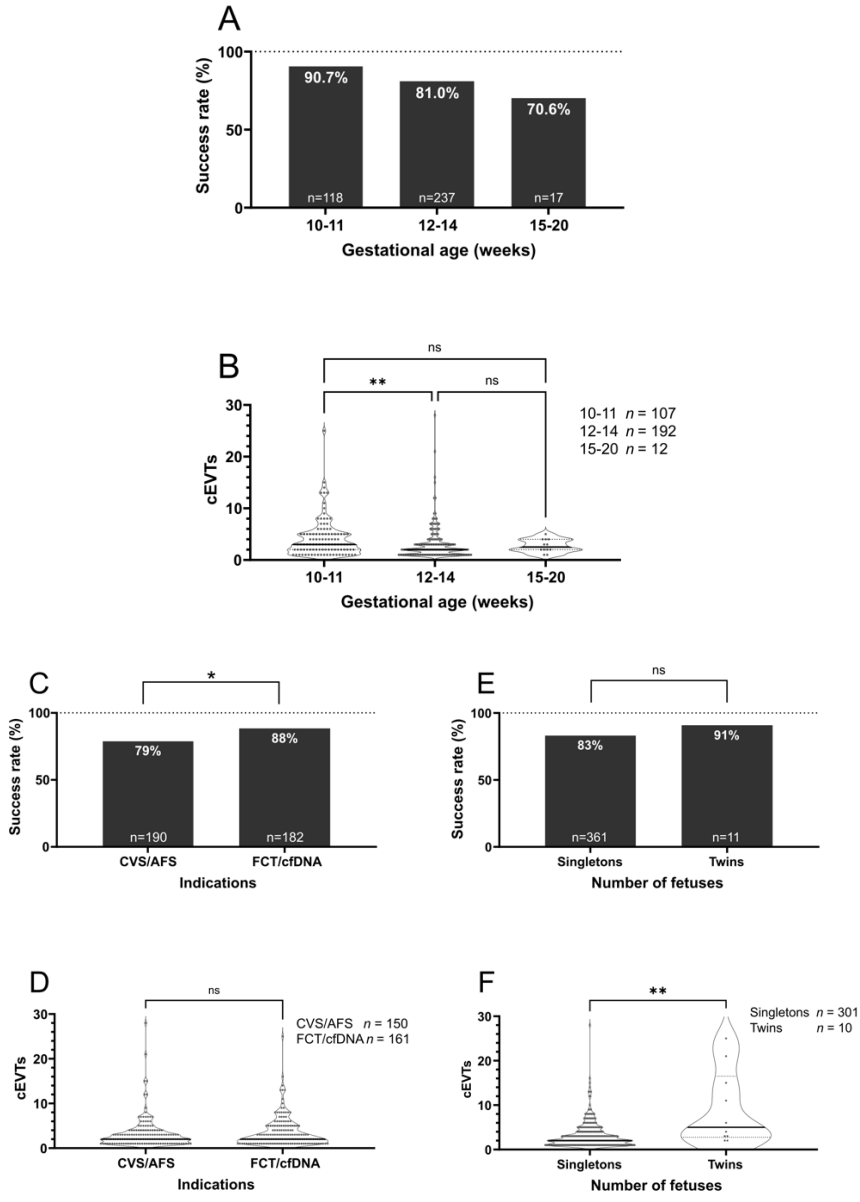


Figure 3. Success rate and number of trophoblasts identified.

A and **B)** Patients were analysed according to gestational age range: 10–11 weeks, 12–14 weeks and 15–20 weeks. **A)** Success rate and **B)** violin plots of cEVTs (solid lines represent median values) in

patients with at least one cEVT isolated. A significant linear trend was observed between gestational weeks and success rate (chi-square test for trend, p <0.01). The number of recovered cEVTs was significantly different comparing 10–11 weeks and 12–14 weeks (Kruskal–Wallis test followed by Dunn's multiple-comparison test, ** p <0.01).

C and **D**) Patients were analysed according to clinical indications: CVS/AFS vs FCT/cfDNA. **C)** Success rate and **D)** violin plots of cEVTs (solid lines represent median values) in patients with at least one cEVT isolated. Success rate is significantly associated with clinical indications (Fisher's exact test, p <0.05).

E and **F)** Patients were analysed according to number of fetuses: Singletons vs Twins. Success rate **E)** and **F)** violin plots of cEVTs (solid lines represent median values) in patients with at least one cEVT isolated. For 365 out of 372 patients, number of fetuses and fetal gender was available. Patients with unknown number of fetuses and/or fetal gender were not included. The number of cEVTs is significantly higher in twins compared to singletons (nonparametric Mann–Whitney U test, p <0.01).

Abbreviations: cEVTs, circulating Extravillous Trophoblasts. CVS, Chorionic Villus Sampling; AFS, Amniocentesis; FCT, First trimester Combined Test; cfDNA, cell-free DNA

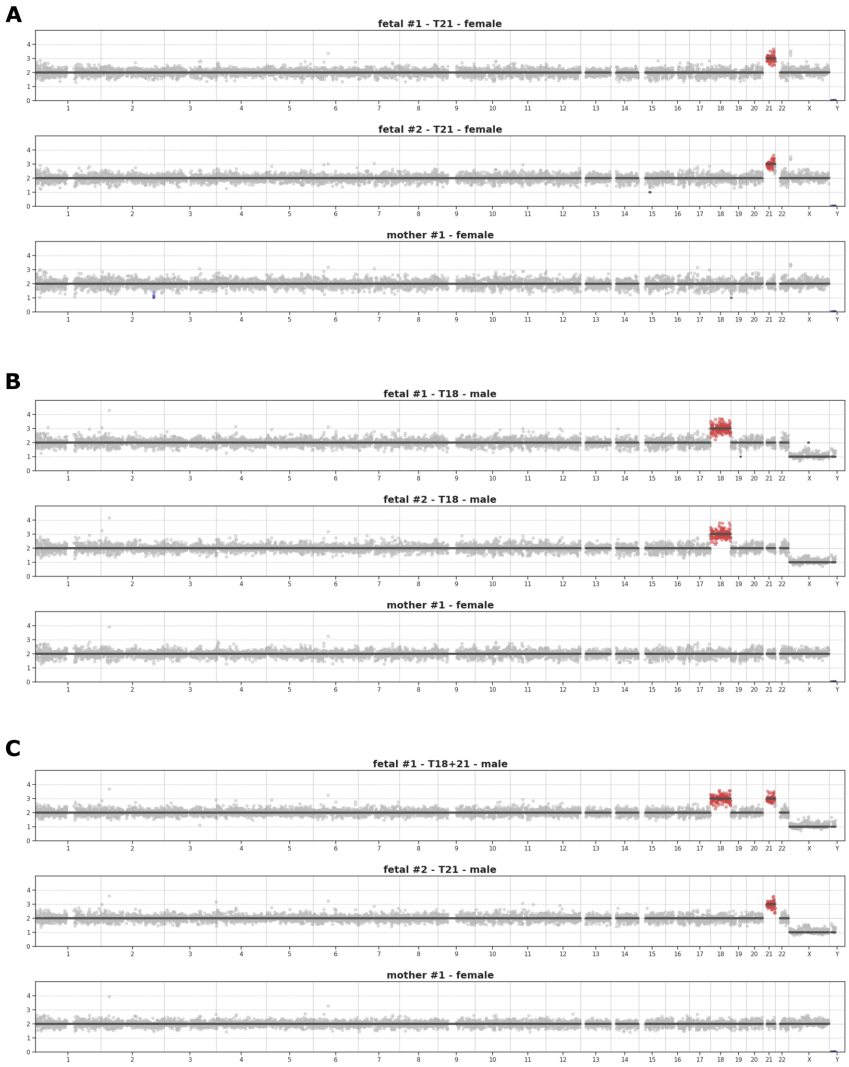


Figure 4. Copy-number profiling of fetal cells.

Absolute copy-number profiles obtained with *Ampli*1 workflow show the detected trisomies (highlighted in red) in the fetal cells isolated from three pregnant women. **A)** The first two tracks represent the copy-number profiles of two different fetal cells of case

M019, showing a normal female diploid chromosomal set with an additional copy of chromosome 21 (T21). The maternal cell, in the last track, shows a normal disomic profile. **B)** Copy-number profiles highlight a trisomy of chromosome 18 (T18) in two male fetal cells of case M057. The normal disomic profile of a maternal cell is displayed in the last track. **C)** Absolute copy-number profiles of two different fetal cells from the same pregnant woman (Case M115) show the presence of T18+T21/T21 mosaicism, confirmed by chorionic villus sampling (direct: 47,XY,+21; long-term: 48,XY,+18,+21). The last track shows the maternal disomic profile.

Abbreviations: T21, trisomy 21; T18, trisomy 18.

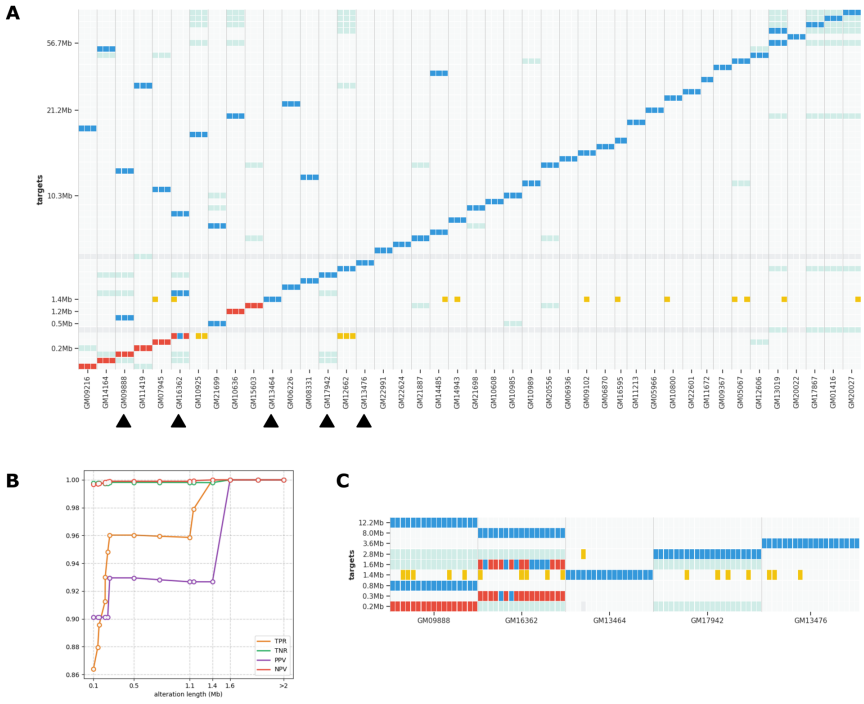


Figure 5. CNV resolution.

A Coriell copy-number variation (CNV) panel, including 43 cell lines and 59 targets, was used to assess the detection resolution of the *Ampli*1 next-generation sequencing approach. A) In this figure, the targets have been arranged on the y-axis in ascending order according to their size, while the cell lines samples (gDNA) - 3 replicates per cell line - have been ordered on the x-axis according to the size of the targets expected in the cell line itself. Each squared cell represents the detection status of the single target in a single replicate (obtained with a 3-million-reads downsampling), with true

positives in blue (the copy-number of the *y* target matches the altered value expected for the *x* cell line), false negatives in red (the copy-number of the *y* target does not match the altered value expected for the *x* cell line), false positives in yellow (the copy-number of the *y* target does not match the normal value expected for the *x* cell line) and true negatives in light grey (the copy-number of the *y* target matches the normal value expected for the *x* cell line). In some cases, some targets were not evaluated, because of a missing signal (light grey boxes) or overlap with other targets (light green boxes). B) This plot shows the trend of sensitivity (true-positive rate; TPR), specificity (true-negative rate; TNR), positive predictive value (PPV) and negative predictive value (NPV) according to increasing target length measured on the data described by the fig. A. C) Single cells isolated from a subset of Coriell cell lines were assessed to confirm the resolution determined with gDNA using samples that simulate real cases (cell line used are indicated by black arrowheads in the figure A). As clearly visible from the figure (same format of fig. A), the results resemble those obtained with gDNA, with a resolution around 1.5Mb and even with 800Kb-sized alterations reproducibly identified by 21 cells. However, in this case it is visible how the alteration size is not the unique factor determining the

correct identification of the expected alterations, but also the specific genome location seems to play a role. In the first case, false positives appear to be concentrated in a specific 1.4Mb target located on chromosome 7, where even true negatives are at the limit of detection, thus suggesting a specific regional bias. In a second case, a 1.6Mb-sized target is partially missed as it is located at the beginning of chr22 in close proximity to the centromere, in a region of low complexity, where the segmentation of the copy-number calling algorithm often fails due to poor copy-number signal. In fact, when evaluating directly normalized read counts in each genomic bin prior to segmentation, the alteration is consistently identified (Fig. S6).

Notably, the cell line affected by the DiGeorge syndrome is correctly identified by all 21 isolated cells.

TABLES

Demographic and Pregnancy related data	
Number of pregnant women	372
Mean whole blood volume (min–max)	17.57 ml (9.5–20.5 ml)
Mean gestational age (min–max)	12.20 weeks (10–20 weeks)
Fetal gender	195 F, 181 M, 7 Not known
Number of fetuses	361 singletons, 11 twins
Mean maternal age (min–max)	35.30 years (20–47 years)
Mean maternal BMI (min–max)	22.79 kg/m^2 (14.7–40.0 kg/m^2)
Referral reason	
Screening test	
FCT	36.83% (*n* = 137)
cfDNA	12.10% (*n* = 45)
Invasive procedure	
CVS	46.51% (*n* = 173)
AFS	4.57% (*n* = 17)
Indications for CVS and AFS	
Advanced maternal age	21.11% (*n* = 42)
Abnormal ultrasound findings	5.26% (*n* = 10)
Positive FCT	53.68% (*n* = 102)
Positive cfDNA	4.21% (*n* =8)
Parent carrier of a chromosomal abnormality, prior affected pregnancy, and risk for monogenic disorders	14.74% (*n* = 28)
Gestational age (range):	
10–11 weeks	31.72% (*n* = 118)
12–14 weeks	63.71% (*n* = 237)
15–20 weeks	4.57% (*n* = 17)

Table 1. Participant information

Abbreviations: BMI, Body Mass Index; FCT, First trimester Combined Test; cfDNA, cell-free DNA; CVS, Chorionic Villus Sampling; AFS, Amniocentesis.

Patient ID	Fetal cells identified by STR	Gender	Indications	Zygosity/chorionicity by US scan and/or cytogenetic result and/or fetal gender	Zygosity/ chorionicity by STR analysis on isolated cells
M012	3	Female; Female	CVS	Dizygotic	Monochorionic - 1 STRs profile detected
M019	6	Female; Female	CVS	Monochorionic	Monochorionic - 1 STRs profile detected
M031	2	Female; Female	CVS	Dichorionic/diamniotic	Dizygotic - 2 STRs profile detected; 2 female cells
M034	21	Female; Female	CVS	Monochorionic	Monochorionic - 1 STRs profile detected
M040	3	Male; Female	CVS	Dizygotic	Dizygotic - 2 STRs profile detected; 2 female and 1
M082	2	Male; Female	CVS	Dizygotic	Monochorionic - 1 STRs profile detected; 2 female cells
M144	0	Male; Female	CVS	Dizygotic	No recovery
M184	15	Male; Female	CVS	Dizygotic	Dizygotic - 2 STRs profile detected; 10 male and 5 female
M226	25	Female; Female	FCT	Monochorionic	Monochorionic - 1 STRs profile detected
M326	11	NA	FCT	Dichorionic/diamniotic	Dizygotic - 2 STRs profile detected; 9 male and 2 female cells
M339	4	Female; Female	cfDNA	na	Dizygotic - 2 STRs profile detected; 2 female cells twin A and 2 female cells twin B

Table 2. Twin pregnancy results

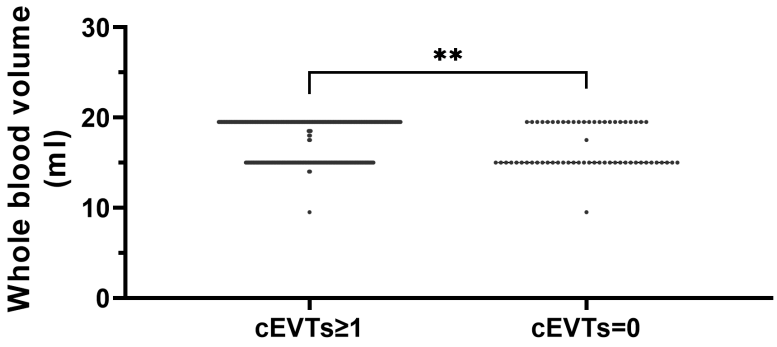
Abbreviations: FCT, First trimester Combined Test; cfDNA, cell-free DNA; CVS, Chorionic Villus Sampling; STRs, Short-Tandem Repeats; T21, trisomy 21; T18, trisomy 18; na, not available

		cEVTs analysis			Invasive prenatal diagnosis	
Patient ID	**Indication for invasive PDx**	**N° cEVTs**	**Autosomes**	**Sex chromosomes**	**Karyotype**	**Prenatal tissue analyzed**
M015	Positive FCT - high NT	6	T21	XY	47,XY,+21	CVSc
M019	Positive FCT - high NT	6	T21	XX	47,XX,+21	CVSc
M038	Positive cfDNA for T21	1	T21	XY	47,XY,+21	CVSc +CVSm
M057	Positive FCT - high NT	2	T18	XY	47,XY,+18	CVSc
M081	Positive FCT - high NT	1	T21	XY	47,XY,+21	CVSc +CVSm
M085	Positive FCT - blood test	2	T21	XX	47,XX,+21	CVSc +CVSm
M094	Ultrasound scan anomalies	2	T21	XX	47,XX,+21	CVSc +CVSm
M096	Positive FCT - high NT	25	66	XXY	69,XXY	CVSc +CVSm
M109	Positive FCT - high NT	1	T21	XY	47,XY,+21	CVSc
M115	Positive cfDNA for T21	3	T21, mosaic T18	XY	47,XY,+21 (CVSc) 48,XY,+18,+21 (CVSm)	CVSc +CVSm
M116	Positive FCT - high NT	2	T21	XY	47,XY,+21	CVSc +CVSm
M124	Ultrasound scan anomalies	2	T21	XY	47,XY,+21	CVSc
M140	Positive cfDNA for T16	3	T16	XX	46,XX	AF
M141	Positive FCT - high NT	1	Euploid	Monosomy X	45,X	CVSc
M191	Positive FCT - blood test	6	T21	XY	92,XXYY,der(14;21), +21,+21 (CVSc) 46,XY,der(14;21), +21 (CVSm)	CVSc +CVSm
M202	Positive FCT - blood test	4	T21	XX	47,XX,+21	CVSc +CVSm
M208	Positive FCT - high NT	2	T21	XX	47,XX,+21	CVSc +CVSm

Table 3. Comparison between aneuploidy detection by sequencing of circulating cEVTs and karyotyping on invasively obtained prenatal samples.

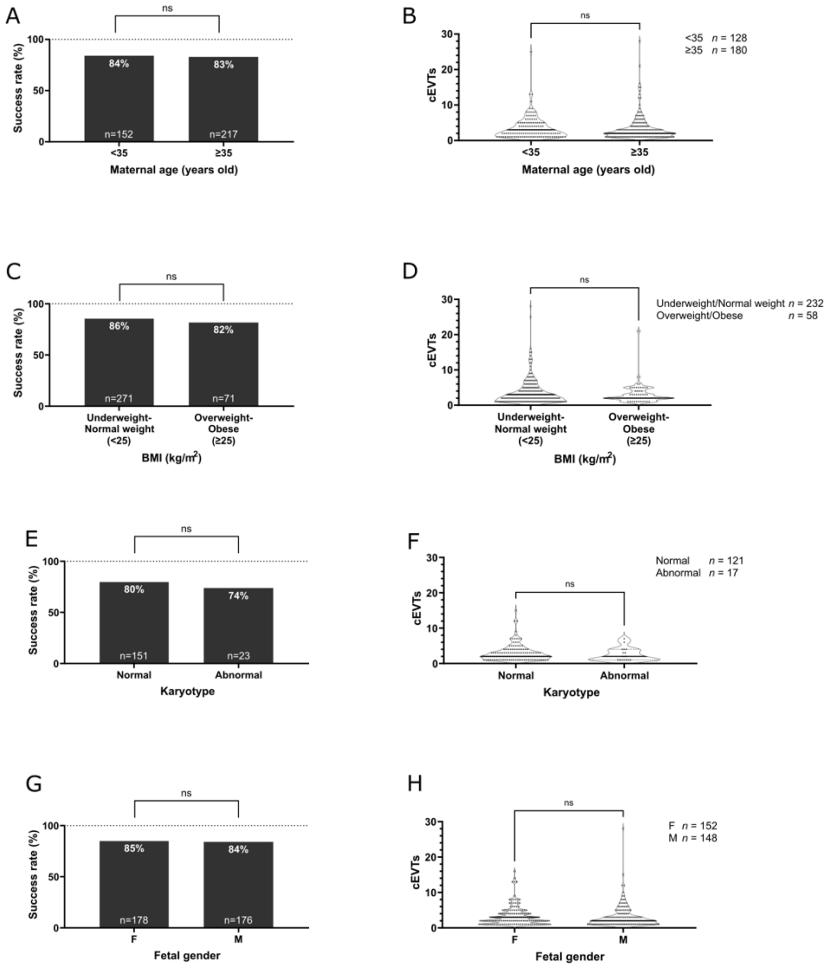
Abbreviations: cEVTs, circulating Extravillous Trophoblasts; FCT, First trimester Combined Test; NT, nuchal translucency; cfDNA, cell-free DNA; T21, trisomy 21; T18, trisomy 18; T16, trisomy 16; CVSc, analysis of cytotrophoblast layer by direct method; CVSm, analysis of mesenchyme by long-term culture; AF, amniotic fluid.

SUPPLEMENTARY FIGURES



Supplementary Figure S1. Whole blood volume in patients with (n = 311) or without (n = 61) cEVTs recovery.

The success in cEVTs recovery is significantly associated with the volume of whole blood drawn (mean[cEVTs≥1] ± SD = 17.73 ± 2.24; mean [cEVTs=0] ± SD = 16.80 ± 2.41; nonparametric Mann–Whitney U test, $p < 0.01$).



Supplementary Figure S2. Success rate and cEVTs of patients classified according to different characteristics.

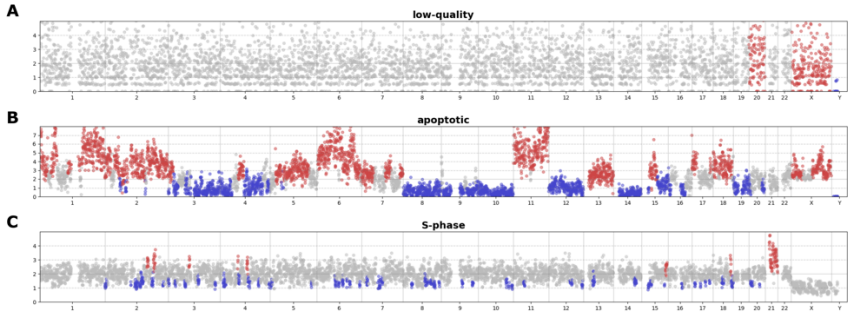
Success rate (A, C, E, and G) and violin plots of recovered cEVTs (B, D, F and H, solid lines represent median values).

A and **B**) Maternal age: <35 years vs ≥35 years; for 369 out of 372 patients, maternal age was available. Patients with unknown maternal age were not included.

C and **D**) BMI: <25 kg/m^2 (underweight/normal weight) vs ≥25 kg/m^2 (overweight/obese). For 342 out of 372 patients, BMI was available. Patients with unknown BMI were not included.

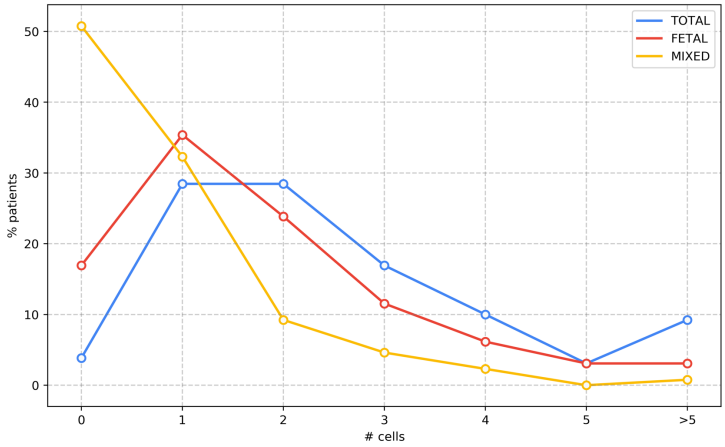
E and **F**) Karyotype: Normal vs Abnormal (MX n = 1; T18 n = 3; T18+T21 n = 1; T21 n = 19). For 185 out of 372 patients, fetal karyotype was available. Patients with unknown karyotype and twins were not included.

G and **H**) Fetal gender: F (female) vs M (male). For 354 out of 372 patients, fetal gender was available. Patients with unknown fetal gender and twins were not included.

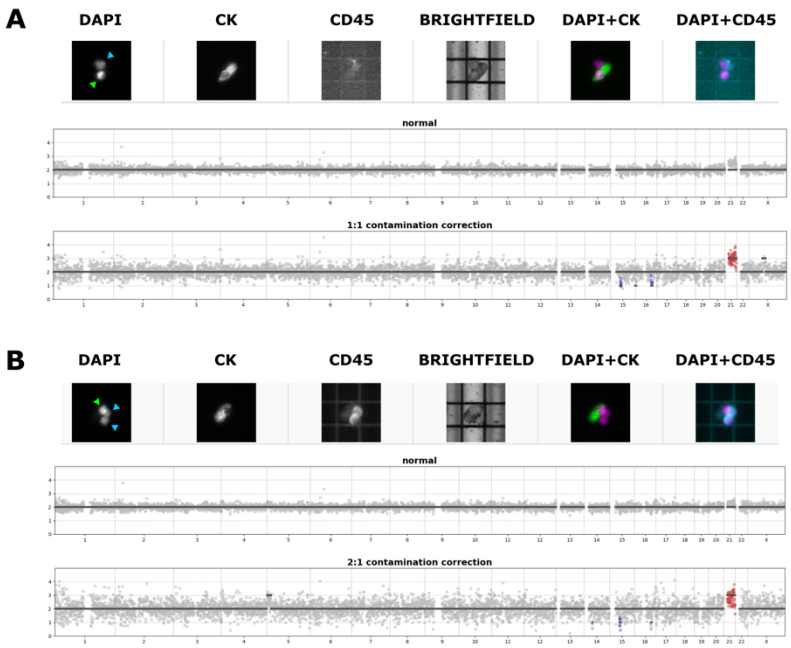


Supplementary Figure S3. Low-quality and anomalous copy-number profiles.

In some cases, the recovered cEVTs showed an uninterpretable profile. A: Low-quality profile, with a considerable background noise (high DLRS). B: apoptotic cEVT, with a high degree of aberration distributed throughout the genome (chaotic profile). C: S-phase cell, characterized by a fairly clean profile with several short deletions located along the entire genome. These small alterations do not prevent the interpretation of the aneuploidies, and for this reason, S-phase single fetal cells were considered in the comparative analysis. Conversely, S-phase profiles in mixed recoveries were discarded, as contamination correction can introduce an additional degree of noise.



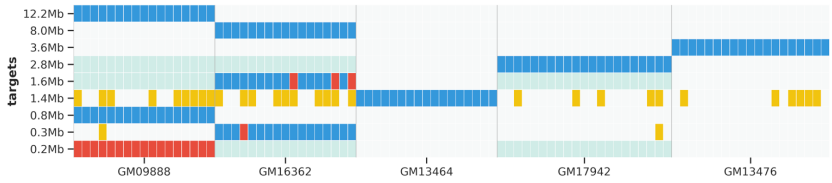
Supplementary Figure S4. cEVTs distribution per single patient. The figure shows the differential distribution of the number of fetal and mixed cells for each patient. The number of cells per subject (x-axis) versus percentage of patients are plotted discriminating the cell type with different colors (red for fetal cells, yellow for mixed recoveries and blue for no-labelled cells). The yellow curve shows with a steep decreasing gradient that slightly more than 50% of patients do not have mixed cells at all and ~80% is made up of at most 1 mixed cell. Conversely, more than 80% of patients are described by at least 1 fetal cell (red curve) and ~50% with more than 1 fetal cell, suggesting a large prevalence of fetal cells in each patient (2.1 fetal vs 0.9 mixed cells on average).



Supplementary Figure S5. Contamination correction in mixed recoveries.

In mixed recoveries, the fetal copy-number signal is partly hidden by maternal DNA contamination. Our pipeline enables correction for possible maternal DNA contamination in mixed cases, rescuing alterations that would otherwise be lost. In the figure, the images of two different mixed recoveries from the same patient (with green and cyan arrowheads indicating fetal and maternal cells, respectively) and their related copy-number profiles (normal and corrected) are reported. A) a doublet case with 1 fetal and 1 maternal cell (1:1), where in the first track a T21 trisomy is undetected. The

second track shows the profile of the same cell corrected by a 0.50 factor that properly identifies the T21. B) a triplet case (1:2) where the T21 is quite invisible in the normal profile, but properly detected in the 0.66x-corrected profile.



Supplementary Figure S6. CNV resolution using no-segmented signal.

This figure reflects the same information contained in the Fig. 5C but the signal for each single 100Kb-sized bin prior to segmentation was used. The correct detection (14/17 single cells) of the 1.6Mb target confirms that for some alterations the genome localization is critical for their proper identification, due to segmentation failure in regions with a poor copy-number signal.

SUPPLEMENTARY TABLES

Automated step	Instrument	Processing time per sample
cEVT enrichment	CellTracks Autoprep	3 h
cEVT staining		
Cell load, scan and selection	DEParray	1.5 h
cEVT confirmation	DEParray – User interaction	10–20 min
Single cEVT recovery	DEPArray	1 h

Supplementary Table S1. Steps and timing of the automated workflow.

Profile quality	Cell type	cEVTs	Percentage	Used for evaluation
High-quality	*fetal*	261	60.4	yes
	mixed	117	27.1	yes
S-phase	*fetal*	25	5.8	no
	mixed	9	2.1	no
Apoptotic	*fetal*	6	1.4	no
	mixed	7	1.6	no
Low-quality	*fetal*	6	1.4	no
	mixed	1	0.2	no
		432	100.0	

Supplementary Table S2. cEVTs distribution by profile and cell type.

		cEVTs by STR		NGS (CVS/AFS)	
		Singleton	**Twin**	**Singleton**	**Twin**
Patients with cEVTs >= 1		301	10	125	6
Patients without cEVTs		60	1	5	0
cEVTs x patient	**mean**	3.3	9.2	2.8	8.2
	median	2	5	2	4.5
	range	1-28	2-25	1-25	2-21

Supplementary Table S3. Statistics on number of cEVTs per patient, stratified by number of fetuses and workflow phase.

	singleton		twin	
cEVTs	**Patients**	**%**	**Patients**	**%**
0	5	3.8	0	0.0
1	37	28.5	0	0.0
2	37	28.5	2	33.3
3	22	16.9	1	16.7
4	13	10.0	0	0.0
5	4	3.1	0	0.0
>5	12	9.2	3	50.0
	130	100.0	6	100.0

Supplementary Table S4. Patient distribution per number of cEVTs, stratified by number of fetuses.

CHAPTER 3

Summary, Conclusions and Future Perspectives

Current methods of prenatal diagnosis require fetal cells to be obtained through invasive procedures including chorionic villus sampling and amniocentesis, which carry associated risks for both fetus and mother. In the last fifty years, numerous efforts have been spent to develop alternative methods for non-invasive prenatal diagnosis (NIPD). Current non-invasive prenatal genetic testing (NIPT) is mainly based on the analysis of cell-free circulating fetal DNA, with the limitation to have a screening value, but not diagnostic[1-2]. Patients with positive NIPT results require confirmation by invasive methods in any case.

The possibility to isolate intact fetal cells offers an exciting alternative to the conventional invasive procedures as they can be used for direct analysis of fetal chromosomes or DNA with minimal risk. However, the identification and isolation of fetal cells in maternal circulation remain challenging, given both the paucity of known surface antigens exclusively expressed on fetal cells and the rarity of the circulating fetal cells in maternal blood[3-6].

Two types of circulating fetal cells can be targeted for this purpose: trophoblasts and fetal nucleated red blood cells (fnRBCs)[7-8]. Trophoblasts derive from the placenta and can be found in the maternal circulation during the implantation, until the second

trimester of pregnancy. The main advantage of these cells is the expression of known specific markers that can facilitate the identification and discrimination against maternal contaminants cells.

Fetal erythroblasts instead, derive directly from the fetus and can therefore represent a real biopsy. Targeting these cells is more complex because they are "differentiating", meaning that also the markers expression change with time. In addition, maternal erythroblasts circulate in maternal blood together with fetal erythroblast and share most of the surface markers with these latter, making the selective isolation very challenging.

Furthermore, both trophoblasts and erythroblasts are extremely rare in the maternal circulation.

The present PhD project has approached and developed many of the matters that have been discussed in the Introduction to this thesis and summarized above, with the major aim to develop an innovative technological platform for the enrichment, identification, isolation and genetic analysis of fetal cells circulating in maternal blood, overcoming the current technical limitations that prevent the use of fetal cells in a clinical context[9]. My major contribution was on the upstream part (from the enrichment to the single cell isolation).

In the reality, this project has begun before my decision to undertake a PhD, when I moved to Singapore with the aim to discover novel biomarkers specific and selective for fetal cells in maternal circulation, in particular fnRBCs. Early protocol development work was focused on fetal cells isolated from cord blood at delivery and later from fetal blood sampling from pregnant women scheduled for surgical abortion. To identify possible differences in the surface markers expression in fetal and adult erythroblast cells, eventually we performed differential gene expression analysis on single erythroblasts isolated from fetal blood and adult bone marrow.

A total of about 800 single adult (from 5 donors) and fetal (from 7 donors) erythroblasts were sorted by FACS and sequenced for transcriptomic analysis, using the Smart-Seqv2 protocol and Illumina NEXTERA XT DNA Kit with minor modifications.

To rank the best potential fetal erythroblast markers, we have applied several criteria of analysis: we first looked in genes exclusively expressed on fetal cells, and among all the 7 biological replicates, we checked for plasma membrane localization, biological function and absolute expression level and we end up with 6 final candidates.

Afterwards, when I moved back to Italy, in order to determine if the differential expression in RNA levels were reflected in the levels of

the respective proteins, we performed immunostaining with the commercial available antibodies.

From FACS analysis we discovered few potential markers that could discriminate between fetal and maternal cells. These antibodies were conjugated with the Ferrofluid particles for preliminary spiking tests, but further optimizations are necessary and under exploration.

In the meantime we also started to investigate the ferrofluid technology with the objective to enrich fetal trophoblasts.

Ferrofluid particles, unlike magnetic beads of microscopic size, are of molecular size and this property allows them to be highly magnetic colloidal nanoparticles. They consists of an iron oxide core which can be conjugated to a specific surface antibody that recognizes and binds to fetal cells, but not to other blood cells.

Based on data from literature, EpCAM and CD105 antibodies were selected and conjugated to the Ferrofluid particles for magnetic capture. Spiking model using different cell lines were developed to evaluate the enrichment yield of each tested condition. The conjugation of CD105 antibody has required several optimizations because no data were available, including:

a. CD105 titration
b. CD105 clone selection

c. Ferrofluid particle size selection

d. Ferrofluid concentration

Other markers (CD146, CD141) have been tested with limited effect on the enrichment yield. Also for fetal cell staining, several antibodies have been tested and eventually pan-Cytokeratin was selected as positive marker, CD45 as negative and DAPI for nuclear staining. The enrichment step was subsequently fully automatized and adapted to the Autoprep machine (from Menarini Silicon Biosystem). In this way the throughput has been increased from 1 to 8 samples per time, with minimal hands-on time. Following the enrichment set-up from whole maternal blood, we were able to obtain a sample suitable for the DEPArray image-based technology analysis. To verify the real origin of the isolated cells (maternal or fetal) the genome was amplified with Ampli1 WGA kit (from Menarini Silicon Biosystems). We optimized then a molecular genotyping assay (Short Tandem Repeats - STR), used in the Forensic field, for our purpose and to be compatible with the WGA product. The fetal profile of the single cells was compared with the fetal genome obtained from amniocentesis leftover (when available), and the maternal genome (from maternal blood). At the end, as soon as we were sure about the real origin of the fetal cells through genetic

confirmation, we also started to explore genome-wide copy number analysis to detect chromosomal aneuploidies (trisomy 18, 13, 21) at single cells level.

Once the optimization of each steps was achieved, we started a clinical evaluation of the automated workflow for cEVTs recovery and their non-invasive copy number profiling comparing the results with the corresponding fetal karyotype.

From this study we demonstrated a 90.7% of recovery rate of at least one fetal cell between 10-11 gestational weeks and of 81.0% between 12-14, showing that gestational age and blood volume are key parameters influencing the recovery rate.

These rates can be easily improved either by increasing the volume at first blood draw (from 20 ml to 40 ml) or by drawing a second sample in the case of no recovery/uninterpretable results, as it is routinely performed with cfDNA testing in cases of no-call results.

Preliminary data show a high concordance rate between isolated single trophoblastic cells and fetal karyotype for common trisomies and normal results deriving from gold standard invasive procedure. In addition, the analysis of genomic DNA and single cells from Coriell cell lines allowed to determine a resolution as low as 1.5Mb

for the detection of micro-imbalances that will likely allow the detection of most of clinically-relevant microduplications and microdeletions.

Overall, the results coming out from this study, support the clinical feasibility of an automated and reproducible isolation of cEVTs for non-invasive prenatal genetic testing, well suited to routine clinical practice: blood samples could be collected before or at the time of routine first-trimester ultrasonographic scan, and stabilized for up 4 days at room temperature, allowing sample transportation to decentralized laboratories. The automated platform enables high throughput and a turnaround time comparable to those of current prenatal genetic methods. This workflow is also minimally operator dependent and therefore standardizable in decentralized laboratories.

These encouraging results have led to a clinical performance evaluation study involving 1500 patients, enrolled from five different Italian Hospitals. Primary endpoints of the study will be the performance evaluation, in terms of sensitivity and specificity, of the developed workflow for fetal aneuploidies and segmental imbalances detection (>8Mb) in a high-risk pregnancies population.

Results will be compared with data resulting from invasive prenatal diagnosis for chromosomal abnormalities obtained on the same women presenting for invasive procedure because classified from the physician as high risk pregnancy. The comparative analysis will determine the false positive, false negative, true positive, and true negative rates of the developed technology. Rare microdeletions/microduplications ranging from 1 to 8 Mb with high penetrance and severe phenotypes will be also investigated. In conclusion, the effectiveness of fetal cell isolation with this technology and the assessment, for each patient, of the number of whole fetal cells suitable for downstream analysis will be investigated on a larger cohort, to confirm data from the first study.

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