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Development and Assessment of Strategies for Non-Invasive Prenatal Diagnosis Using Fetal Cells in Maternal Blood

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Développement et évaluation de méthodes pour le diagnostic prénatal non-invasif à partir des cellules fœtales circulant dans le sang maternel

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Thèse présentée à la Faculté de Médecine et des Sciences de la Santé en vue de l'obtention du diplôme de *Philosophiae* Doctorat (Ph.D) en Biochimie, Faculté de Médecine et des Sciences de la Santé, Université de Sherbrooke, Sherbrooke, Québec, Canada, J1H 5N4

Le diagnostic prénatal résulte encore aujourd'hui de procédures invasives, qui présentent des risques pour la grossesse. Le développement du diagnostic prénatal non-invasif (DPNI) changerait le rapport risque : bénéfice, rendant le diagnostic prénatal plus intéressant pour les femmes enceintes. Plusieurs chercheurs ont montré la présence de cellules fœtales dans le sang maternel et des travaux ont été entrepris afin de les cibler et de les utiliser éventuellement en DPNI. Toutefois, la faible concentration des cellules fœtales dans le sang maternel réduit les possibilités d'isolement ainsi que celles de leur utilisation en clinique. Un autre aspect technique du DPNI, le balayage manuel, est très laborieux, surtout en terme de temps technique. Il y a donc un besoin certain pour des études approfondies afin d'évaluer et d'améliorer la faisabilité du DPNI.

La détection d'évènements rares dans une grande population cellulaire offre un potentiel pour le diagnostic en oncologie mais aussi en diagnostic prénatal. Dans cette thèse, la première étude était dédiée à l'optimisation d'une stratégie pour détecter les cellules rares. Nous avons développé une méthode d'étalement sur lame d'un nombre précis de cellules cibles parmi des centaines de milliers de cellules. Cette stratégie a permis d'évaluer le taux de détection d'évènements rares et de comparer l'efficacité des techniques d'enrichissement en connaissant le nombre exact et la localisation de cellules cibles sur les lames. De plus, il a été possible d'évaluer les problèmes d'hybridation des évènements manqués. Nous avons, par la suite, développé un algorithme robuste pour la détection de cellules rares en utilisant la plateforme de microscopie automatisée MetaSystems et utilisé cette approche dans la validation des balayages manuel et automatique d'un nombre précis de cellules mâles parmi une large population de cellules femelles marquées avec la technique FISH. Nous avons testé ce classificateur avec des échantillons de sang de femmes enceintes de grossesses normales et aneuploïdes et évalué la fréquence de cellules fœtales isolées par différentes méthodes d'enrichissement au cours des premier et second trimestres de grossesse. Les données accumulées ont confirmé la présence de cellules fœtales chez toutes les grossesses et leur fréquence plus élevée dans les grossesses aneuploïdes. Le nombre de cellules fœtales est dynamique tout au long de la grossesse. De plus, un nombre plus élevé de cellules fœtales peut être obtenu en optimisant le moment du prélèvement et les méthodes d'enrichissement. De plus, le balayage automatique s'est avéré plus sensible et constant que le balayage manuel, ce qui permet de balayer un grand nombre de cellules et devient plus approprié pour une application clinique. Nous avons aussi montré la faisabilité d'utiliser des cellules fœtales dans le cadre du DPNI. Cinq cellules amniotiques microdisséquées, provenant de grossesses normales et aneuploïdes, ont suffi pour poser un diagnostic prénatal par une combinaison de l'amplification du génome complet et de la technique QF-PCR (réaction quantitative en fluorescence d'amplification entraînée par une polymérase) permettant la détection d'anomalies chromosomiques. Nos résultats ouvrent la voie à l'utilisation de cellules fœtales dans le sang maternel pour le DPNI.

Mots-clés: Cellules fœtales – Diagnostic prénatal – Hybridation in situ observée en fluorescence – Balayage automatique

Development and Assessment of Strategies for Non-Invasive Prenatal Diagnosis Using Fetal Cells in Maternal Blood

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Current prenatal diagnosis depends on invasive procedures and is thus offered only to high-risk pregnancies. Development of non-invasive prenatal diagnosis (NIPD) would change the risk-benefit ratio and make it likely that more women would benefit from prenatal testing. Scientists have documented the presence of rare fetal cells in maternal blood and envisioned targeting them with specific markers and their use in NIPD. Considering their extremely low frequency in maternal blood, fetal cells have been difficult to retrieve and use in clinical practice. Therefore, there is a pressing need for systematic sequential studies to evaluate their feasibility in NIPD.

Generally, detection of rare cells within a large cell population carries great potentialities for the prospects of cancer management and NIPD. Manual scanning is very cumbersome and time-consuming Therefore; the first part of our project was, dedicated to the optimization of an effective strategy to evaluate retrieval of rare cells. We have developed a way of distributing a controlled number of target cells among hundreds of thousands of other cells on microscope slides. This strategy allows the precise evaluation of the retrieval of rare events and the comparizon of the efficacy of different techniques and enrichment approaches by knowing the definite number and locations of target cells on the slides. Furthermore, it allows the evaluation of hybridization of missed events. We have also developed a robust custom-made detection algorithm for rare cells using the MetaSystems automated platform and have used this strategy in the validation of manual and automatic scanning of 60 slides with a pre-defined number of rare male cells among a pure population of female cells using XY-FISH. Consequently, we tested the developed classifier for the detection of real fetal cells from maternal blood in both normal and aneuploid pregnancies with Down syndrome. We further evaluated the number of fetal cells with different methods of enrichments in the first and second trimesters. The data collected confirmed the early presence of fetal cells in all of the pregnancies tested and their frequencies were higher in cases of aneuploidies. Fetal cells are in a state of dynamic change throughout the pregnancy. Higher numbers of these cells can be obtained by optimizing the harvest time and methods of enrichment. We found that automatic scanning is more sensitive and reliable than manual detection. Furthermore, it alleviates the burden of scanning large numbers of cells and thus is more suitable for clinical application. We also demonstrated the feasibility of using rare cells in NIPD. Five microdissected amniotic fetal cells from 26 cases of normal and aneuploid pregnancies were quite enough to provide accurate NIPD through using whole genome amplification coupled with QF-PCR. Our findings laid the ground for the use of rare fetal cells in maternal blood for NIPD.

Keywords: Fetal cells – Prenatal diagnosis – Fluorescence *in situ* hybridization – Automatic microscopy

Dedications

Firstly I would like to thank God ALLAH ALMIGHTY for giving me the audacity and sanctioning me with acquaintance and confidence to fulfil this task.

I dedicate this work to my loving wife and to my parents in acknowledgment of their courage, effort, faith and enormous sacrifice.

Preface

This thesis is submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy at the Université de Sherbrooke. Work was supported by a PhD studentship provided by the Egyptian government. The thesis is the result of many years of struggle and labour work, filled with concerns and hopes for good results, sadness and tiredness with each failed attempt and finally joy when everything came together and the work was completed.

I started my doctorate working full-time on a project with Dr. Régen Drouin, which was related to the identification and quantification of rare fetal cells in the peripheral blood of pregnant mothers. The objective of the research was to develop non-invasive prenatal diagnostic tools through using these rare cellular events in the identification of common chromosomal aneuploidies. In four years of dedicated work, I developed new techniques for this research and my results enabled me to be the first author of four research articles related to this project (articles 1 to 4). These were the articles on which this thesis is based. In the last two years, I extended the concept of using rare cells to cancers and developed two articles on the use of these cells in the diagnosis of bladder cancer through the identification of specific chromosomal abnormalities. Unfortunately, we were unable to include these articles in this thesis because of unexpected change of the project director.

I have learned a lot in the process of writing this thesis, as well as the primary version of all the articles, and my initial conceptions have certainly been changed. I have dealt with a lot of subjects, in an attempt to give this thesis a broader perspective. I did the major part of the experimental work. However, this research would not have been possible without the contribution of other people, who have worked with me over the years, in particular the co-authors including the medical team who provided me with the clinical samples, the associate researchers who were always there to help and of course my fellow researchers and colleagues for their help, useful discussions, comments and suggestions.

During my doctorate, I also developed competences in other disciplines and benefitted from the dynamics of exchanges and interdisciplinary interactions within medical genetics. I have learned a lot of concepts about ethics and medical anthropology from Dr. Chantal Bouffard, who is a medical anthropologist. I enjoyed very much our daily exchange of ideas and thoughts, and the fun moments together. Moreover, I improved my clinical knowledge and challenged my medical competence in preparation for entry into supervised clinical practice in postgraduate training programs. I have successfully passed the Medical Council of Canada for both the evaluating (Part I) and qualifying Examination (Part II) and anticipate application for the 3rd part in the 2014 session.

The knowledge, which I have acquired, and the competences which I have developed during my doctoral work, would not have been possible without the support of many people, who I would like to acknowledge and thank all of them. Today, I am about to finish my PhD but I will continue to challenge myself with what I have learned during this period of my life.

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AFP	*Alpha-fetoprotein*
ACOG	*American Congress of Obstetricians and Gynecologists*
aCGH	*Array comparative genomic hybridization*
AuSc	*Automatic scanning*
BAC	*Bacterial artificial chromosome*
bp	*Basepair*
CIHR	*Canadian Institute for Health Research*
cffDNA	*Cell-free fetal DNA*
°C	*Celsius temperature*
CHUS	*Centre Hospitalier Universitaire de Sherbrooke*
CEP	*Centromeric enumeration probe*
CEP	*Centromeric enumeration probes*
CVS	*Chorionic villous sampling*
Ch	*Chromosome*
CD	*Cluster differentiation*
CD	*Cluster differentiation*
CCMG	*College of Medical Geneticists*
CGH	*Comparative genomic hybridization*
C.C	*Correlation coefficient*
Cy	*Cyanine dye*
Cy2	*Cyanine dye 2*

Cy3	*Cyanine dye 3*
Cy5	*Cyanine dye 5*
CK	*Cytokeratins*
DAPI	*4',6'-diamidino-2-phénylindole*
DOP-PCR	*Degenerate oligonucleotide-primed-polymerase chain reaction*
DGC	*Density gradient centrifugation*
DNA	*Deoxyribonucleic acid*
ε	*Epsilon*
NRBC	*Erythroblasts or nucleated red blood cells*
FN	*False negative*
FBS	*Fetal bovine serum*
FC	*Fetal cell*
FCs	*Fetal cells*
FITC	*fluorescein isothiocyanate*
FACS	*Fluorescence activated cell sorting*
FISH	*Fluorescence in situ hybridization*
FSH	*Follicle-stimulating hormone*
γ	*Gamma*
gDNA	*Genomic desoxyribonucleic acid*
g	*Gram*
Hb	*Haemoglobin*
HBSS	*Hank's balanced salt solution*
h	*Hour*

HASH-2	*Human Achaete-Scute Homologue 2*
hCG	*Human chorionic gonadotropin*
HLA	*Human leucocytes antigen*
HPL	*Human placental lactogen*
ID	*Identification code*
ISIS	*In situ imaging system*
kb	*Kilobase*
LCM	*Laser capture microdissection*
LSI	*Locus specific probe*
MACS	*Magnetic Activated Cell Sorting*
MnSc	*Manual scanning*
MB	*Maternal blood*
MB	*Maternal blood*
µg	*Microgram*
µl	*Microlitre*
mg	*Milligram*
ml	*Millilitre*
mM	*Millimole*
min	*Minute*
M	*Mole*
M-FISH	*Multicolour - Fluorescence in situ hybridization*
MoM	*Multiple of median*
ng	*Nanogram*

NIFTY	*National Institutes of Health Fetal Cell Study*
NIPD	*Non-invasive prenatal diagnosis*
N/A	*Not acquired*
p	*Short arm of chromosome*
PBS	*Phosphate-buffered saline solution*
PAC	*Plasmid P1-derived artificial chromosomes*
PCR	*Polymerase chain reaction*
KCl	*Potassium chloride*
PAPP-A	*Pregnancy associated plasma protein A*
PGD	*Preimplantation genetic diagnosis*
PRINS	*PRImed In Situ labelling*
PEP	*Primer extension pre-amplification*
P	*Probability value*
q	*Long arm of chromosome*
QF-PCR	*Quantitative fluorescence - Polymerase chain reaction*
RhD	*Rhesus D antigen*
RNA	*Ribonucleic acid*
RT	*Room temperature*
SSC	*saline-sodium citrate buffer*
SCs-WGA	*Single-cells whole genome amplification*
STRs	*Sort tandem repeat*
SKY	*Spectral karyotyping*
SD	*Standard deviation*

SAS	*Statistical Analysis Software*
SSPS	*Statistical Package for the Social Sciences*
TRITC	*Tetramethyl Rhodamine Iso-Thiocyanate*
TCs	*the total number of cells*
TP	*True positive*
UV	*Ultraviolet*
uE3	*Unconjugated estriol*
W	*Weeks*
WCP	*Whole chromosome painting probes*
WGA	*Whole genome amplification*
YAC	*Yeast artificial chromosome*
ζ	*Zeta*

INTRODUCTION

1. General Information

The burden of genetic disorders is heavy in all parts of the world, particularly in under-resourced settings, which lack specialized health and social services to care for affected individuals. Large numbers of infants with genetic disorders are born each year from families in underserved populations as a result of the high birth rate, consanguinity and late procreation. As a result, most pregnant women would wish to be reassured that their unborn babies are healthy. Access to safe, accurate and affordable screening and diagnostic tests at a time that avails the mother an option of pregnancy termination is therefore essential.

Prenatal diagnosis of aneuploidies is usually performed by collecting fetal samples through either amniocentesis or chorionic villous sampling (CVS). These procedures are invasive and are associated with a significant risk of miscarriage. Therefore, in recent years, considerable effort has been made to develop non-invasive prenatal diagnostic procedures (Finegan et al., 1990).

One potential non-invasive approach is to utilize the fetal cells (FCs) within the maternal circulation. Cell's trafficking between the fetus and its mother provides indirect clues as to the underlying pathophysiology during pregnancy. It also provides a source of fetal materials for non-invasive prenatal diagnosis. Many questions remain about the feasibility of using FCs from maternal blood (MB) for prenatal diagnosis.

Although the retrieval of FCs from maternal blood (MB) is an attractive concept, many questions remain regarding the feasibility of using FCs from maternal blood (MB) for prenatal diagnosis. In fact, the very low abundance of FC in MB is a technical challenge (Bianchi et al., 2002; Ariga et al., 2001; Krabchi et al., 2001) and there is currently no application for these methods in clinical practice. Additional work is needed to isolate FC early in pregnancy in order to provide, if necessary, pregnancy termination options to the parents.

2. Historical background

The presence of the FCs in maternal circulation during the pregnancy has been known for a long time. Indeed, the existence of FCs in MB was shown more than one century ago. Many authors in this field recognize the work of Georg Schmorl, a German pathologist, which was published in Leipzig in 1893,, to document the first description of feto-maternal cellular trafficking, as well as the first clues for the presence of retained FCs in maternal body organs (Schmorl, 1893). His elegant and pioneer work has been recently translated in English and critically re-evaluated from a 21st century perspective (Lapaire et al., 2007). Schmorl had noted for the first time the presence of multinucleated syncytial giant cells sequestered in the thin capillaries of the pulmonary parenchyma in the autopsies of 14 of 17 women who died of eclampsia. Schmorl assumed that the only source of the multinucleated cells could be the decidua or the placenta. Furthermore, he was the first physician to emphasize the importance of the placenta in the etiology of pregnancy complications such as eclampsia and preeclampsia (Schmorl, 1893). More than 60 years later, the placental origin of these trophoblastic cells was established. Other authors reported the presence of these cells in other tissues including the uterine veins and peripheral venous blood in both normal and complicated pregnancies (Wagner et al., 1964; Douglas et al., 1959).

Many investigators, in late 1950s, began to demonstrate that fetal erythrocytes were also present in the maternal peripheral circulation. Kleihauer found FCs in the blood of pregnant women by using a specific stain for fetal haemoglobin (Kleihauer et al., 1957). Clayton et al. noted that the proportion of fetal red cells was higher in women with pregnancies complicated with preeclampsia or vaginal bleeding, and after abortion or amniocentesis (Clayton et al., 1964).

The first cytogenetic study to identify FCs in maternal circulation was done in 1969 by Walknowska et al (Walknowska et al., 1969). These authors treated MB with a lymphocyte mitogen and then examined the metaphase cells. They clearly showed the presence of XY metaphases in maternal peripheral blood of 19 out of 21 pregnancies with

male fetus. De Grouchy and Trebuchet (de Grouchy and Trebuchet, 1971) as well as other groups confirmed these results (Schindler and Martin-du-Pan, 1972; Takahara et al., 1972).

Cell sorting techniques became available in late 1970s which allowed isolation and characterization of cells based on their antigenic characteristics. In 1979, Herzenberg et al added, to the step of analysis of Y chromatin of male FCs, a preliminary technique of enrichment using a flow cytometer (FACS: fluorescence-activated cell sorting) on the basis of cellular class I major histocompatibility antigens. They exploited the human leucocytes antigenic differences (HLA) between the mother and her fetus. Using blood from HLA-A2 negative women whose partners were HLA-A2 positive, cells were sorted based on the presence of the HLA-A2 antigen. The sorted cells, most likely lymphocytes, were then analyzed for the presence of Y-chromatin fluorescence. Y-chromatin positive cells were found more frequently in women carrying male fetuses than in those carrying female fetuses. Five out of twelve pregnancies with male fetuses presented cells with Y chromosome (0.3 to 1.6% of the sorted cells). For the other seven, there was in fact no cells with Y chromosome (Herzenberg et al., 1979). Iverson and co-workers (Iverson et al., 1981) used for the first time this technique to enrich FC in order to determine the sex of the foetus. The heterochromatin of the long arm of the Y chromosome was used as independent marker in pregnancies as early as 15 weeks of gestation. Y chromatin-containing cells were found among the sorted cells from prenatal MB specimens in eight pregnancies out of eight subsequently produced male infants. The presence of positive HLA-A2 with the heterochromatin of Y confirmed the fetal origin of the cells.

Molecular techniques became available in late 1980s, which allowed reliable and significant analysis of genetic markers to confirm the presence of the FCs in MB circulation. In 1989, Lo and co-workers (Lo et al., 1989) proposed the use of polymerase chain reaction (PCR) to identify Y-specific DNA sequences in MB. Y-chromosome specific sequence could be detected in MB as early as six weeks of gestation. Four years later, the same group reported performing PCR on unsorted first trimester MB and were able to correctly identify six of seven pregnancies with male fetuses and five of six pregnancies with female fetuses. The detection rate was at least as good in the first trimester as in the second or third. Serial

dilutions of male DNA were also performed to calculate that this method has the sensitivity to detect one male cell dispersed in 300,000 female cells (Liou et al., 1994; Lo et al., 1993; Lo et al., 1990). These PCR results were also confirmed with the use of enrichment procedures of cellular sorting using either fluorescent monoclonal antibody by FACS (Bianchi et al., 1990; Mueller et al., 1990) or magnetic balls by Magnetic Activated Cell Sorting MACS (Wachtel et al., 1991). The recognition of FCs by specific antibodies and their isolation using cell sorter or magnetic beads were the subjects of intense search. It is however necessary to point out that the sensitivity and the specificity of these methods remain insufficient to allow a reliable prenatal diagnosis.

Following these reports, the potential use of FCs, for a non-invasive prenatal diagnosis (NIPD), was strongly reconsidered. Many research teams studied the nature of these FCs. These studies raised much controversy, in particular, on the origin of these cells, their lifespan and antigenic specificities that could distinguish them from maternal cells. Other questions regarding their time of appearance and relative frequency throughout the gestational age were also the subject of intense studies.

Several authors reported a quantification of the number of FCs (Krabchi et al., 2001; Bianchi, 1998). The frequency of FCs circulating in maternal peripheral blood in normal pregnancy is very low. In the pathological situations, such as for example in the preeclampsy or fetal aneuploidies, the fetomaternal cellular transfusion is apparently increased (Zhang et al., 2008; Krabchi et al., 2006b; Krabchi et al., 2006c).

Various categories of FCs have been proposed. The possible cell types that can be isolated from MB and used for prenatal diagnosis include trophoblasts, lymphocytes, erythroblasts, granulocytes, and thrombocytes (Goldberg, 1997). All these cells are nucleated except for the blood platelets (thrombocytes) which loss their genomics DNA during the process of differentiation. The presence of rare lymphoid progenitor cells of fetal origin in MB from former pregnancies has been also reported (Bianchi et al., 1996). Given the extreme scarcity of these events and their antigenic specificities, they are not regarded as major handicap for the development of NIPD. Many attempts of isolation of the FCs, for the sake of NIPD, focus mainly on the erythroblasts and cytotrophoblasts. Indeed, these two

types of cells have the advantage of being highly differentiated and are known to have a short life span and thus unlikely to persist in MB postpartum.

3. Aneuploidies and screening modalities

Prenatal care has existed for over 100 years as an approach to improve maternal and newborn outcomes. Screening tests are usually non-invasive and may help delineate which patients are at high risk and should be offered invasive testing. Diagnostic tests on the other hand have the benefit of providing a definite answer about the presence of a genetic disorder. Since these tests carry varying risks of pregnancy loss, they are usually reserved for high-risk women with positive screening.

3.1. Trisomy 21 and other aneuploidies

Trisomy 21, also known as Down syndrome, is the leading cause of prenatal chromosome abnormalities, accounting for more than half of all reported chromosomal aneuploidies (Hook et al., 1983). The incidence of trisomy 21 in the general population is 1.3 per 1000 live births (1/770). Incidence rate increases significantly with maternal age (Table1) (Huether et al., 1998).

Table 1 Maternal age and risk of Down's syndrome

Maternal age at term	Risk of Down's syndrome	Maternal age at term	Risk of Down's syndrome	Maternal age at term	Risk of Down's syndrome
20	1:1450	30	1:940	40	1:85
21	1:1450	31	1:820	41	1:70
22	1:1450	32	1:700	42	1:55
23	1:1400	33	1:570	43	1:45
24	1:1400	34	1:460	44	1:40
25	1:1350	35	1:350	45	1:35
26	1:1300	36	1:270	46	1:30
27	1:1200	37	1:200	47	1:30
28	1:1150	38	1:150	48	1:30
29	1:1050	39	1:110	49	1:25

Table 1 shows that risk of Down's syndrome increase with advancing maternal age. J Med Genet. Jun 1998; 35(6): 482–490

Down syndrome individuals have a distinct phenotype and show various degree of intellectual disability (Sherman et al., 2007). Patients with trisomy 21 have a slightly reduced life expectancy (Thorpe et al., 2012; Baird and Sadovnick, 1990). Indeed, they are often victims of medical complications and developmental disorders (Tenenbaum et al., 2012; Hayes and Batshaw, 1993; Carey, 1992). They account for 20 to 30% of the subjects with moderate to severe mental retardation. More than 15% of the adult subjects will develop Alzheimer's disease around the age of forty (Strydom et al., 2013).

In Canada, there are approximately 400,000 births with more than 600 new cases of trisomy 21 per year. A prenatal screening program has been created in Quebec in 1976 (Okun et al., 2008; Forest et al., 1995; Baird and Sadovnick, 1990). The majority of mothers with affected fetuses, after being informed of the prognosis and postnatal therapeutic options, choose medical interruption of the pregnancy (Grant and Flint, 2007; Fletcher, 1981).

Other trisomies, such as trisomy 18 (Edward syndrome) and trisomy 13 (Patau syndrome), are associated with fatal congenital malformations. Survival is very poor with approximately 50% miscarriages prior to birth and, most liveborns die within the first month of life (Hutaff-Lee et al., 2013).

3.2. Screening modalities

Screening is conventionally described as the evaluation of asymptomatic people to detect unsuspected disease or risk in order to improve health outcome in a defined population (Henry and Bronson, 1996).

Reproductive genetic screening is performed to assist reproductive decision-making and to give parents the opportunity to avoid the birth of an affected child. Because the decision to terminate a pregnancy is highly personal, prenatal screening is considered optional, in sharp contrast to the mandatory newborn screening after birth. In keeping with this difference, genetic professionals have developed a counselling approach to assist couples to determine their own preferred course of action (Mahowald et al., 1998).

3.2.1. Genetic counselling

Genetic counselling begins with a thorough medical and family history of the patient and her partner to identify high-risk patients who may benefit from diagnostic or therapeutic procedures. Historically, the first and most important risk factor for trisomy 21 is advanced maternal age. Incidence increases gradually up to the age of 35 years then rises very sharply. The reason for this is not entirely known but may have to do with abnormal function of the meiotic spindle during female meiosis, resulting in nondisjunction (Zournatzi et al., 2008).

Because, at the age of 35, the risk of having a newborn with chromosomal aneuploidy approximately equals the risk of pregnancy loss with invasive testing (0.5%, or 1/200), it is standard of practice to offer invasive testing to women who will be 35 years or older at the time of delivery. However, this strategy is not very efficient because two third of the trisomic 21 children are born from women having less than 35 years old (Zournatzi et al., 2008). For this reason, different screening modalities were developed and implemented in order to re-evaluate the risk and precisely identify those that would benefit from invasive diagnostic tests.

3.2.2. First trimester ultrasound

Anatomical ultrasound has been used since the 1980s for detection of major structural abnormalities. With advances in technology, prenatal ultrasound has expanded to include detection of soft markers more commonly found in fetuses with chromosomal abnormalities (Getz and Kirkengen, 2003). During the first trimester, between 10 and 14 weeks, thickening of an area behind the fetal neck (nuchal translucency) is associated with an increased risk of Down syndrome. This fluid-filled area of the posterior neck normally resolves by the second trimester (Snijders et al., 1998).

Other soft markers linked to Down syndrome include echogenic bowel, renal pelvic dilation, absence of the nasal bone, ventriculomegaly, clinodactyly, and sandal gap toe (Cicero et al., 2001). The presence of one of these markers increases the risk by two-fold while three or more markers increases the risk by 100-fold (Benacerraf, 2010; Nyberg and

Souter, 2001). While these criteria help in determining relative risk, they remain limited by the quality of the ultrasound and the expertise of the sonographer.

3.2.3. Maternal serum biomarkers

Maternal serum biomarkers are substances secreted by the fetus or placenta during pregnancy and that can be measured in the MB. The level of these markers can be useful to predict congenital anomalies and chromosomal abnormalities, particularly trisomy 21. The expected amount of these substances found normally in the mother's bloodstream changes weekly during pregnancy, so it is important to to accurately determine the gestational age, usually using ultrasound at 10-12 weeks.

The level of each marker is expressed as multiples of the median (MoM), obtained by dividing the serum concentration at a particular gestational age by the population median concentration at the same gestational age (Spector et al., 2005). Combined values of different markers provide a risk estimate rather than a definitive diagnosis.

Alpha-fetoprotein (AFP) is produced by the fetal liver, but is transported to the MB across the placenta. High level of AFP is frequently associated with neural tube defects. Other possible causes of a high AFP include incorrect dates, multiple pregnancies, fetomaternal bleed, and other fetal malformations, such as defects of the abdominal wall. This may be due to leaky placental barrier or placental dysfunction associated with these pathologies. Low levels of AFP are associated with Down syndrome. Any abnormal AFP measurement should be followed by a detailed fetal ultrasound (Guibaud et al., 1998; Rose and Mennuti, 1993).

Human chorionic gonadotropin (hCG) is also known as the ‘pregnancy hormone’. It is produced by the placenta very early in pregnancy. It is made of α and β chains. The rate of synthesis of the total hCG is dependent on the rate of synthesis of the free β-hCG fraction. This hormone peaks early in pregnancy at 8–10 weeks. After that peak, it progressively declines to reach a plateau at 18 to 20 weeks of gestation. Levels are increased in Down syndrome, and decreased in trisomy 18. Elevated mid-trimester levels

have been associated with congenital abnormalities, placental dysfunction and adverse pregnancy outcome (Rose and Mennuti, 1993; Bogart et al., 1987).

Unconjugated estriol (uE3) is the dominant form of estrogen during pregnancy. This hormone is derived from precursors from the fetal adrenal and liver that are processed in the placenta. Low estriol may be associated with Down syndrome and anencephaly, the most severe neural tube defect. Other syndromes associated with low estriol include congenital adrenal hypoplasia, and X-linked icthyosis (Guibaud et al., 1998; Canfield and O'Connor, 1991).

Inhibins are placental hormones that inhibit the secretion of follicle-stimulating hormone (FSH). There are two forms: inhibin-A and inhibin-B; however, only the former is found in pregnant women. Inhibin-A has been found to be increased in Down-syndrome pregnancies, and has most recently been added as a fourth serum marker for second trimester screening (Gagnon et al., 2008; Lockwood et al., 1997).

Pregnancy associated plasma protein A (PAPP-A) is produced by the placenta and thought to have several functions, including prevention of recognition of the fetus by the maternal immune system. A PAPP-A level was found to be low in pregnancies with Down syndrome and other chromosomal defects. Recent studies support an association between low PAPP-A levels in first trimester and risk for adverse pregnancy outcomes as prematurity and growth retardation (Smith et al., 2002).

3.3. Routine prenatal screening

3.3.1. Second trimester screening

Several studies showed that measurements of biochemical markers in the maternal serum between 15 and 17 weeks of gestation could be useful to identify complicated pregnancies. The possibility of prenatal screening, using maternal serum markers, was reported for the first time in 1984 by Merkatz et al. (Merkatz et al., 1984). AFP was the earliest serum marker used to detect open neural tube defects and abdominal wall defects

and with time it was extended to screen for Down syndrome (Cuckle et al., 1984; Wald et al., 1977).

In 1987, Bogart and co-workers (Bogart et al., 1987) showed that serum concentrations of hCG are higher than normal in the pregnancies affected by trisomy 21. Continued advancements in research resulted in the introduction of a multiple markers screening panel, or the" triple test", in 1991 (MacDonald et al., 1991). In addition to AFP, the panel included uE3 and total hCG. The triple screen was widely employed in obstetrical practice to detect neural tube defects and chromosomal aneuploidies. The detection rate for Down syndrome varies from 30% to approximately 69%.

The quadruple test was introduced in 2000, when inhibin-A was added to the triple test panel (Hackshaw and Wald, 2001; Haddow et al., 1998b; Aitken et al., 1996). The introduction of the quadruple test has significantly increased the detection rate. By combining maternal age with the quad screen, the detection rate is roughly 75% for Down syndrome in women younger than 35 years and 80% in women 35 years and older with an approximately 5% false positive rate (Benn et al., 2001).

3.3.2. First trimester screening

It was not until the late 1990s that first trimester screening was introduced as an earlier screening option for the detection of Down syndrome. First trimester screening incorporates maternal age, nuchal translucency, and measurement of specific serum markers. A Collection of blood for biochemical analysis and ultrasound assessment is typically performed between 11 and 14 weeks. The most effective first-trimester biochemical markers are PAPP-A and free hCG in maternal serum (Biagiotti et al., 1998; Haddow et al., 1998a).

However, there is no single marker can detect all the pathological pregnancies. The echographic signs and the serum markers together can increase the detection rate. First trimester biochemical markers alone have only 60% sensitivity (Cuckle and van Lith, 1999). Combined with nuchal translucency, the detection rate is around 80%, with a 5% false–positive rate (Krantz et al., 2000).

Large collaborative, prospective studies have validated the clinical application of first-trimester screening, and showed that it could be superior to second-trimester screening (Nicolaides et al., 2005). In addition, it reduces both physical and psychological trauma related to late interruption of pregnancy as well as therapeutic costs.

3.3.3. Combined first and second trimester screening

Several investigators studied different ways of incorporating the results of first and second trimester serum screening to obtain the most accurate estimation of Down syndrome risk. Many modalities have been created to help maximize the sensitivity, while maintaining a low false-positive rate.

Integrated screening in which a patient's first trimester screening results are not disclosed until second trimester screening is performed and a combined risk can be calculated, has been ethically debated. This option precludes patients who are at high risk based on first-trimester screening from being offered early CVS or other available options (Knight et al., 2005).

Independent sequential first and second-trimester screenings, with separate individualized risk assessments increases the detection rate from 80% to 90% but it also increases the false-positive rate from 5% to 11%. In contrast, stepwise sequential screening in which only patients who screen negative in the first trimester are offered second-trimester screening, increases the detection rate to more than 90% while still maintaining a low false-positive rate of 6% (Aagaard-Tillery et al., 2009; Platt et al., 2004).

Contingent screening method is similar to stepwise sequential screening. However, the contingent screening uses the first-trimester results to classify patients into three groups, i.e., screen-positive, screen-negative, and borderline. Second-trimester screening is only offered to patients who fall into either the screen-negative or the borderline group. The detection rate for this method is 95%, with a 5% false-positive rate (Palomaki et al., 2006; Wright et al., 2004).

4. Prenatal Diagnosis

In most developed countries the option of having prenatal diagnosis is discussed as part of routine antenatal care. Testing strategies, guidelines, and diagnostic options have expanded from their conception in the 1970s. At that time, any woman aged 35 years or older was considered to be of advanced maternal age, and this was the sole criterion used by the American Congress of Obstetricians and Gynecologists (ACOG) to define pregnancies that should be offered amniocentesis or CVS.

As of 2007, the ACOG has extended the definition of a “high-risk” pregnancy that justifies prenatal cytogenetic diagnosis to include advanced maternal age, parental chromosome rearrangements, previous pregnancy with autosomal anomaly, abnormal fetal ultrasound findings during the current pregnancy and increased risk calculated from non-invasive screening (ACOG, 2007b).

However, current ACOG guidelines stated unequivocally that neither age 35 years nor any specific age should be used as a threshold for invasive testing: ‘All women, regardless of age, should have the option of invasive testing’. The guidelines specifically elaborate that ‘patients informed of the risks, especially those at increased risk of having an aneuploid fetus, may elect to have diagnostic testing without first having screening’ (ACOG, 2007a). Younger women may elect an invasive procedure because they wish to achieve the near 100% detection, possible only with an invasive procedure; detection by an invasive procedure exceeds by 10–15% that of any non-invasive screening protocol.

4.1. Routine prenatal diagnosis

4.1.1. Invasive diagnostic procedures

The Prenatal diagnosis of the chromosomal anomalies generally requires collection of fetal tissue and chromosomal analysis of FCs. Fetal tissues can be obtained by either CVS, amniocentesis or less commonly cordocentesis through puncture of the umbilical cord. Valenti and co-workers, in1969, (Valenti et al., 1969) reported the first prenatal diagnosis of Down's syndrome three years after the achievement of amniotic cell growth by

Steele and Berg (1966) (Steele and Breg, 1966). Amniocentesis is usually performed through puncture of amniotic sac to obtain amniotic fluid for karyotyping and other biochemical tests at 16-18 weeks of gestation. Amniocentesis performed before 15 weeks is referred to as ‘early amniocentesis’. Early amniocentesis is not a safe alternative to second-trimester amniocentesis because of increased pregnancy loss, limb reduction defects and clubfoot (CEMAT, 1998).

The development of CVS in the early 1980's has allowed anticipation of diagnosis in the first trimester (Brambati and Simoni, 1983). CVS is usually performed between 9 and 13 weeks of gestation and involves aspiration or biopsy of placental villi. CVS can be performed using either a transabdominal or a transcervical approach. Several randomised trials show almost identical miscarriage rates after transcervical CVS compared with the transabdominal approach (Jackson et al., 1992; Brambati et al., 1991). Only one trial demonstrated the transabdominal approach to be significantly safer (Jackson et al., 1992).

Hundreds of thousands of amniocentesis and CVS after 10 weeks of gestation have been done without causing any complications or an increase in fetal malformations. However, both procedures are sometimes difficult and associated with some risks mainly to the pregnancy, but in certain circumstances to both the mother and the fetus. They also require the expertise of a specialized medical team and present a risk of iatrogenic fetal loss estimated between 0.5 and 2% (Tabor et al., 1986). The clear advantage of an early procedure like CVS over amniocentesis is the avoidance of a prolonged period of uncertainty and the availability of less stressful options in cases in which termination of pregnancy is desired after an abnormal result (Bindra et al., 2002). However, the disadvantage is the increased risk of miscarriage after first trimester CVS. Some authors even reported higher rates of limb reduction abnormalities and subsequent development of preeclampsia with CVS carried out at 9 weeks or earlier (Grobman et al., 2009; Philip et al., 2004). Other fetal risks include intrauterine fetal death and premature birth (Vigliani, 2009). Severe sepsis, including maternal death, has been reported following invasive prenatal procedures. The level of risk cannot be quantified as case report literature does not provide denominator information but the risk of severe sepsis is likely to be less than 1/1000

procedure (Bodner et al., 2011). Infection can be caused by inadvertent puncture of the bowel, skin contaminants or organisms present on the ultrasound probe or gel. The procedures also increase the risk of maternal isoimmunization provoked by fetomaternal hemorrhage. Therefore, maternal RhD status should be obtained and prophylaxis with anti-D immunoglobulin must be offered following each procedure to RhD negative women in line with international recommendations (ACOG, 2006).

Currently, these invasive procedures are offered only to small group of women who are in a higher risk of having an offspring with a chromosomal defect in comparison to the general population. This high-risk group constituted less than 5% of the pregnant population. In addition, only one out of 20 procedures performed will reveal aneuploidy (Crossley et al., 2002). Development of non-invasive methods would obviate this risk and change the risk-benefit ratio of prenatal diagnosis. Such a change would make it likely that more women presently eligible for prenatal diagnosis would choose to undergo testing. In addition, genetic testing could even be offered to women who are not considered at high risk. One of the most promising non-invasive sources of fetal genetic materials is the peripheral MB. In this view, analysis of FC represents a major objective of many researches.

4.1.2. Routine fetal karyotype

With invasive tests such as amniocentesis or CVS, FCs are obtained for culture. For routine fetal karyotype, culture of FCs is essential prior to analysis as chromosomes are only visible in dividing cells. The application of strategies for improving cell culture and chromosome banding has expanded the number of laboratories that may perform successfully fetal chromosome analysis (Cheung et al., 1987; Brackertz et al., 1983; Porreco et al., 1980). The standard analysis implies the study of the number and structure of the 23 chromosome pairs. The most common chromosome anomalies, are related to non-disjunctional errors, result in an extra copy or loss of one chromosome. Trisomy 21 is by far the single most common cause of aneuploidies. Other identified abnormalities involve trisomies of chromosomes 13, 18, or sex chromosomes.

Although high resolution banding could allow diagnosis of small structural anomalies, these anomalies are relatively uncommon, accounting for less than 1% of all chromosomal abnormalities. Furthermore, balanced translocations and inversions, which are the commonest identified structural anomalies, are clinically irrelevant for the current pregnancies (Warburton, 1984; Jensen et al., 1982). However, there is a general consensus among cytogeneticists and physicians that the extra knowledge provided by a full karyotype is beneficial and thus, a full fetal karyotype is the gold standard of prenatal diagnosis.

4.1.3. Rapid diagnostic techniques of fetal chromosomal anomalies

The time needed to culture FCs and complete the analysis ranges from 10 to 21 days, which is generally considered to be a psychological burden and results in late terminations following a pathological diagnosis. In the early 1990s, FISH (Fluorescence *In Situ* Hybridization) (Kuo et al., 1991) and, more recently, QF-PCR (quantitative fluorescence polymerase chain reaction) (von Eggeling et al., 1993) entered the field of prenatal diagnosis to overcome the need to culture FCs, and hence allowed a rapid diagnosis of some selected chromosomal anomalies. FISH and QF-PCR provide a rapid diagnosis of aneuploidies within 24–48 hours. Although both techniques could be applied to identify all chromosomes, only chromosomes 13, 18 and 21, as well as the sex chromosomes, are routinely tested (Divane et al., 1994). The result was, and still is, sufficient to take action if a chromosome anomaly is thus identified, but is usually considered only a preliminary step while awaiting the result of full karyotype.

Array comparative genomic hybridization (CGH) has been proposed as a genome-wide assessment approach for prenatal diagnosis of chromosomal abnormalities. Array CGH is a molecular cytogenetic method for analysing copy number variations relative to ploidy level in the DNA of a test sample compared to a reference sample, without the need for culturing cells. Many reports have demonstrated the sensitivity, specificity and accuracy of this methodology detecting large and small-size imbalances (Pickering et al., 2008; Shaffer et al., 2008; Shaffer et al., 2007). Although different types of chromosomal abnormalities have been successfully identified by array CGH, the CGH traditionally is costly and requires advanced equipment (Lao et al., 2008). Another disadvantage of an

array CGH system is the time required for analysis and interpretation of the results, especially with the many incidental findings of unknown clinical significance, which creates an ethical dilemma and raises the maternal anxiety (Keren et al., 2010). However, array CGH has particular importance in investigating cases with strong history of intellectual disability or congenital abnormalities despite a normal conventional karyotype.

Although these techniques hasten the process of prenatal diagnosis, they did not overcome the risk associated with invasive sampling of fetal tissues, which, therefore, limits offering prenatal diagnosis only to women with high risk pregnancies, as estimated by increased maternal age, abnormal biochemical markers and ultrasonographic findings.

4.2. Non-invasive prenatal diagnosis

The long-term goal of modern prenatal genetics is the development of definitive NIPD. That is, an analysis of MB that can detects fetal aneuploidy and other disorders without the need for an invasive procedure. Currently, prenatal diagnosis safety is limited by the need of invasive means to obtain fetal tissues. This limits its application to only small group of high-risk patients, which constitute less than 5% of all pregnancies. However a fairly good non-invasive method to obtain fetal tissues would obviate this risk and extend prenatal testing to include wider portion of pregnant population. It is currently agreed that genetic fetal material including both cells and cell-free fetal DNA (cffDNA) pass into maternal circulation during pregnancy. Non-invasive fetal diagnosis could therefore be possible from their isolation and analysis from peripheral MB. Although the cffDNA is increasingly used with massively parallel sequencing (Jensen et al., 2013; Liang et al., 2013; Liao et al., 2012; Ashworth, 1869) or targeted deep sequencing (Nicolaides et al., 2013; Zimmermann et al., 2012) to test for aneuploidy and single-gene disorders, it is still consider as a screening test and its application in clinical practice is very cumbersome and expensive. Although, cffDNA is currently a topic of great interest, this review will focus on the discussion of intact FCs in MB.

Intact FCs have considerable advantage over cffDNA as the whole fetal genomic DNA can be purely recovered without maternal contamination and consequently simplify

the analysis and enables more women to undergo prenatal diagnosis without a significant increase in health expenditure. Analysis of pure fetal DNA from intact FCs would allow not only readily diagnosis of aneuploidy but also other small structural genetic defects concurrently. Even if one or few FCs were recovered, the approach would merely be analogous to that already routinely carried out on blastomeres or polar bodies in pre-implantation genetic diagnosis. Analysis of intact FCs rather than cffDNA should facilitate provision of information about Mendelian disorder, and other chromosomal abnormalities.

An array allows comparative genomic hybridization (CGH) detection of aneuploidy for all chromosomes on a single cell is already available. This approach is proposed for analysis of a polar body or blastomere. The same approach could be applied for analysis of FCs. Furthermore; genetic diseases where the mother does not have the genetic alteration can be diagnosed by analyzing cffDNA in the maternal plasma. However, plasma analysis cannot be used for prenatal diagnosis of maternally inherited genetic diseases.

However, even if several diagnostic perspectives could theoretically be realized, the use of these FCs for non-invasive prenatal diagnosis is currently far from being achievable in routine. Many interrogations persist on the types of FCs, physiology of trans-placental passage to the maternal circulation and the feasibility of their use in NIPD. Original efforts involved recovering intact FCs from MB. Various types of FCs in MB were recovered, primarily trophoblastic, erythroblastic and leucocytic cells. Each cell type has its own unique cellular characteristics and antigenic specificity. Consequently, various types of enrichments and cellular sorting have been tried. Given the fragility and rarity of these cells in MB (1 fetal cell for 10^6 to 10^8 maternal cells), translation into clinical utility remained elusive. Adapted techniques and new technologies are required to enable cellular recovery and accurate single cell analysis.

Clearly, these newly developed techniques that could retrieve FCs from MB MB need additional improvements and clinical validation before being proposed in clinic for aneuploidy detection. Admittedly, though the harmlessness of these techniques seems attractive, but this does not mean they should necessary lead to NIPD.

5. Types of fetal cells in maternal blood

The possible cell types that can be isolated from MB and used for prenatal diagnosis include erythroblasts, trophoblasts and lymphocytes.

5.1. Erythroblasts

Fetal erythroblasts or nucleated red blood (NRBC) appear to be the ideal candidates for detection and enrichment of FCs in MB. For multiple reasons, they are the cellular category most commonly studied and, probably, well characterized in MB. Fetal erythroblasts are abundant in fetal blood especially in early gestation, comprising approximately 10% of the red blood cells in the 11-week fetus. This proportion declines as pregnancy progresses reaching approximately 0.5% at the 19 weeks of gestation.

The trafficking of fetal red blood cells into the maternal circulation is clinically evident by cases of 'silent' rhesus isoimmunisation in rhesus negative women (MacKenzie et al., 1999). They have a nucleus with full complement of genetic information and a limited lifespan of approximately 90 days. In contrast to lymphocytes, they are unlikely to persist more than few days' post-partum and are rare in the peripheral blood of a normal adult (except in clinical circumstances of increased haematopoiesis such as pregnancy). Fetal NRBC can be recognised in a number of different ways and many groups have convincingly sorted this fetal cell type using a variety of strategies. These cells were identified by their morphological characteristics and positive coloration for fetal hemoglobin (Clayton et al., 1964).

Nucleated erythrocytes express several unique antigens, such as the transferrin receptor, which is recognize by the AntiCD71 antibody, which recognizes the transferrin receptor (Ganshirt-Ahlert et al., 1992). Unfortunately, NRBC in the maternal circulation are not exclusively fetal, as was previously thought. AntiCD71 antibody recognizes also other nucleated fetal and maternal cells. Slunga-Tallberg has demonstrated that a definite population of maternal NRBC can be found during pregnancy and others have shown that only half of the erythroblasts in MB are of fetal origin (Troeger et al., 1999a; Slunga-Tallberg et al., 1995).

Enrichment of erythroblasts can be improved with the use of several specific markers at the same time (e.g., CD71, size of the cells, granularity and positivity of the glycophorine A). The glycophorine A is a major glycoprotein of the erythroid membrane, which appears later than the CD71 and is not present in monocytes or lymphocytes. In a study carried out by Wachtel and co-workers (Wachtel et al., 1991), PCR technique could identify a sequence of the Y chromosome in 12/12 samples of enriched MB from male fetuses. In addition, researchers of the same team combined two markers, glycophorine A and CD71, with FISH technique and could successfully detect fetal chromosomal anomalies by analysis of MB. Using this technique, they were able to detect both trisomy 18 (Price et al., 1991) and trisomy 21 (Elias et al., 1992).

Other approaches of enrichment encountered more difficulties. The detection of the fetal erythroblasts using antibodies directed against the gamma (γ) globin chain was not conclusive because of the presence of this same globin in maternal erythroblasts. However, other antibodies directed against the chains of embryonic zeta and epsilon (ζ or ε) globins were shown to be more specific. The expression of these globins is limited to early stages of pregnancy. The expression of the gene, which codes for the globin ζ decreases in a drastic way after six to seven weeks of pregnancy. It is present in only less than half of the erythroblasts at 11-12 weeks and becomes practically undetectable after the 15th week of gestation (Choolani et al., 2001).

Many studies suggest that most nucleated erythrocytes that are present in first trimester MB samples are maternal in origin (Slunga-Tallberg et al., 1995; Slunga-Tallberg et al., 1994). Enrichment of nucleated erythrocytes in general will enhance the concentration of maternal cells as well as fetal. Therefore, a unique marker for fetal nucleated erythrocytes continues to be sought.

5.2. Trophoblastic cells

Trophoblastic cells represent the key element of the human placenta and are essential for its development and function. It has long been recognized that trophoblasts circulate in MB in pregnancies complicated by pre-eclampsia (Attwood HD, 1958).

Trophoblasts would seem ideal targets for fetal cell sorting efforts because of their abundance at the feto-maternal interface. Evidences suggest their early passage into the maternal circulation. They have also a particular morphology and apparently are present only during the period of pregnancy. Indeed, one highly specialized group of trophoblastic cells, the extravillous trophoblast, actually migrate into the walls of the spiral arteries which supply the maternal stroma and epithelium to bring about a process of remodelling of these blood vessels. It is these mononuclear cells that van Wijk and colleagues believe to be the trophoblasts that are most frequently found in MB (Van Wijk et al., 1996).

Initial concerns that trophoblasts could only be found in MB in pregnancies affected by pre-eclampsia (Chua et al., 1991) have been dismissed in the light of results achieved subsequently by a number of groups (Vona et al., 2002; Lim et al., 2001; Van Wijk et al., 1996). Nonetheless, targeting trophoblasts is not a straightforward process. While a considerable number of trophoblastic cells escape into the MB, very little stay in the peripheral circulation. In fact, the majority of the multinucleated syncitio-trophoblasts are filtered and sequestered by maternal lungs. In addition, their multinucleated nature may also limit subsequent genetic analysis (Benirschke, 1994).

Furthermore, enrichment of trophoblastic cells is difficult. The membrane markers specific to the trophoblastic cells are rare. Several erroneous observations of trophoblasts were published from mixing up with maternal leucocytes containing either trophoblastic or erthroblastic antigens. In 1984, Covone and co-workers were the first to successfully isolate trophoblastic cells from maternal peripheral blood at various stages of the pregnancy (Covone et al., 1984). They used a monoclonal antibody, anti-H315, directed against a placenta's isoform of alkaline phosphatase of placental type. In fact, it has been found that the isolated cells were maternal cells on which the H315 antigen had been adsorbed. These observations did not prevent the study of this cellular category from ensuing with much enthusiasm in order to develop a NIPD (Bertero et al., 1988; Covone et al., 1988).

One of the major characteristics, which differentiate the trophoblastic cells, is their expression of the cytokeratins. Whereas all hematopoietic as well as endothelial cells are keratin negative, the trophoblasts are keratin positive. This difference in the expression and

the development of the specific monoclonal antibodies provided a base for their isolation and enrichment. The most sensitive and specific method of recognising trophoblasts has been developed by Vona who sorted trophoblasts purely on the basis of size and then used either staining with haematoxylin and eosin or immunohistochemical localisation with KL1 antibody (recognises a cytokeratin found in cytotrophoblasts) or anti-placental alkaline phosphatase antibody (recognises syncytiotrophoblasts) (Vona et al., 2002).

Other trophoblastic markers such as HLA-G (Moreau et al., 1994), human placental lactogen (HPL) (Van Wijk et al., 1996) and Human Achaete-Scute Homologue 2 (HASH-2) (Alders et al., 1997) have been tested. The major disadvantage of the use of cytokeratins and other antigens for separation by FACS or MACS remains the permeabilization of the cellular membranes. The latter is, indeed, necessary to make it possible for the antibodies to penetrate and reach their antigens inside the cells. Also the antibodies directed against the epithelial cytokeratins, in particular CK 7 and CK 17, and other antigens are not ultimately specific. Bruch used trophoblast specific antibodies to detect sequences of the Y chromosome from newly enriched samples (Bruch et al., 1991). However, the cells resembled leucocytes more than trophoblasts.

Other concerns remain over the possibility of confined placental mosaicism and variability along the fetal-placental karyotypic axis. Confined placental mosaicism represents a discrepancy between the chromosomal makeup of the cells in the placenta and the cells in the baby. The extra-embryonic placental tissue does not always reflect the fetal genotype and 1-2% of placental cells differ in their karyotype from that of the fetus due to placental mosaicism (Farra et al., 2000; Kalousek and Vekemans, 1996). This also limited the use of trophoblasts in MB and led many investigators to conclude that trophoblasts are not the ideal cells to use for prenatal diagnosis (Bianchi, 1999).

However, despite these biological and technical problems, some diagnostic success has been achieved. Several groups have successfully isolated trophoblastic cells from maternal peripheral blood and could carry out some morphological and genetic confirmation (Vona et al., 2002; Lim et al., 2001; Van Wijk et al., 1996).

5.3. Lymphocytes

Fetal lymphocytes are a potential source of cells for prenatal diagnosis. Walkowska and co-workers showed the presence of the Y chromosome in metaphases of lymphocytic cells of fetal origin in maternal peripheral blood stimulated with phytohemaglutinine (Walknowska et al., 1969). This was also confirmed by quinacrine, which highlights the heterochromatin of the long arm of Y chromosome (Iverson et al., 1981).

The analysis of the fetal lymphocytes is limited by several factors, in particular the lack of monoclonal antibodies specific to this cellular type. The antigens of surface of the fetal lymphocytes are not very different from those of the maternal ones. The technique used to isolate the fetal lymphocytes consists of identifying the specific paternal HLA, so that if the mother and the father have distinct HLA, cells may be sorted by flow cytometry and enriched for paternal antigen expressing cells. However, this approach requires paternal HLA typing, and is impossible when paternal and maternal HLA antigens are shared (Herzenberg et al., 1979). This approach is further limited because fetal production of lymphocytes does not begin until the 20th weeks of gestation after the development of fetal bone marrow.

Another important limitation is the misinterpretations of the subsequent pregnancies as the fetal lymphocytes can persist in the maternal circulation for many years after a pregnancy. Bianchi et al., flow sorted cells based on hematopoietic stem cell markers and found Y-chromosome-specific sequences in six of eight non-pregnant women who were not pregnant who had previously had a male child and were 6 months to 27 years post-partum (Bianchi et al., 1996). This confirmed findings by Schroder et al and Ciaranfi et al, who, two decades earlier, had demonstrated male lymphocytes in MB in the first year after the birth of a male child (Ciaranfi et al., 1977; Schroder et al., 1974).

5.4. Granulocytes

They were studied in a limited number of cases and their presence in MB is uncertain. Only one group claimed of having successfully isolated these cells from MB and

raised the possibility of using fetal granulocytes as target cells for NIPD (Wessman et al., 1992).

This group used a Ficoll gradient followed by a cellular sorting by FACS and suggested early presence of these cells in the maternal compartment. Using FISH technique, they detect a Y signal among eight women of which one did not give birth to a male child. The reported explanation for this case was either a technical error or the presence of fetal male cells from a previous pregnancy (Bianchi, 1995). The granulocytes account for 0.02 to 0.04% of the mononucleated cells. But, these cells are unlikely to be potential candidates (Geifman-Holtzman et al., 1994; Simpson and Elias, 1993).

5.5. Haemopoietic progenitor cells

The presence of fetal haematopoietic progenitor cells in maternal peripheral blood was shown by the use of surface antigen CD34 (Little et al., 1997). The application of FISH followed by immunohistochemistry using anticytokeratin antibody AE1/AE2 (markers of the epithelial cells), anti-CD45 antibody (leucocytic markers) and heppar-1 (hepatocytic marker), made it possible to confirm the presence of these totipotent FCs (Khosrotehrani et al., 2004).

Under appropriate conditions, these cells can be cultured and would provide enough material for the genetic analysis of the fetal genome (Jansen et al., 2000). However, the inherent problem of maternal cellular contamination makes this analysis difficult. Tutschek et al., found that only 0.8% of colonies were purely fetal in origin (Tutschek et al., 2000).

Furthermore, there is evidence that these cells can persist in the MB from previous pregnancies (Guetta et al., 2003). This was shown by the presence of male cells in pregnancies with female fetuses (Bianchi et al., 1996). Therefore, it appears that these cells are not ideal candidates for prenatal diagnosis.

5.6. Thrombocytes (platelets)

The blood platelets do not have a nuclei or DNA material to facilitate their detection by molecular cytogenetics. Consequently, they were excluded from fetal cell researches.

6. Biological Parameters of feto-maternal cell trafficking

The detection and characterization of FCs in the maternal circulation is a rapidly expanding field with important implications for both prenatal diagnosis and for a better understanding of the physiology of feto-maternal interactions. Relatively little is known about the biological parameters governing the passage of FCs into maternal circulation. For example, it is still not known, for sure, whether the existence of FCs in maternal circulation represents a normal physiological phenomenon present in all pregnant women or whether it is merely a consequence of spontaneous feto-maternal hemorrhage. This key issue has a direct consequence on the general applicability of prenatal diagnosis using FCs in MB. Further research is still required before any clinical application can be made.

6.1 Anatomy of the placenta

The placenta is a gestational organ of very complex structure and function. It is made up of both fetal and maternal components. During the pregnancy, the stromal cells of the uterine endometrium are differentiated into bulky decidual cells, known as decidua basalis, which forms the maternal component of the placenta. The fetal component is derived from a tissue that arises from the conceptus, called the chorion. The chorion comprises a chorionic plate and finger-like projections of chorionic villi. The intervillous space lies between the fetal and maternal parts of the placenta and is filled with MB. The two portions are held together by anchored chorionic villi, which are attached to the decidua basalis at the site of implantation. Chorionic villi are separated from MB by a double layer of trophoblastic cells: the cytotrophoblasts, which form the internal layer and the multinucleated syncytiotrophoblasts, which form the external layer (Huppertz, 2008).

Maternal blood flows into the intervillous space through spiral endometrial arteries and leaves through endometrial veins on the surface of the decidua basalis. The fetal blood is separated from the MB by a thin membrane, so fetal blood and MB are in close proximity but do not intermix. This allows bidirectional exchange of gases, nutrients, metabolites and endocrine secretions (Figure 1) (Lightner et al., 2008). During the pregnancy, the bulky

decidual cells play an important role in the control of the invasive potential of the trophoblast and provide immunomodulatory function (Lisman et al., 2007).

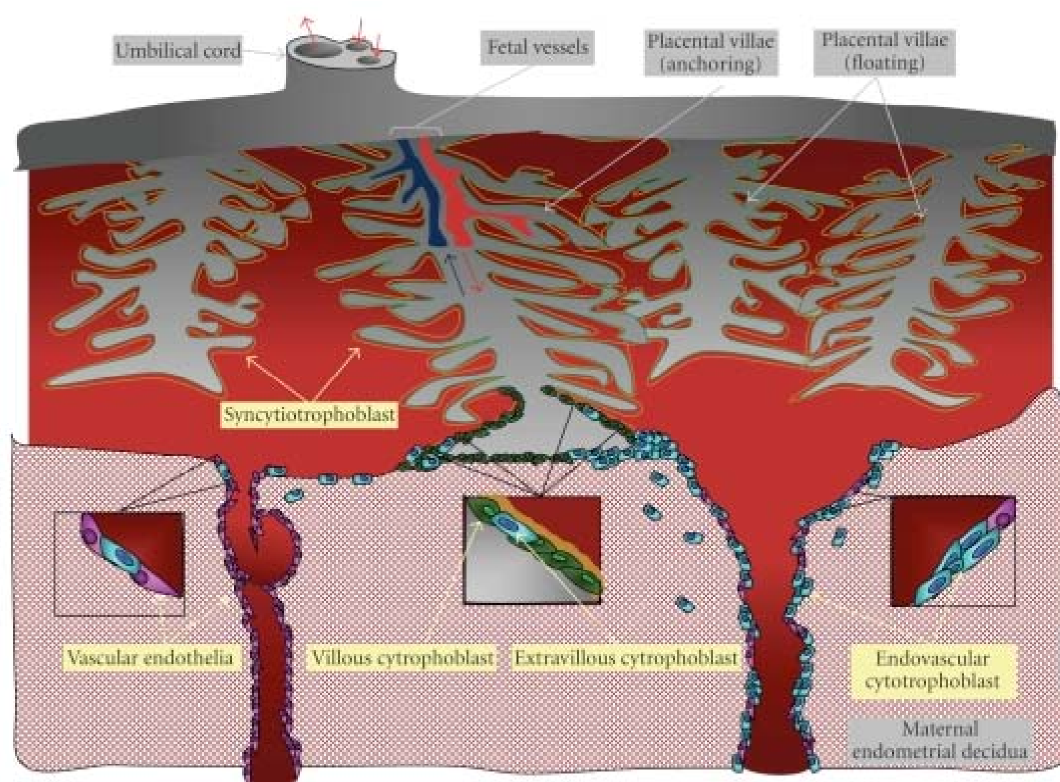

Fig. 1 shows a diagrammatic presentation of the human maternal-fetal interface

Fetal blood enters and exits the placenta via the umbilical cord. Fetal vessels lie at the core of each placental villae and all villae are lined externally by trophoblast cells. The cells of the inner layer are called villous cytotrophoblast cells. Those of the outer layer develop from the villous cytotrophoblast through syncytialization and are called syncytiotrophoblast. Floating villae are completely bathed in maternal blood; anchoring villae traverse the intervening blood-filled space to attach to the maternal decidua. Some extravillous cytotrophoblast cells will invade the maternal uterine arteries and become endovascular trophoblast cells Clin Dev Immunol. 2008;2008:631920.

Immunologically, the relationship between the uterus and the placenta is comparable to that between a recipient and a clinically transplanted graft, which is partially foreign for it. Immunologists have never found it easy to explain why the semi-allogeneic fetus is not attacked by the maternal immune system. Most researchers agree that local effects at the maternal–placental interface are important to tolerate paternal antigens. The geneticists consider it as special organ of embryonic origin, which presents unique characteristics, in

particular, the inactivation of paternal X chromosome, and is sometimes the seat of confined placenta mosaicism, which is frequently associated with intra-uterine growth restriction (Lestou and Kalousek, 1998). Lastly, the placenta represents for the cellular biologist a single model of limited and controlled pseudo-tumor invasion, and which in addition constitutes one of the rare models in human biology of cellular fusion leading to the formation of a syncytium (Cox et al., 2009).

6.2 Factors affecting passage of fetal cells to maternal blood

Bi-directional trafficking of cells between the mother and the fetus is a component of maternal-fetal tolerance. Thus, alterations in trafficking may be related to the breakdown of tolerance between the mother and the fetus (Starzl, 2004). The mechanism by which cells are exchanged across the placental barrier is unclear. Possible explanations include deportation of trophoblasts, micro-traumatic rupture of the placental blood channels or that specific cell types are capable of adhesion to the trophoblasts of the walls of the fetal blood channels and migration through the placental barrier created by the trophoblasts. Intervillous thrombi containing mixed maternal and FCs occur in the fetal placenta (Batcup et al., 1983). Histological defects in the continuity of the trophoblasts lining the vasculature of the placenta are also reported (Jauniaux and Hustin, 1992). Together these observations suggest the possibility that fetomaternal hemorrhage may allow exchange of cells between the fetal and maternal circulation (Figure 2) (Dawe et al., 2007).

The factors, which can influence the frequency of the FCs in MB, are numerous. One can mention among others, the type of placentation, multiple pregnancies (Al-Mufti et al., 2003), feto-maternal incompatibility (Batcup et al., 1983), gestational age (Hamada et al., 1993), bimanual pelvic examination (Simpson and Elias, 1993), invasive prenatal diagnostic procedures (Adinolfi, 1995), and abortions. The trafficking of FCs has been demonstrated to be greater in fetuses with abnormal karyotypes (Krabchi et al., 2006b; Krabchi et al., 2006c; Ganshirt-Ahlert et al., 1993; Elias et al., 1992; Bosso and Al-Mulla). Mechanisms for this increase in fetal cell trafficking remain speculative. Trisomic placentae demonstrate immaturity and hydropic change and it is suggested that the passage of trophoblastic FCs across such placentae are made easier as a result of these changes. It is

possible also that the compromised fetus has more NRBC secondary to the chronic stimulation of hypoxic bone marrow. Alternatively, placental damage may result in greater fetal cell trafficking (Holzgreve et al., 1998).

Increased amounts of FCs and cell-free fetal DNA have been also seen in maternal serum after fetal intervention (Wataganara et al., 2005), certain maternal complications such as diabetes (Al-Mufti et al., 2004), and may be a marker in prenatal complications as preeclampsia and preterm labor (Farina et al., 2005). However, the most important potential application remains to be the NIPD of fetal chromosomal abnormalities.

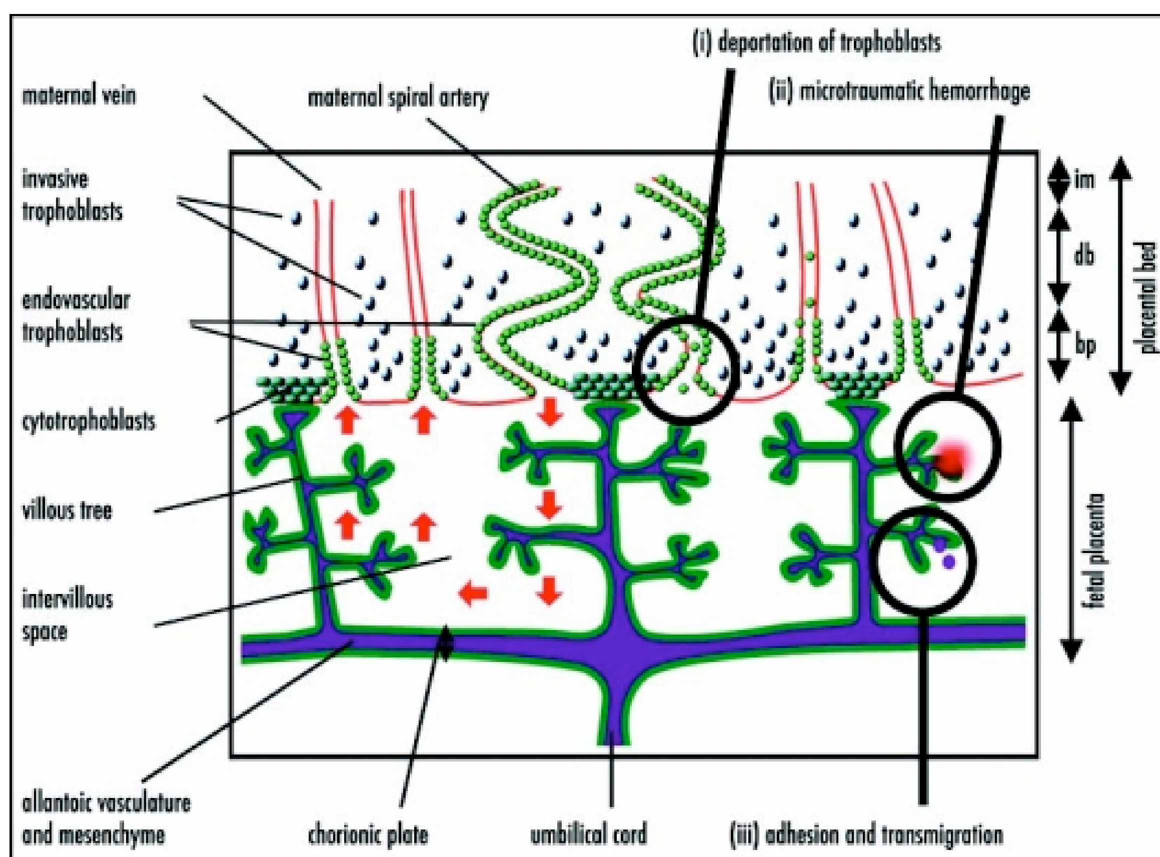

Fig. 2 Simplified diagram of the hypothesized mechanisms of fetomaternal cell traffic

Fetal blood enters the placenta via the umbilical cord passing through the intervillous space bathing the branches of the villous trees to exit through the maternal veins. Hypothesized mechanisms of fetomaternal cell traffic include (i) deportation of trophoblasts lining the maternal vessels and intervillous space; (ii) microtraumatic hemorrhage; and (iii) cell adhesion and transmigration across the placental barrier. Cell Adh Migr. 2007 Jan-Mar;1(1):19-27.

6.3 Microchimerism

Microchimerism is the presence of a small population of genetically distinct and separately derived cells within an individual. This usually occurs following transfusion or transplantation (Reed et al., 2007; Adams and Nelson, 2004). Microchimerism can also occur between a mother and her fetus. Recent studies showed the presence of male cells, proposed to be of fetal origin, in up to 50% of normal healthy women after delivery of male infant (Lambert et al., 2002). Microchimeric FCs are found in various maternal tissues and organs. Some of these cells can persist for decades in MB (Bianchi et al., 1996). Their presence creates a cellular state of microchimerism considered as an epiphenomenon of pregnancy with potential pathological consequences.

The FCs are semi-allogenic for the maternal immune system. Thus, microchimerism may have some implications regarding the immune status of women such as influencing autoimmunity and tolerance to transplantation. Bianchi reported an association between the persistence of this kind of FCs and the later development of autoimmune diseases like Sjogren syndrome. Greater numbers of cells were found in women suffering from autoimmune diseases like scleroderma (Nelson et al., 1998) and thyroiditis (Srivatsa et al., 2001) as well as non-autoimmune pathologies, such as hepatitis C (Johnson et al., 2002) and cancer (Cha et al., 2003). Increased maternal microchimerism has been also described in patients with type 1 diabetes mellitus (Nelson et al., 2007), neonatal lupus syndrome-congenital heart block (Stevens et al., 2003), and biliary atresia (Muraji et al., 2008). Little information is available on the phenotype of the FCs present in non-hematopoietic maternal tissues. The long-term consequences of the cellular microchimerism hardly start to be considered and its biological significance remains unclear.

It is recognized that the cellular exchange through the placental barrier is bidirectional (Lo et al., 1996). Trafficking of cells between the mother and the fetus is mostly considered from the feto-maternal side. Researchers focus on the implications of FCs on maternal health. However, trafficking in the other direction (maternal into fetal) is less well understood. The passage of the maternal cells to fetal tissues occurs in all species with hemochorial placentation, like mouse and humans (Piotrowski and Croy, 1996). Recent

studies re-examined the assumption that persistent maternal cells in offspring may play a key role in the pathogenesis of certain diseases like severe combined immunodeficiency (Pollack et al., 1982) and juvenile myopathies (Artlett et al., 2001). More detailed understanding of the biology of microchimerism is required (Klonisch and Drouin, 2009).

6.4. Problems linked to persistence of fetal cells from former pregnancies

One of the main criticisms for using FCs in MB for NIPD is a concern about the presence of remanent FCs from a former pregnancy or miscarriage. Hamada and co-workers followed up the FCs from day one to 24 months postpartum of 25 primiparous women. Fetal cells were detected shortly after childbirth at a rate of an average one fetal cell for 17500 maternal cells. This frequency decreased with time reaching an undetectable level three months after childbirth (Hamada et al., 1994).

The FCs present in maternal circulation are mainly of hematopoietic origin. Studies report diversities of the expressed hematopoietic markers. Several publications have shown the persistence of CD34+ cells several years after the childbirth (Adams et al., 2003; Guetta et al., 2003). These progenitor cells have the ability of self-activation by fusion or trans-differentiation in maternal tissues. FCs were also identified among the mononuclear subsets of B and T lymphocytes, natural killer cells and T-cells which express the antigens CD4/CD8 (Artlett et al., 2002; Evans et al., 1999). This indicates that these cells are able to be grafted and to be differentiated in hematopoietic cell lines. Thus, certain hematopoietic FCs (lymphoid progenitors; B and T lymphocytes) can be found for a long time after pregnancy, and thus preclude their use in NIPD.

Contrary to the fetal lymphocytes, fetal trophoblasts and erythroblasts are unlikely to persist more than few days post-partum and are rare in the peripheral blood of a normal adult. They have a full complement of genetic material yet have a limited lifespan and therefore seem ideal targets for the screening of fetal aneuploidies. Furthermore, they appear at an earlier stage of gestation in contrast to fetal lymphocytes. In summary, the duration of persistence of FCs seems to depend on the cellular type and the long-lived FCs cannot, consequently, be used for possible systematic screening of fetal aneuploidies.

7. Frequency of fetal cells

The analysis of several publications has made it possible to know the percentage of FCs at various stages of the pregnancy. The FCs circulating in MB can be detected as early as the 4-5th week after implantation (corresponding to 6-7th weeks of amenorrhea) in nearly 30% of the pregnancies. The rate of detection clearly increases between 8th and the 13th week, to reach 80% of pregnancies. More optimistic results were also published. Thomas and co-workers using PCR technique of Y chromosome (Thomas et al., 1994) detected male FCs in 18 pregnancies out of 18 at the 6th week of gestation. Liou et al., (Liou et al., 1994; Liou et al., 1993) found specific sequences of male fetuses in 19 out of 19 pregnancies analyzed at 10th to 11th weeks. It is necessary however to consider these figures with great caution because they depend on a limited number of cases.

The frequency of FCs varies with the gestational age, which may influence fetal cell trafficking. Ganshirt et al., (Ganshirt-Ahlert et al., 1993) showed an increasing trend in the frequency of NRBC with advancing gestation from 10th to 40th weeks. De Alba et al (Rodriguez de Alba et al., 2001) found a slight, but nevertheless significant, increase in fetal NRBC between the first and second trimesters. Sohda et al., (Sohda et al., 1997), however, demonstrated a non-significant reduction in the number of presumed fetal NRBC after 19th weeks gestation. Neither Lim et al., (Lim et al., 2001) nor Shulman et al., (Shulman et al., 1998) saw a change in the number of fetal NRBC as gestation advanced. Lim et al., (Lim et al., 2001) found a decreasing trend for the mean number of trophoblasts and Taniguchi et al., (Taniguchi R, 2001) found the optimal time for trophoblast RNA isolation to be from 9th to 13th weeks of gestation.

Estimates of absolute fetal cell numbers circulating in MB have varied significantly. The rate varies widely from less than 1 to more than 150 FCs per 1 ml of MB. Studies, which count specific types of FCs based on staining or morphological criteria probably, overestimated the total number of cells because no fetal cell recognition system is completely specific. The majority of the studies of fetal NRBC have found between 1 and 10 FCs per ml of MB (Kitagawa et al., 2002; Rodriguez de Alba et al., 2001; Campagnoli et

al., 2000; de Graaf et al., 1999; Rodriguez De Alba et al., 1999; Bischoff et al., 1998; Simpson et al., 1995; Ganshirt-Ahlert et al., 1993). Nevertheless, Lim apparently sorted 30–40/ml (Lim et al., 2001), Troeger 40–50/ml (Troeger et al., 1999b), Sohda 60–70/ml (Sohda et al., 1997) and Wachtel 150 NRBC per ml of MB (Wachtel et al., 1998). The later concludes that more than 30% of the erythroblasts circulating in MB were of fetal origin using a technique of cellular separation based on physical differences between fetal and maternal cells. It is however extremely probable that most of these cells are not fetal in origin. Lesser numbers of trophoblast sorting reports have produced similar yields (Vona et al., 2002; Lim et al., 2001; Van Wijk et al., 1996).

On the other hand, less generous predictions are usually given by studies, which use universal marker that recognise cells as fetal only by the presence of a Y chromosome FISH signal in male pregnancies. Bianchi et al., (Bianchi et al., 1997) found a mean of 19 male fetal cell DNA equivalents per 20 ml sample of MB using quantitative PCR suggesting that each millilitre of MB contains one fetal cell. More recently, Ariga et al., (Ariga et al., 2001) found between 2 and 40 genomic equivalents of male cells in the cellular fraction of 1 ml of MB using real-time PCR. Quantitative PCR work carried out by Bianchi did not support a gestational effect unlike that of Ariga, which suggested increased trafficking as pregnancy progresses. All these data did not take into account a well-known risk of PCR technique, which is that the wrong amplification of parasitic sequences may give falsely positive results. It should however be noted that the recent improvements in this technique have made it possible to reduce this risk to great extent,.

Other studies have used a laborious but far more accurate approach. Small samples of unsorted MB have been fixed onto slides and all mononuclear cells examined for their FISH signals. Hamada et al., (Hamada et al., 1993) used specific sequences of Y chromosome (DYZ1) on samples collected directly without enrichment. They observed two slides of approximately 150,000 cells, representing the equivalent of 0.01 to 0.03 ml of MB per patient. The frequency of the FCs observed was estimated as $0.27/10^5$ cells during the 1st and 2nd trimesters and $8.5/10^5$ cells during the 3rd trimester. In our laboratory, Krabchi et al., used FISH and PRINS techniques to investigate a larger volume of MB (1 ml) in

twelve cases of male pregnancies (Krabchi et al., 2001). An average of 2 to 6 FCs was found per ml of MB during the 2nd trimester. Our results are in agreement with those of Ariga et al., (Ariga et al., 2001) and that of Kolvraa et al., (Kolvraa et al., 2005).

Although the number of FCs in MB is variable and no consensus has been reached, all are agreed that number is very low. The rate probably ranges from 1 per 500,000 maternal cells to 1 per 1.5 million maternal cells. In the light of the many false results reported by certain investigators, it becomes essential that accurate and specific techniques with suitable controls be used to enrich the samples for FCs and to confirm their nature. Unfortunately, these conditions were not always respected in the literature (Wachtel et al., 1998; Adinolfi, 1995; Tharapel et al., 1993).

8. Techniques for isolation and analysis of fetal cells

The original motivation for developing NIPD for chromosomal abnormalities was the low sensitivity and positive predictive values of existing screening tests and the subsequent need for invasive procedures for confirming the diagnosis. Although enrichment and isolation of FCs from MB has dominated the work in the field of NIPD, improvements in the detection and analysis of the FCs may overcome the problems of impurity and high contamination with maternal cells.

8.1 Techniques for enrichment and isolation of specific cell type

The strategy generally adopted for enrichment was not to obtain a pure sample of FCs, but to generate a sample with which one will be more likely to find FCs. Fetal cells can be harvested from the maternal circulation in a variety of ways. Negative enrichment techniques deplete unwanted maternal cells whereas positive enrichments actively select for FCs.

It is well recognized that even after enrichment, the majority of the cells are of maternal origin. Reaching a concentration of 1 fetal cell to 50,000 maternal cells, would allow more comfortable detection and analysis by FISH technique. The problem of enrichment is that there is no unique marker of different types of presumed FCs and,

consequently, one cannot enrich all FCs at a time and should target a specific type of FCs. Furthermore, many of these fragile cells could be lost or destroyed in the process of enrichment itself decreasing the chance of prenatal diagnosis.

8.1.1. Density gradients enrichments

The principle of this method consists of a centrifugation of the MB across a continuous or discontinuous gradient to isolate the mononuclear cells and to separate cells according to their densities. The gradients are specific solutions of different densities, which superimpose according to its individual densities. The cellular suspension is deposited on the surface of the gradient and then the cells migrate during centrifugation according to their own density. The solutions used can be of Ficoll, Percoll or Histopaque. These solutions are made up of polysucrose and a radiopaque medium (sodium diatrizoate). One can use a simple gradient of only one layer or a multiple gradients containing two or more layers (Samura et al., 2000; Troeger et al., 1999b; Johansen et al., 1995; Ganshirt-Ahlert et al., 1993).

The yield of FCs tends to be inversely related to the purity of the final sample and, inevitably, a compromise must be reached between target cell losses and contamination with maternal cells. The optimum gradient, which minimises target cell losses but also limits contamination by non-target cells is unclear for both trophoblasts and NRBC although some comparative work has been carried out. The cytoplasmic-to-nuclear ratio of fetal NRBC varies as gestation progresses and the embryonic Hb drops from 90% to 10% to be superseded by gamma globin (HbF). The cells progressively become smaller and have a lower cytoplasmic-to-nuclear ratio (Choolani et al., 2003). This variability in size and density causes them to sediment over a wider density range with more than 80% overlapping with that of maternal red blood cells at a given density.

Ganshirt-Ahlert et al., used a triple gradient of 1.077 g/ml, 1.110 and 1.119 Ficoll-Histopaque. Most NRBC settled between 1.077 and 1.110 (Ganshirt-Ahlert et al., 1993). Subsequently, a gradient of 1.090 g/ml was found to deliver a superior cell yield than 1.083 g/ml (Sekizawa et al., 1999). Later, the yield of heavier gradient, 1.119 g/ml, was found to

be even higher (Samura et al., 2000; Troeger et al., 1999b). The trophoblasts are said to sediment between 1.051 and 1.064 g/ml (Johansen et al., 1995). However, narrowing the ‘density window’ from 1.053 to 1.060 g/ml was found to reduce the final yield but increase the cell purity (Van Wijk et al., 1996).

Overall, the separation by density gradient was often used as preliminary stage in the isolation of FCs. Few groups argued that prenatal diagnosis is possible after simple enrichment by one gradient (Oosterwijk et al., 1998) whereas the majority believe that a more thorough enrichment is required.

8.1.2. Cellular sorting

Further improvement in FC enrichment can be achieved using antibody-based methods. Two principal methods have been used to exploit distinct cell antigens for the purposes of cell separation. Fluorescence activated cell sorting (FACS) and magnetic activated cell sorting (MACS) are cell separation techniques, which are significantly more selective than density gradients.

8.1.2.1. Cellular sorting by fluorescence flowcytometry or FACS

The technique takes advantage of the differences between maternal and FCs, in particular, regarding the expressed intracellular and surface antigens. FACS selects cells using fluorescent antibodies. If a monoclonal antibody which recognises the fetal-specific antigen is fluorescently labelled and then incubated with the cells, only those bearing the particular antigen will bind antibody, fluoresce and be recognised during flow cytometry. The isolated cells are put in contact with monoclonal antibodies to these antigens. The cell suspension is then passed through a vibrating jet, which changes continuous liquid flow into droplets that contain individual cells. After generation of droplets, the liquid intercepts a laser beam. The laser excites the fluorescent molecules that are present on the surface of target cells, which consequently acquire an electrical charge. The positive droplets are then deviated into a collecting tubule. This method allows simultaneous evaluation of several parameters including cell size, nuclear shape and surface antigenicity (Lewis et al., 1996; Herzenberg et al., 1979).

A number of different antigens have been exploited, depending on the target fetal cell type. Fetal NRBC have been sorted by recognizing CD71, the transferrin receptor (Ganshirt-Ahlert et al., 1992), glycophorin-A (Elias et al., 1992; Price et al., 1991), CD36, the thrombospondin receptor (Zhao et al., 2002; Sohda et al., 1997), gamma-globin (DeMaria et al., 1996) and epsilon-globin (Choolani et al., 2001). Finding trophoblastic specific antigens has been more difficult. The majority of work on trophoblasts has either used negative depletion (van Wijk, 1998; Van Wijk et al., 1996) or has sorted cells according to size (Vona et al., 2002).

Apart from its complexity, the flow cytometry is time consuming and expensive. FACS is also difficult to carry out, requires specialised equipment and considerable expertise. Furthermore, cell sorting may reduce target cell numbers if cell losses are significant or cell recognition is inadequate.

8.1.2.2 Cellular sorting by immunomagnetic beads or MACS

MACS technique is the most widely used technique in fetal cell sorting. Tiny magnetic beads are labelled with the monoclonal antibody, which recognise specific fetal antigen. They are incubated with the cell mix and then extracted using a magnet. The beads fixed at the FCs will retain them at the time of their passage through a distillation column attached to a magnetic field creating positive selection of FCs. The cells, which do not present specific antigen, will not attach to the beads and will pass freely through the magnetic field (Miltenyi et al., 1990). After release of the magnetic field, the cells attached to the beads remain hung along the column. An elution will then detach them from the column so they can be collected and analysed (Simpson and Elias, 1995). Unfortunately, none of the antigens are unique to FCs and this inevitably results in a lot of contamination from maternal cells (Zhao et al., 2002; Ganshirt-Ahlert et al., 1993).

The question of which fetal enrichment technique is best is an extremely complex one as very few comparisons have been made. Evidence suggests that MACS is favoured over FACS as it is simpler and cheaper. Furthermore, the cellular yield with MACS is usually greater than that from FACS although at the expense of purity. These conclusions

were reached by Johansen and colleagues (Johansen et al., 1995) and have been supported by the findings of the National Institutes of Health Fetal Cell Study (NIFTY), trial. The NIFTY trial represents the largest assessment of fetal cell sorting made to date. The results were published in 2002 (Bianchi et al., 2002) and have been discussed in depth subsequently at the National Institute of Child Health and Human Development conference on FCs entitled ‘Sharpening the Tools’. Jackson reported a general feeling that new ideas and technologies will be necessary if significant improvements are to be made (Jackson, 2003). Unless specific fetal cell identifiers become available the sorting techniques will remain too laborious and inconsistent for clinical practice.

There is no doubt that FCs are present in MB but it remains unclear as to whether they will ever be sorted and analysed with sufficient efficacy and accuracy for the techniques to be applied for screening and diagnosis of common chromosomal abnormalities in the general population.

8.2 Techniques for detection and analysis of fetal cells

Isolating FCs from MB is only of value if these cells can be meaningfully analyzed. In view of the difficulties in finding a truly fetal-specific antigen, which detects all FCs and persists throughout the pregnancy, alternative approaches to enhance detection and analysis of FCs in samples highly contaminated with maternal cells have been pursued. It is hoped that further new concepts and technological advances will hasten the development of this field and lead to the introduction of NIPD into routine clinical practice. The major techniques used to detect and analyse FCs are:

- Cytogenetic analysis of the FCs: FISH or PRINS

- Molecular evaluation of fetal genotype: PCR technique

Unfortunately, these tests have limitations, which add further to the challenges of fetal cell sorting. Their usage is critic when cell numbers and purity are low, as is the case with FCs sorted from MB.

8.2.1 Fluorescence *In Situ* Hybridization (FISH) technique

Refinements in cytogenetic techniques over the past 30 years have allowed an increasingly sensitive detection of chromosome abnormalities. Banding techniques, though historically pivotal in cytogenetics, are limited to mitotically active cells. The introduction of FISH in the late 1980s, has revolutionized cytogenetic analysis at the molecular level (Pinkel et al., 1988). FISH technique is used to detect numerical and structural anomalies, for which there is an appropriate probe, either in the metaphase spread of dividing cells or in the interphases nuclei of non-dividing cells. The high sensitivity and specificity of FISH and the speed with which the assays can be performed have made FISH a powerful technique with numerous applications, and it has gained general acceptance as a clinical laboratory tool (Gozzetti and Le Beau, 2000). The use of interphase FISH in the study of FCs in maternal circulation allowed detection and even quantification of these cells. The presence of a Y chromosome hybridization signal (in a pregnancy with a male fetus) or of three chromosome 21 hybridization signals within one nucleus (in a pregnancy affected by Down syndrome) was taken as reliable evidence of fetal cell sorting.

8.2.1.1 Principle

FISH is essentially based upon the same principle as a Southern blot analysis, a cytogenetic equivalent that exploits the ability of single-stranded DNA (probe) to anneal to its complementary DNA (target). In the case of FISH, the target is the nuclear DNA of either interphase cells or metaphase chromosomes fixed to a microscope slide. The nucleic acid probes are a small sequence of DNA (or RNA) whose normal site is known in the genome and is chemically marked in order to be located thereafter (Figure 3) (Speicher and Carter, 2005). This anneals to its complementary sequence in the specimen DNA and is detected either directly or indirectly through a reporter molecule (Kearney, 2001).

For direct detection, an attached fluorochrome of FITC, Rhodamine, Texas Red, Cy2, Cy3, or Cy5 is used as reporter molecule. This enables direct visualization of the probe as coloured fluorescent signal at the hybridization site by fluorescence microscopy. In the indirect detection method, hapten molecules such as biotin, digoxigenin and dinitrophenol

are used as reporter molecules. This enables indirect visualization of the probe after an additional step in the process in which there is binding with an antibody coupled to a fluorochrome (Ramos-Vara, 2005).

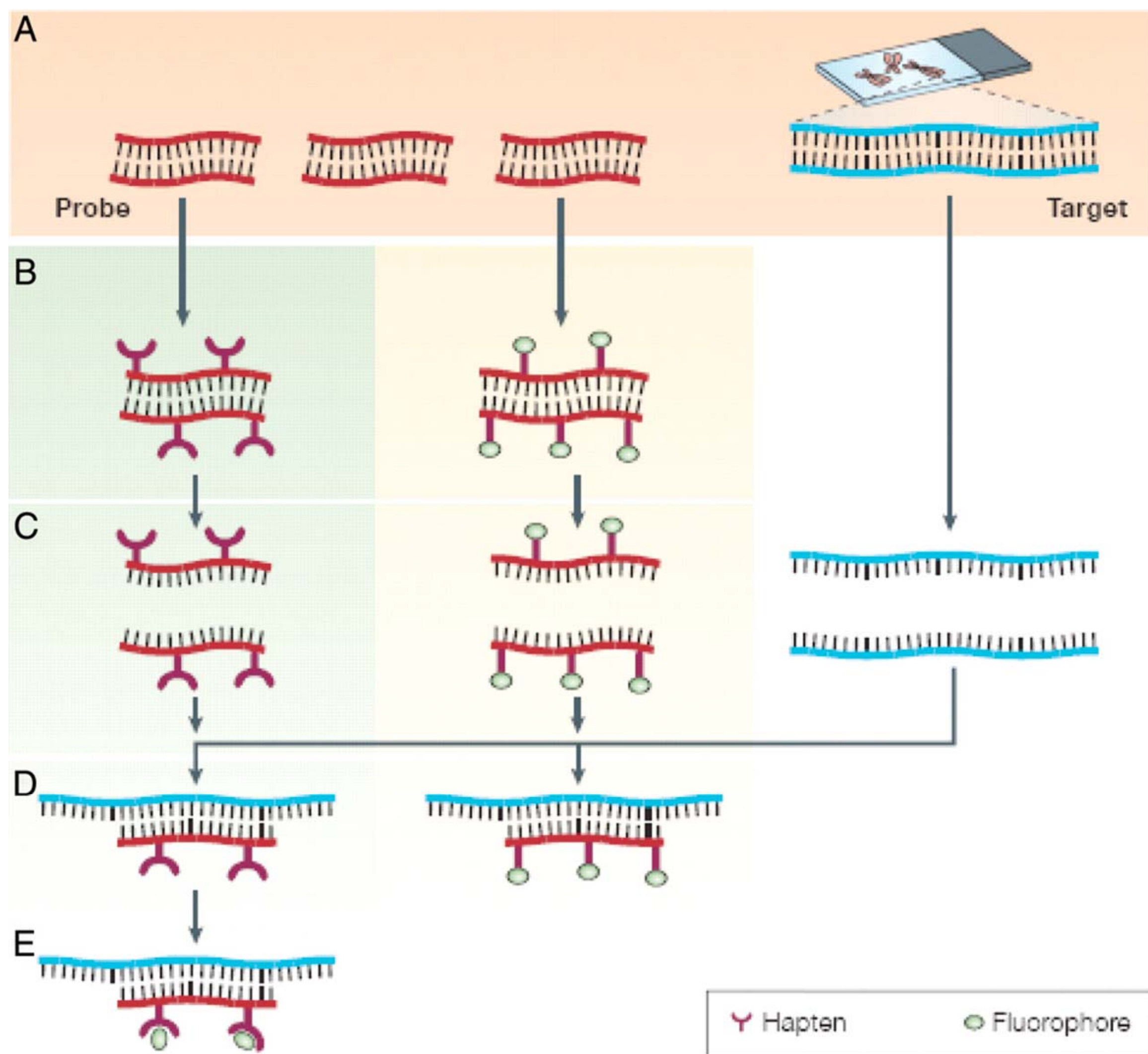

Fig. 3 shows a diagrammatic presentation of the principles of the FISH technique

The principles of fluorescence *in situ* hybridization; (A) The basic elements are a DNA probe and a target sequence. (B) Before hybridization, the DNA probe is labelled indirectly with a hapten (left panel) or directly labelled via the incorporation of a fluorophore (right panel). (C) The labelled probe and the target DNA are denatured to yield single-stranded DNA. (D) They are then combined, which allows the annealing of complementary DNA sequences. (E) If the probe has been labelled indirectly, an extra step is required for visualization of the non-fluorescent hapten that uses an enzymatic or immunological detection system. Finally, the signals are evaluated detected by fluorescence microscopy. Nat Rev Genet 6: 782–792, 2005.

8.2.1.2 Types of probes

One of the most important considerations in FISH analysis is the choice of probe. A wide range of probes can be used, from whole genomes to small cloned probes. There are broadly three types of probes, each with a different range of applications:

- Chromosome painting probe (WCP: Whole Chromosome Painting): The probe consists of a set of small probes, which cover the whole chromosome. These probes are obtained after isolation and labelling of the DNA of an entire chromosome without necessarily knowing the sequence of this DNA. They provide intense and specific fluorescent staining of entire human chromosomes, allowing the distinctive identification of chromosomes involved in complex rearrangements. There are also specific painting probes for chromosomal arms or even certain bands. This type of probe is most useful for clarifying cytogenetically visible structural or numerical chromosome rearrangements in metaphase and to determine precisely the origin of an unidentified fragment. Painting probes are not otherwise helpful in the analysis of interphase cells because the signal domains are too large and diffuse (Ried et al., 1998).

Whole chromosome painting is technically available for every human chromosome. Application of all WCP probes together allows the simultaneous painting of the entire human genetic complement in 24 colours. This promptly led to the developments of two independent FISH techniques, spectral karyotyping (SKY) (Veldman et al., 1997) and multicolour FISH (M-FISH) (Azofeifa et al., 2000) that have both been invaluable for diagnostic and research applications.

- Centromeric probes (CEP: Chromosome Enumeration Probe): This approach targets the α and β satellite repetitive sequences which are present as thousands of copies localized in the centromeric area of human chromosomes. The signal obtained is thus very intense making CEPs particularly suitable for the detection of monosomy, trisomy and other aneuploidies. In most cases, these sequences are distinct, such that an α-satellite probe derived from one chromosome will hybridize only to that chromosome, however pan-centromeric probes, which target all human centromeres, are also available.

Other type of probes which target the repetitive sequences is the pan-telomeric probe targeting the tandemly repeated (TTAGGG) sequences present on all human chromosomal ends (Kearney, 2001).

- Locus-specific probes (LSI: locus specific identification), are usually clones of small sized and highly specific sequence, make it possible to identify a very precise area of the genome. These probes obtained by marking of the DNA cloned in various vectors and their size vary depending on the nature of the cloning vector, from plasmids (1–10 kb) to the larger PAC, YAC and BAC vectors (80 kb to 1 Mb). Probes of this classification are particularly useful for detecting precise structural rearrangements such as specific chromosomal translocations, inversions or deletions in both metaphase and interphase. They can be used alone or in combination to allow multicolour deciphering of complex chromosomal rearrangements. These unique sequence probes also allow specific recognition of chromosomes whose centromeric sequences have a strong homology like the one between CEP13 and CEP 21 (Gozzetti and Le Beau, 2000).

8.2.1.3 Applications and limitation of FISH

The potential of almost all applications of *in situ* hybridization is greatly enhanced by multicolour detection of simultaneously hybridized probes. This is particularly useful when structural chromosome aberrations involving different chromosomal regions are to be diagnosed, or when several numerical aberrations should be detected in parallel. A particular advantage of FISH techniques is the possibility of also studying chromosomal aberrations in non-dividing cells, which is useful for the direct visualization of chromosomal aberrations in cytological preparations and tissue sections. However, FISH techniques have a major downfall because it can only target pre-suspected genetic aberrations, providing that specific probes are available. In other words, a probe for a known genetic aberration has to be hybridized to the specimen in order for the FISH technique to indicate the presence or absence of that specific genetic aberration alone. Therefore, FISH cannot serve as a screening test for all chromosomal rearrangements (Kearney, 2001).

Technically, non-specific binding of the probe, or failure of the probe to bind, may result in extra signals or missing signals and false results. Furthermore, a lone signal may split and appear as two distinct signals, or two separate signals may overlap and appear as one. G2 nuclei can also give rise to four signals for each chromosome. The possibility for diagnostic error is clear when so few target cells are available for analysis. Because of these potential problems, careful quality control has been introduced to avoid false signals. Therefore, the quality control must ensure scoring a minimum number of cells (often 50) to ensure that aberrant binding becomes inconsequential (Gozzetti and Le Beau, 2000).

8.2.2. Primed *In Situ* Labelling (PRINS) Technique

8.2.2.1 Principal

The PRINS technique of human chromosomes uses primers for repeated DNA sequences from centromeric alpha-satellite motif. The length of the primers ranges from 18 to 35 nucleotides, which greatly facilitates their accessibility to genomic target sequences. Based on the use of chromosome-specific primers, the PRINS reaction combines the high sensitivity of the PCR with the cytological localization of DNA sequences as in FISH technique (Lebo et al., 1992). The chromosomal identification is performed by *in situ* annealing of specific and unlabeled oligonucleotide primers to complementary sites of target sequence on interphase and metaphase spreads. The annealed primers provide initiation sites for chain elongation catalysed by Taq DNA polymerase in the presence of free nucleotides, of which at least one is labeled with fluorochrome. The *in situ* visualization of generated fragments results from the incorporation of the labeled nucleotides. The complementation process between the primer and its centromeric target will be so specific that a simple mismatch between the 3’-end of the primer and the genomic sequence will prevent initiation of the *in situ* elongation by the Taq DNA polymerase. Thus it has been possible to define specific alpha-satellite primers for some chromosomes indistinguishable by FISH with centromeric probes, such as chromosomes 13 and 21, which share 99.7% homology in their alpha-satellite DNA sequences (Pellestor et al., 1994).

The use of automatic thermocyclers allows for optimization of both annealing and extension conditions. An additional improvement was the direct use of fluorochromes in sequential PRINS reactions (Hindkjaer et al., 1994). Recently, our group has developed a new multicolour PRINS protocol, allowing the performance of ultra-rapid detection on several chromosomes by only mixing different fluorochromes during the chain elongation reaction (Yan et al., 2001). Each PRINS reaction consists of a unique four minutes step for annealing and elongation of each chromosome-specific primer. This new sequential procedure simplifies the PRINS technique and provides an easy way to carry out multi-site labelling.

8.2.2.2 Applications

PRINS has successfully been tested for the assessment of aneuploidy in lymphocytes, amniocytes (Pellestor et al., 1995) and pre-implantation embryos (Findlay et al., 1998). The use of PRINS has also been reported for analysis of structural aberrations such as translocations, marker and ring chromosomes (Hindkjaer et al., 1995). More recently, the PRINS protocol has been tested to detect fetal cells from MB (Krabchi et al., 2006a; Orsetti et al., 1998a; Orsetti et al., 1998b). It is also worth mentioning here that PRINS technique can be used in combination with or as an alternative to FISH technique for detection of FCs. The PRINS technique allows for detection of FCs and appears to have comparable sensitivity and specificity to that of FISH technique at a much lower cost (Krabchi et al., 2006b; Krabchi et al., 2006c; Krabchi et al., 2001).

8.2.3. Polymerase Chain Reaction (PCR) Technique

PCR consists of an exponential amplification of DNA sequences through repeated cycles of DNA replication. Each cycle involves DNA denaturation, primer annealing and primer extension. All cells, maternal and fetal, obtained by enrichments or through sorting procedures are lysed to release their DNA and analysed by PCR (Durrant et al., 1996; Van Wijk et al., 1996). PCR technique was employed to amplify particular sequences of DNA of the fetal genome for recognition by a labelled DNA probe. Theoretically, PCR technique can diagnose chromosomal abnormalities and even detect polymorphisms of certain genes

related to genetic disorders providing a pure population of FCs are available and the genes are clearly identified (Saiki et al., 1988). Despite the sorting process, the majority of the DNA obtained was maternal in origin because of the many maternal cells that escape through the sorting process. This technique, therefore, is mainly useful for the detection of fetal DNA sequences not shared with the mother and this seriously limits its clinical application for the detection of aneuploidies and maternally inherited mutations. The development and the improvement of the sensitivity and the specificity of this technique will allow its use in a more reliable way.

Within the framework of research on FCs, the sequences most frequently used are those specific for the Y chromosome when the fetus is male to indicate the presence of some cells of fetal origin in the MB. Successful amplification of fetal cell specific DNA sequences, such as those found on the Y chromosome was also used to assign fetal gender and taken as evidence of successful fetal cell sorting (Durrant et al., 1996; Van Wijk et al., 1996). They were also used for rough estimation of the frequency of FCs throughout the pregnancy (Ariga et al., 2001; Bianchi et al., 1997). Specific sequences of the Rhesus gene and HLA of paternal origin were also used. The amplification of rhesus-D DNA sequences from rhesus-D negative woman can be taken as a sign that the fetus is rhesus-D positive while failure of amplification would indicate a rhesus negative fetus with no threats from maternal antibodies (Hahn et al., 2000).

8.3. Advanced technologies and clinical applications: automatic detection and microdissection

Although QF-PCR and other techniques can be used to test for karyotypic abnormalities, a pure population of FCs would be needed. The very low number of FCs after enrichment or sorting techniques limits these clinical applications. Prenatal diagnosis of most single gene or chromosomal disorders in this way would, however, require a pure sample of FCs, otherwise, DNA simultaneously released from maternal cells would confound the results (Mansfield, 1993). Fluorescence *in situ* hybridization circumvents this problem because cells are fixed onto a slide and each cell provides its own result.

Manual detection of FCs requires laborious examination of the nucleus of each and every cell in the final mix to determine if any FCs had been sorted. The use of automated image cytometry may help to overcome this problem of low purity and non-specific cell identifiers. This technology can acquire data from hundreds of cells in a short period of time and has already been applied to amniocentesis samples with great success (Hennerbichler et al., 2003; Merchant and Castleman, 2002).

If a fetal cell can be reliably identified and detected under the microscope, it can then be removed and placed in isolation, making a 'pure' fetal cell sample. Laser-capture microdissection instruments are able to lift individual cells off the slide in this way. It is then possible to use the technique of single cell PCR, which has been developed for whole genome amplification, to test for karyotypic abnormalities and also for single gene mutations. Provided the fetal origin of the cell can be guaranteed, maternally inherited mutations and aneuploidies can be tested by simple techniques like QF-PCR. Furthermore, it opens the way to genome-wide screening using techniques like comparative genomic hybridization (CGH) array. One of the main advantages of CGH is as its use as a discovery tool, in that it requires no prior knowledge of the target chromosome imbalance that is sought. Such techniques require significant time and skills; however, the effort may be justified for the sake of NIPD especially for precious pregnancies where the risks of genetic diseases are high such as those occur after in-vitro fertilization. This technique is particularly important in investigating cases with strong family history of hereditary genetic disorders of unexplained etiology. In such cases, a thorough look throughout the genome without prior knowledge of expected chromosomal abnormalities is required. Array CGH in postnatal diagnosis allows accurate diagnosis, characterization of syndromes, phenotype and genotype correlation, prevention, prognosis and better clinical management. The detection rate of array CGH in postnatal diagnosis was estimated between 7-11% in patients with intellectual disability or multiple congenital abnormalities despite normal conventional karyotype (Pickering et al., 2008; Shaffer et al., 2008; Shaffer et al., 2007).

9. Objectives of research

9.1 General Objectives

Currently, prenatal diagnosis of fetal genetic traits relies on invasive procedures associated with increased risk of pregnancy loss. A long sought goal of prenatal diagnosis has been the replacement of current invasive procedures by non-invasive methods. Data generated in different laboratories led to the conclusion that few intact FCs are present in MB (Liou et al., 1994; Simpson and Elias, 1993). NIPD through using FCs would permit accurate prenatal diagnosis for aneuploidies and single gene disorders without attendant risks associated with invasive procedures.

Fetal cells could be identified by targeting specific genetic marker exclusive for the FCs by immunologic or molecular techniques such as FISH or PRINS. Manual scanning was commonly used for retrieval of these rare FCs from MB (Krabchi et al., 2006b; Krabchi et al., 2006c; Krabchi et al., 2006a; Feldman et al., 2002; Krabchi et al., 2001; Orsetti et al., 1998b). Although there is general agreement about the presence of FCs in the MB, their routine use in clinical practice is not yet feasible due to their extreme low frequency in the maternal circulation. Most workers agree that the presence of FCs in MB is a rare event, only one FC per 10^5-10^9 of maternal cells, which makes their isolation difficult but not impossible (Hamada et al., 1993; Price et al., 1991; Ganshirt-Ahlert et al., 1990).

In quest for the development of NIPD using FCs, two strategies have emerged; the enrichment of rare circulatory FCs from MB and the analysis of fetal DNA from single cells.

Considering their extreme low frequency in MB and relative abundance of contaminating maternal cells, various purification and enrichment procedures have been proposed over the last two decades (Kitagawa et al., 2002; de Graaf et al., 1999; Wachtel et al., 1998; Ganshirt-Ahlert et al., 1992; Wachtel et al., 1991). Efforts were made to improve the development and evaluation of enrichment protocols. Despite all these efforts, a simple and efficient procedure is not yet available in clinical practice for routine testing using FCs.

Array-based comparative genomic hybridization has been proposed as a genome-wide assessment after whole genome amplification from single cells. However, traditionally comparative genomic hybridization requires DNA from large number of cells in addition to the high cost and complicated equipment (Fiegler et al., 2007; Hu et al., 2007).

In summary, through the last several decades, considerable effort has been done but no conclusive progress has been made in the field of FCs. Many controversies persist about the type and frequency of target cells as well as the best recovery time and enrichment protocol. A thorough assessment of the available protocols and innovative technologies are required before implementation of using these rare FCs in NIPD. Therefore, we assigned and committed ourselves in this work to the following general objective:

"Development and assessment of strategies for non-invasive prenatal diagnosis using fetal cells in maternal blood"

9.2. Specific objectives

The specific objectives of my thesis projects were carried out in terms and appear in the various chapters of this thesis.

9.2.1. Chapter 1: Development of a protocol that allows accurate evaluation of detection of rare cellular events through controlled spreading of a pre-determined number of target cells on slides within different cellular populations and using this strategy in measuring of the efficacy of manual scanning used in retrieval of fetal cells from maternal peripheral blood.

The number of FCs in MB is very low compared to that of maternal cells. Therefore, an accurate identification of circulating FCs is essential for their reliable use in prenatal diagnosis. Many groups identified FCs by molecular and cytogenetic techniques and used manual scanning for retrieval of FCs from MB (Krabchi et al., 2006b; Krabchi et al., 2006c; Krabchi et al., 2006a; Krabchi et al., 2001; Orsetti et al., 1998b; Hamada et al., 1993). Although these studies yielded important information concerning the number of circulating FCs in MB, the results were possibly skewed by the fact that the methodology of detection of these rare cellular events by manual scanning has never been evaluated. Assessing the efficacy of detection of extremely rare cellular events, although required for different applications, remained problematic. Artificial sample mixtures (spiked samples) could be acceptable up to certain limits but were not suitable for extreme rare events like FCs (Ntouroupi et al., 2008; Johnson et al., 2007). An accurate establishment of the frequency of FCs in the MB remains a prerequisite to determine the feasibility of using FCs in NIPD and is critical before optimizing any enrichment procedures. Therefore, we assigned the following objectives for this study:

1. Development of a procedure of sequential spreading of two different groups of cells and allowing identification of shape and coordinates of the rare target (true positive) cells on the slides by Giemsa staining after the initial spread.
2. Using the previously mentioned strategy to spread a determined number of XY cells ranging from 0-10 cells in pre-defined areas on slides among a pure population of XX cells to evaluate the efficacy of detection of rare XY cells.
3. Optimization of FISH protocol for the detection of rare cellular events.
4. Measuring the efficacy of manual scanning for the detection of rare cellular events.

9.2.2. Chapter 2: Development of a robust custom-made detection algorithm for the detection of rare cellular events using an automated platform and validation of its efficacy on slides with a pre-defined number of rare events. We compared manual with automatic scanning as well as FISH with PRINS techniques. We have also tested this classifier for detection of fetal cells in maternal blood samples from normal and aneuploid pregnancies.

Detection of rare cellular events is required for different applications in cancer and NIPD (Maheswaran and Haber, 2010; Wei et al., 2007; Thornhill and Snow, 2002). When target cells are present in very low frequency, manual scanning is very cumbersome, time-consuming and unsuitable for clinical applications (Krabchi et al., 2006b; Krabchi et al., 2001). Innovative technologies have been invented to allow detection of cellular events. Although they proved efficient for routine clinical tasks, their validation for rare cellular events remained questionable. Therefore, we used the aforementioned strategy for the optimization of automatic scanning using MetaSystems automated platform. We developed a robust custom-made detection algorithm and validated its efficacy on the retrieval of rare XY cells among a nearly pure population of XX cells. Slides were scanned for presence of predefined XY cells after FISH and PRINS techniques. After optimization of the classifier, it has been used for retrieval of FCs from MB of normal and aneuploid pregnancies. We assigned the following objectives for this study:

1. Development of a robust custom-made algorithm for the detection of rare cellular events using an automated platform.
2. Optimization of FISH and PRINS protocols and evaluation of their efficiencies for detection of rare cellular events.
3. Comparison between the efficacy of FISH and PRINS techniques for the detection of rare cellular events.
4. Comparison between the efficacy of manual and automatic scanning for the detection of rare cellular events.
5. Evaluation of the frequency of FCs in both normal and aneuploid pregnancies.

9.2.3. Chapter 3: Evaluation of the impact of enrichment of fetal cells from maternal blood by density gradient centrifugation which is used as an initial step of FC enrichment in the vast majority of enrichment protocols published to date and the development of an alternative version of the procedure that reduces fetal cell loss.

Over the past two decades, investigators have devised and pursued different strategies that depend on the combination of two or more successive steps of enrichments to provide efficient isolation of FCs from MB (Zhao et al., 2002; de Graaf et al., 1999; Simpson and Elias, 1995). The physical separation by density gradient centrifugation is by far the most common initial step of enrichment protocols published to date (Al-Mufti et al., 2003; Kitagawa et al., 2002; Vona et al., 2002; Rodriguez de Alba et al., 2001). Many protocols were designed but no single approach was efficient enough to provide NIPD using FCs. Procedures and methods were difficult to compare due to non-uniformity of protocols among different groups and a comparative analysis has been limited by the fact that the samples were being processed across the entire protocol rather than determining the efficacy and impact of each single step. Recovery of FCs is jeopardized by their loss during the process of enrichment. It would have been more appropriate to evaluate each step to devise the most efficient protocol for enrichment of FCs from MB. Therefore, we assigned the following objectives for this study:

1. The main task of this study was to evaluate the impact of the density gradient centrifugation step on fetal cell loss during enrichment.

2. We analyzed the FC frequency before and after enrichment by density gradient centrifugation in samples of MB from both normal and aneuploid pregnancy.

3. We also optimized an alternative version of the procedure that reduce fetal cell loss to be used in subsequent studies.

9.2.4. Chapter 4: The purpose of this study was to assess the feasibility of using a few fetal cells to determine the fetal sex and major chromosomal abnormalities by quantitative fluorescence-polymerase chain reaction (QF-PCR) as a proof of concept of the feasibility of using fetal cells in non-invasive prenatal diagnosis.

The detection and molecular characterization of rare cellular events was first proposed more than one century ago, but has only been recently realized and used in different applications. It is now clear that these cells can provide novel approaches for cancer management, pre-implantation genetic and non-invasive prenatal diagnosis (Maheswaran and Haber, 2010; Wei et al., 2007; Thornhill and Snow, 2002). Scarcity of DNA in rare cells, such as FCs in MB, is a major limiting factor for their routine use in clinical diagnosis. Array-CGH has been proposed as a genome-wide assessment and was successfully used for the detection of chromosomal abnormalities from single cells following whole genome amplification. However, the current protocol is costly, time-consuming and does not seem to fit into clinical schedule (Fiegler et al., 2007; Hu et al., 2007). Looking for an alternative approach that is clinically practical and has the potential to detect chromosomal abnormalities and single gene disorders, this study focuses on evaluating the fidelity of DNA from few cellular events in terms of detection of fetal sex and major chromosomal aneuploidies using rapid and cost-effective multiplex QF-PCR. Therefore, we outlined the following specific objectives:

1. Optimization of the protocol of whole genome amplification from a few microdissected FCs and evaluation of their efficacy to determine fetal sex and major chromosomal abnormalities by quantitative fluorescence-polymerase chain reaction.

2. Determination of the required number of FCs that can be quite enough to provide accurate and reliable non-invasive prenatal diagnosis.

This application must demonstrate that the detection and utilization of as few as 5 FCs can be quite enough to provide accurate and reliable non-invasive prenatal diagnosis.

RESULTS

Chapter I:

Development of protocol that allows accurate evaluation of extreme rare cellular events detection within different populations of cells on slides and using this strategy in measuring of the efficiency of manual scanning used in retrieval of fetal cells from maternal peripheral blood.

Article 1:

Efficiency of manual scanning in recovering rare cellular events identified by fluorescence in situ hybridization (FISH): simulation of the detection of fetal cells in maternal blood.

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My contribution in work:

I made the experimental design and developed sequential spreading approach, carried out 70% of the experiments, and wrote the first full version of the article.

Résumé

L'hybridation *in situ* observée en fluorescence (FISH) et le balayage manuel en microscopie font partie d'une stratégie couramment utilisée dans la détection de cellules rares comme les cellules fœtales circulant dans le sang maternel. Afin de déterminer l'efficacité de ces techniques, des lames ont été préparées avec un nombre connu (0-10) de cellules XY dans une population de cellules XX. Après l'hybridation FISH, les lames ont été balayées par différents observateurs à l'aveugle pour la détection des cellules XY. L'efficacité moyenne de détection était de 84% (125/148). L'évaluation de la qualité d'hybridation de la sonde en ce qui concerne les évènements non détectées a montré que 9% (2/23) des évènements n'ont pas été hybridés, 17% (4/23) sont mal hybridés tandis que l'hybridation n'était pas en cause pour les autres évènements manqués (74%; 17/23). En conclusion, le balayage manuel est une méthode relativement efficace dans la détection d'évènements cellulaires rares mais environ 16% des évènements ont été ratés. Ainsi, le nombre de cellules fœtales par unité de volume de sang maternel a probablement été sous-estimé par l'utilisation du balayage manuel.

Abstract

Fluorescence in situ hybridization (FISH) and manual scanning is a widely used strategy for retrieving rare cellular events such as fetal cells in maternal blood. In order to determine the efficiency of these techniques in detection of rare cells, slides of XX cells with predefined numbers (1-10) of XY cells were prepared. Following FISH hybridization, the slides were scanned blindly for the presence of XY cells by different observers. The average detection efficiency was 84% (125/148). Evaluation of probe hybridization in the missed events showed that 9% (2/23) were not hybridized, 17% (4/23) were poorly hybridized, while the hybridization was adequate for the remaining 74% (17/23). In conclusion, manual scanning is a relatively efficient method to recover rare cellular events, but about 16% of the events are missed, therefore, the number of fetal cells per unit volume of maternal blood has probably been underestimated when using manual scanning.

1. Introduction

Detection of rare cellular events has enormous potential in both cancer [1-3] and prenatal diagnosis [4-7]. The presence of fetal cells in maternal circulation generates a great amount of interest as a source of genetic material for non-invasive and risk-free diagnosis of aneuploidies and single gene disorders [8]. Instead of cell-free fetal DNA (cffDNA) in maternal plasma, fetal cells in the maternal blood can be an alternative approach for the development of a non-invasive method for prenatal diagnosis that accurately detects chromosome anomalies for two major reasons: 1) to work with pure fetal DNA material, which will allow specific characterization of fetal genome, and 2) to have the whole genome of the fetus and not just part of it.

The number of fetal cells is extremely low in maternal blood [9,10], therefore, enrichment, accurate identification and optimal timing of recovery are essential for their reliable use in prenatal diagnosis [11-15]. Fetal cells could be identified by targeting specific genetic marker exclusive for the fetal cells by molecular cytogenetic techniques such as fluorescence in situ hybridization (FISH) and primed in situ labelling (PRINS). Manual scanning is a commonly used strategy for retrieving these rare fetal cells from maternal blood.

Recent studies have indicated that fetal cells can be detected directly from the maternal blood without prior enrichment, to avoid losing fragile fetal cells, using techniques such as FISH and PRINS. Low frequency predictions were given by these studies that recognized fetal cells only by the presence of a Y chromosome signal in male pregnancy [4,5,9,16]. Although these studies yielded important information concerning the number of circulating fetal cells in maternal blood, the results are possibly skewed by the detection efficiency of these rare events by cumbersome and time consuming manual scanning. Therefore, automation will be required for widespread clinical use of fetal cells in prenatal diagnosis. Precision of manual scanning is crucial to validate adequately any automatic scanning device. However, the accuracy and reliability of locating these rare cells by manual scanning has never been evaluated.

The purpose of this study was to develop a robust protocol to assess the detection efficiency of rare events such as fetal cells in the maternal blood. We developed a strategy to spread a known number of XY cells in pre-defined areas on the slide and to detect these XY cells amongst thousands of XX cells. This strategy allows for evaluation of the detection efficiency of the manual scanning by knowing the exact number of XY cells and their exact location on the slides. In addition, the efficiency of the FISH technique in recovering rare cells can be evaluated by verification of the missed events and evaluation of the hybridization signals after scanning. Furthermore, this strategy has various potential applications as it could be used in the validation of automatic scanning and comparisons between different detection techniques.

2. Materials and Methods

2.1. Sampling

Three millilitres (3mL) of heparinised peripheral blood were obtained from both male and female donors and rendered anonymous. Donors were healthy, non-pregnant adults between 20 and 35 years of age. Immediately after sampling, whole blood samples were dispensed into 250μL aliquots, washed with Hank's balanced salt solution, and harvested by standard cytogenetic techniques. For both XX and XY cells, small aliquots of fixed cell suspensions were prepared and stored at −20°C until needed.

2.2. Spreading and Counting

Spreading of 2μL of diluted fixed XY nucleus suspension at one, two, or three predefined spots onto cleaned slides was performed in a modified Thermotron environmental control unit (CDS-5, Thermotron, Amsterdam, Netherlands) at 25°C and 36% humidity. All slides were encoded and stained with 4% Giemsa solution (Harleco; EMB, Gibbstown, NJ) containing 4% of Sorensen's phosphate buffer [17].

Two different observers scored the number of XY cells on each slide, blindly. Slides with more than 11 cells or with no concordance of cell counts between observers were excluded. Each Giemsa-stained target cell was located, imaged using the 100x objective on

an Olympus BX-61 microscope and coordinates were registered. The selected slides were then subjected to secondary spreading with XX nuclei suspension of 1.5×10^5 nuclei on top of XY spreading areas. In addition, 100% XX and XY cell slides were spread as controls. A total of 148 XY cells were distributed on 60 slides. For statistical analysis, the slides were divided in two categories: 30 slides with a range of 2 to 11 XY cells on each slide and 30 slides with either 0 or 1 XY cell per slide.

2.3. FISH Procedure

Slides were first aged overnight at 37°C, then, immersed in 2xSSC at 37°C for 30 minutes. Slides were dehydrated through a series of ethanol baths (70%, 80%, 100%). Conventional dual-color FISH was performed, using probes specific for chromosomes X and Y (CEP X: spectrum orange alpha-satellite and CEP Y: spectrum green satellite-III; Vysis/ABBOTT Diagnostics, Downers Grove, IL) diluted 1:100 and 1:300, respectively, in cDenHyb-1 (Insitus Biotechnologies, Albuquerque, NM). The slides and the probes were co-denatured at 75°C for 5 minutes before being sealed with rubber cement and placed in a humid chamber for hybridization at 37°C for 16 hours. Coverslips were then carefully removed and the slides were washed with a solution of 0.4xSSC/0.3% NP-40 at 72°C for 2 minutes. A second wash was performed in a solution of 2xSSC/0.1% NP-40 at room temperature for 3 minutes. After a final wash with distilled water, the slides were mounted in DAPI II (0.1M Tris pH 8.0, 90% glycerol, 1mg/mL p-phenylenediamine, 0.01% 4,6-diamidino-2-phenylindole).

2.4. Microscopic Observation

Microscopy equipped with appropriate filter sets. Fluorescence nucleus pictures were taken using a CCD camera and ISIS-2 software (Metasystems, Altlussheim, Germany). All slides were analyzed using the appropriate single band pass filter. Primary search was performed for Y-signal using spectrum green band filter (FITC). Suspected events were subsequently verified for the presence of single X chromosome-specific signal on a DAPI-stained nucleus under the appropriate spectrum red (TRITC) and blue (DAPI) filters. The number of detected XY cells per slide, cellular location, and imaging were

recorded, along with time required for the scoring of each slide Manual scoring was performed blindly on an Olympus BX-61 fluorescent microscope. All manual microscopy was performed at 1000X magnification. We also scored the hybridization status of the Y signals in 5000 cellular events distributed in the 5 control slides of 100% XY cells. The slides were stored in dark at −20°C after the scanning process to avoid bleaching of signals.

In all cases, cells were considered to be positive if the following criteria were met: nuclei had two different fluorescent signal colours representing both the X and Y chromosomes, an intact nuclear border as indicated by DAPI staining, and presence of fluorescent signals only through appropriate filters. Cells in direct contact with each other were excluded. The hybridization signals were usually bigger and brighter than the background signals such as debris, fluorescent materials, or air bubbles that are not blocked by the filter. Most of these background signals could be excluded by bleed through signals that is, signals which appears in all filters.

2.5. Rehybridization Procedure (Re-FISH)

A reverse color FISH was done for all recovered cells to confirm the identity of the cells. Evaluation of its reliability in confirming positive events was also recorded. For the re-FISH procedure, coverslips were removed by dipping the slides in a prewarmed 2xSSC bath, at 37°C for 10 minutes. The existing FISH probes were then removed by denaturing the slides with 70% formamide/2xSSC at 73°C for 2 minutes 30 seconds. Slides were then dehydrated in successive ice-cold ethanol baths (70%, 80%, 100%) and air-dried. Finally, slides were processed through a second round of FISH procedure using the opposite fluorochrome labelling (X probe in green and Y probe in orange) to produce the reverse color FISH pattern.

2.6. Analysis of Cellular Scanning

The slides were scanned blindly (without knowing the number of XY cells or their location on the slide) by one investigator, then the analysis was performed within 48 hours after scanning by another investigator. Following the scanning, the location (coordinates) and the shape of captured events were compared with that of the previously recorded

Giemsa-stained photos. Depending on the results of the scanning, three different possibilities were observed (Section 3).

2.7. Statistical Methods

The statistical analysis was performed using “proc reg” and “proc mixed” procedures of the Statistical Analysis System (SAS) software, version 9.1.3 (SAS Institute Inc., Cary, NC). Spearman's correlation was used to assess the process of the manual scanning and regression analysis curve was plotted to demonstrate the detection efficiency of the target events. Kruskal-Wallis test was done for the analysis of variance of status versus different observers. The odds ratio was calculated for determining the effect of hybridization on the detection efficiency. Index of Youden was used for assessment of the methodology.

3. Results

As our manual scanning approach was based on the finding of the Y signal first, we first tested the hybridization efficiency of the Y chromosome centromere probe. We found that the hybridization efficiency of the Y probe in 5,000 pure XY cells, processed by conventional FISH, was 99.1% (4,955/5,000). The hybridization was adequate in 97.3% (4,865/5,000) of cells whereas the remaining 1.8% (90/5,000) showed poor hybridization signals.

Next, we evaluated the retrieval of rare cellular events by a manual scanning based FISH method, using an approach, which is summarized in Figure 1 and Table 1. All slides were screened at 1000X for the Y-signal for primary detection, with subsequent confirmation of an X chromosome-specific signal on a DAPI stained nucleus, using the appropriate band pass filters. Comparison of the location (coordinates) and the shape of captured events with that of the previously recorded Giemsa stained photos resulted in three possible results (Figure 1 and Table 1).

The first one is when a captured fluorescent image matched with a previously taken Giemsa photo, in which case the cell was scored as a recovered event (Figure 2). The

second possibility is when no corresponding fluorescent image was found for a previously recorded Giemsa one, in which case the cell was scored as a missed event. In these circumstances, retrieval of the cell, using the coordinates and shape of the recorded Giemsa image, and evaluation of its hybridization efficiency were performed. Cells were scored as either a missed event or a hybridization failure. In the third scenario, a captured fluorescent image had no corresponding Giemsa one. Re-FISH was then used to score the cell as either a true positive or false positive.

Table 1 Interpretation of detected cellular events by manual scanning according to the concordance of FISH images with those previously taken with Giemsa.

Interpretation of Cellular event	FISH photo	Giemsa photo
Retrieved XY target (true positive cell)	Present	Present
Missed event	Absent	Present
Extra-cell detected	Present	Absent

Comparison of location and shape of captured events with previously recorded Giemsa images brought up three possibilities. The first one is when a captured fluorescent image matched with a Giemsa photo, in which, the cell is scored as recovered event. The second possibility is when no corresponding fluorescent image found for a Giemsa one, in which, the cell scored as a missed event. In this case, retrieval of the cell, using the coordinates and shape of the Giemsa image, and evaluation of its hybridization efficiency were performed. For the third scenario, a captured fluorescent image had no corresponding Giemsa one. The re-FISH was used to score the cell as true positive or false positive

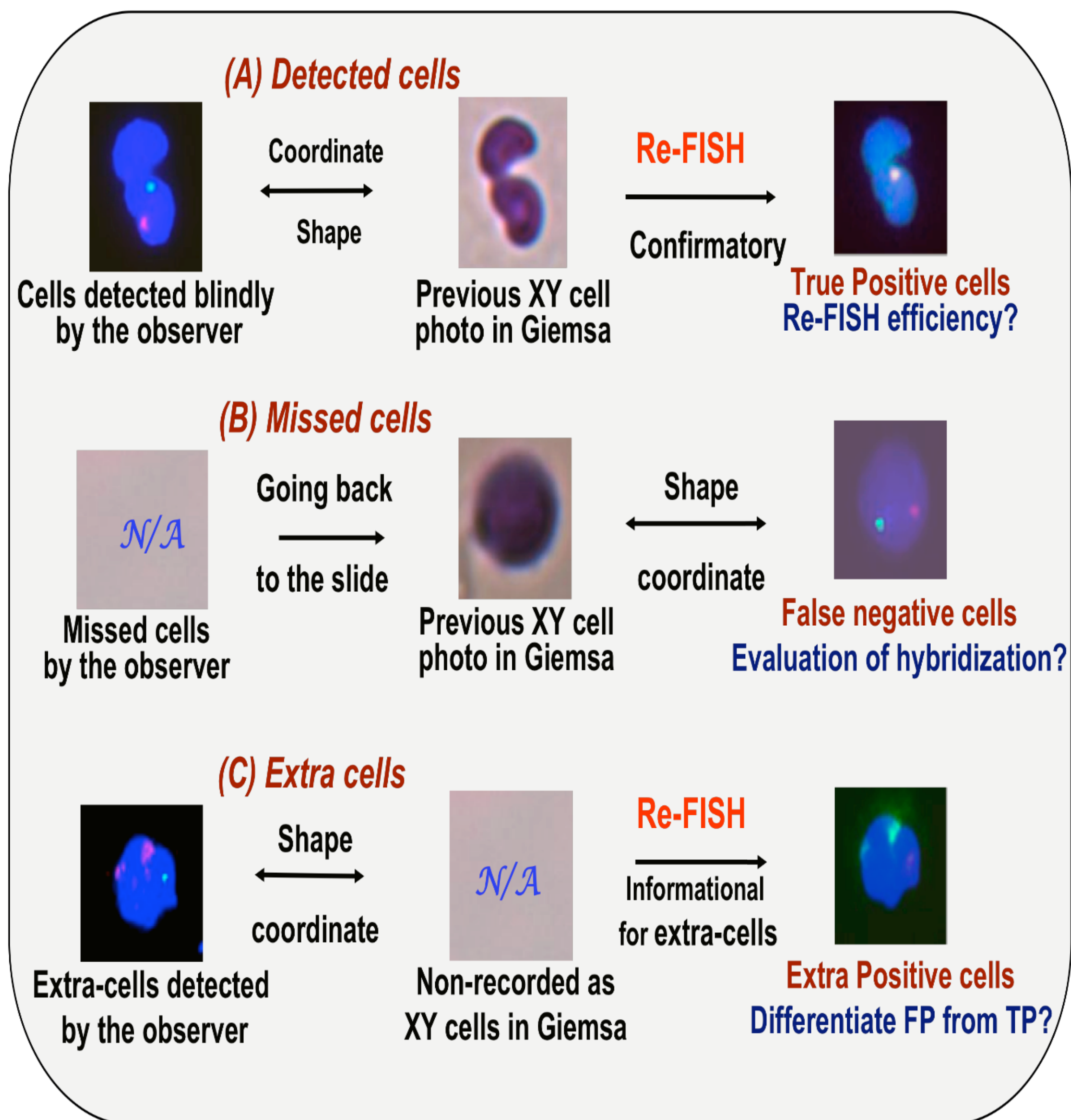

Figure 1 Schematic management of detected (A), missed (B), and extra cells (C).

FP: false positive, TP: true positive, N/A: not acquired. Panel A: cells scored as detected when a captured fluorescent image matched with a previously taken Giemsa photo, in which re-FISH is done as confirmatory to evaluate its efficiency. Panel B: cells scored as missed when no corresponding fluorescent image was found for a previously recorded Giemsa one, in this case, the cell was retrieved and hybridization was evaluated. Panel C: cells scored as extra-cells when captured fluorescent image had no corresponding Giemsa one. Re-FISH was then used to score the cell as either a true positive or false positive.

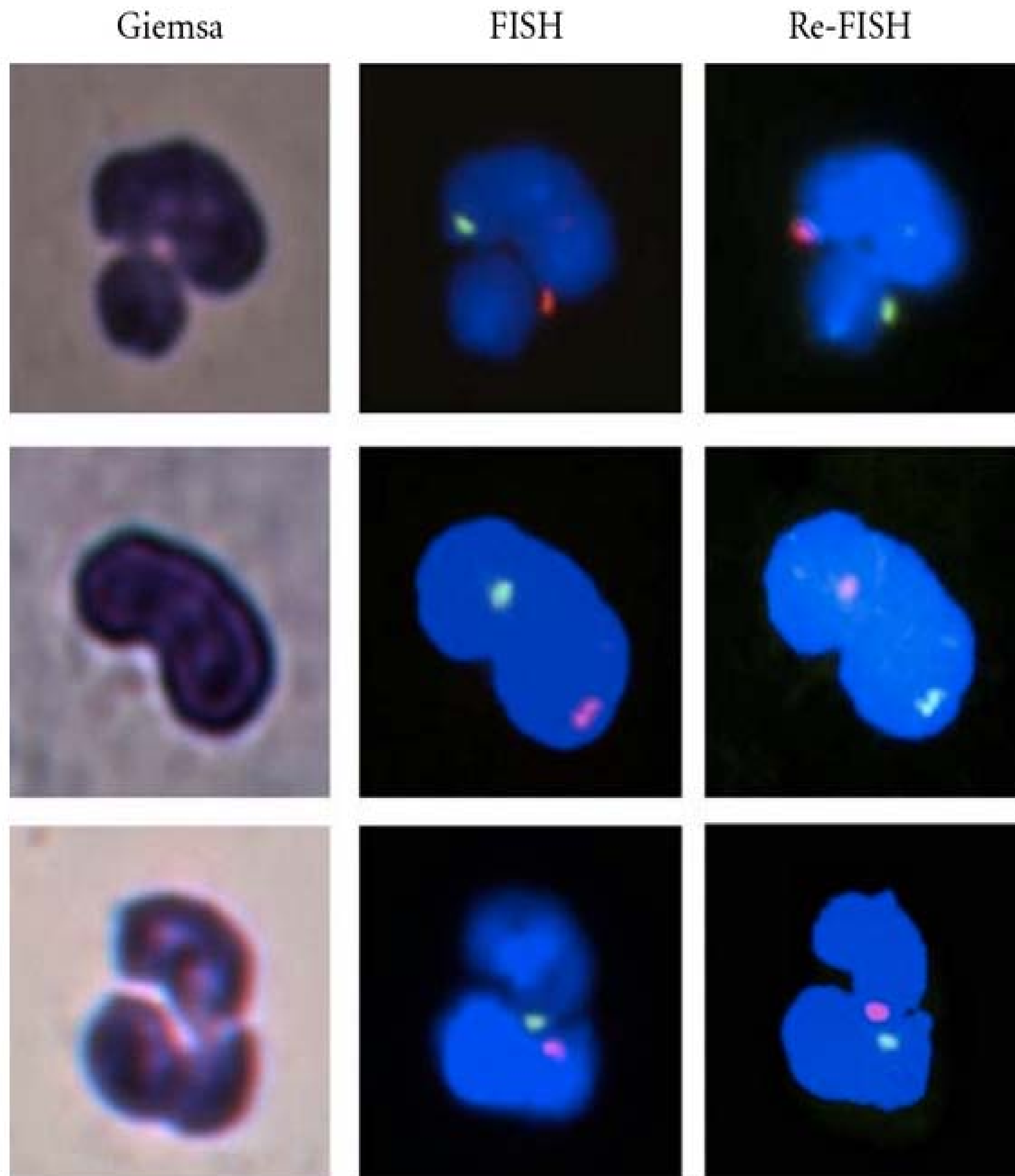

Figure 2 Example of Giemsa, FISH and re-FISH images of three detected events.

Figure demonstrates three examples of cells scored as retrieved when a captured fluorescent image matched with a previously taken Giemsa photo. FISH showed positive XY cells (Y probe in green and X probe in red) and cell identity confirmed by reverse color FISH (Y probe in red and X probe in green).

Table 2 summarizes the results obtained by manual scanning. Two observers A and B blindly scanned 18 and 42 slides, respectively, in order to retrieve 148 XY positive cells among around 90 x 10^5 XX cells, distributed on 60 slides, with an average of 1.5 x 10^5 XX cells per slide. The overall detection rate of the true positives was 84.5% (125 out of the 148 XY cells). The remaining 23 missed cells were considered false negatives. Individually, observers A and B detected 100 out of the 113 XY (88.5%) and 25 out of the 35 XY cells (71.4%), respectively (Figure 3A). The variance analysis did show a statistical difference between the two observers (p=0.606). Therefore, the combined findings of these two observers were used to assess the sensitivity, specificity and efficiency of rare events detected by the manual scanning and FISH technique.

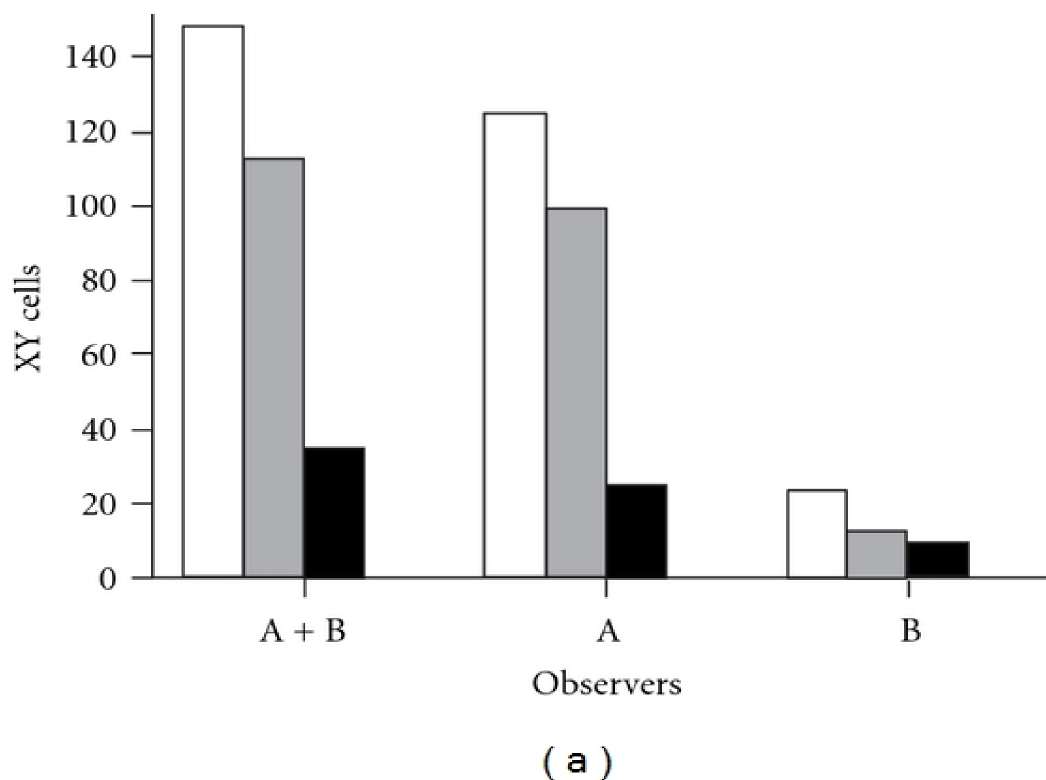

Figure 3 Comparison between detected cells and real number of XY cells. (a) Analysis of detection efficiencies of the two observers by comparing detected cells to real number of XY cells.

Bar charts represent the total number of target cells (white bars), detected (grey bars) and missed (black bars) cells reported for observer A and observer B. Although the overall detection efficiency of manual scanning is acceptable, analysis show marked inter-observer variability.

Table 2 Results obtained by manual scanning of rare cellular events hybridized by FISH technique.

Slide ID	Spread XY cells	Detected cells (TP)	Missed cells (FN)
AMP-9	1	1	0
AMP-10	4	4	0
AMP-11	2	0	2
AMP-12	5	4	1
AMP-13	5	2	3
AMP-14	2	2	0
AMP-18	3	3	0
AMP-19	0	0	0
AMP-20	1	1	0
AMP-21	1	1	0
AMP-22	3 + 2E*	4	1
AMP-23	0	0	0
AMP-24	1	1	0
AMP-25	1	0	1
AMP-26	3	1	2
AMP-27	0	0	0
AMP-28	1	1	0
AMP-29	0	0	0
SMP-10	4	3	1
SMP-11	11	10	1
SMP-12	2	2	0
SMP-13	11	9	2
SMP-24	5	5	0
SMP-25	3	3	0
SMP-26	2	2	0
SMP-27	3	3	0
SMP-28	1	1	0
SMP-29	5	4	1
SMP-30	6	6	0
SMP-31	3	3	0
SMP-32	6	5	1
SMP-33	7	6	1
SMP-34	6	5	1
SMP-35	4	3	1
SMP-36	2	2	0
SMP-37	1	1	0
SMP-38	3	3	0
SMP-39	2	2	0
SMP-40	5	5	0
SMP-41	4	3	1
SMP-42	0	0	0

SMP-43	3	3	0
SMP-44	1	1	0
SMP-45	0	0	0
SMP-46	1	1	0
SMP-47	0	0	0
SMP-48	2	2	0
SMP-49	0	0	0
SMP-50	1	1	0
SMP-51	1 + 1E*	2	0
SMP-52	0	0	0
SMP-53	0	0	0
SMP-54	0	0	0
SMP-55	3	3	0
SMP-56	0	0	0
SMP-57	1	1	0
SMP-58	0	0	0
SMP-59	1	0	1
SMP-60	1	0	1
SMP-61	1	0	1

TP: true positive, FN: false negative, ID: identification code, E*, extra-true positive cells confirmed by Re-FISH. Spread XY cells represent the target rare cells on the slides within pure population of XX cells. Detected cells represent the true positive events detected by manual scanning with FISH technique and missed cells represent the false negative events missed by the process of scanning.

We found that the specificity and sensitivity for detection of XY cells were 99.9% and 84.5%, respectively, with a positive predictive value of 97%. In the same vein, by using the Spearman's correlation, we found high correlation between the detected cells and the number of predefined target cells per slide (C.C= 0.947, $P < 0.001$). Regression analysis was plotted to demonstrate the relation of the detected cells versus the true number of XY cells (Figure 3B). Furthermore, a high index of Youden at 0.85 confirmed the efficiency of our approach. In summary, the manual scanning is a reliable method to detect rare events hybridized using the FISH technique.

To gain deeper insight into the causes of the occurrence of false negative events, we evaluated missed cells after scanning. Of the 23 missed cells, 2 cells (8.7%) were not hybridized for the Y chromosome, 4 cells (17.4%) were poorly hybridized and the hybridization was adequate in the remaining 17 cells (73.9%). Thus, manual scanning and

the related FISH procedure were responsible for the occurrence of 73.9% and 26.1% of the false negative events, respectively.

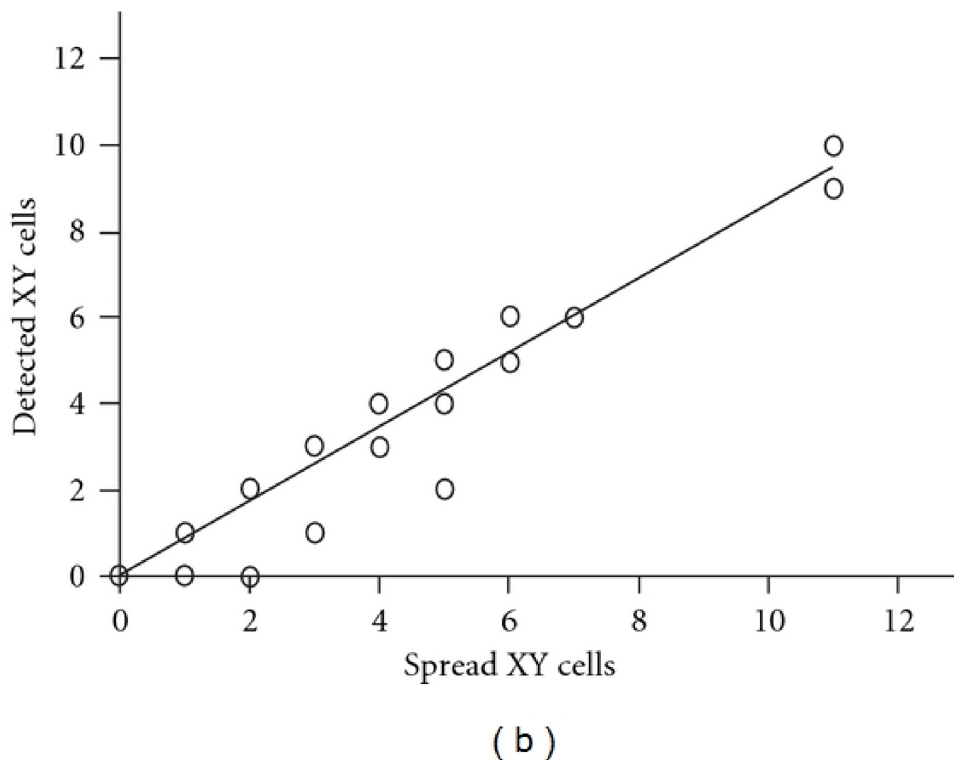

Figure 3 Comparison between detected cells and real number of XY cells. (b) Regression analyses represent correlation between detected cells by manual scanning and real number of XY cells on the slides.

Regression analysis plotted to demonstrate relation between numbers of detected cells by FISH technique and real numbers of XY cells and Spearman's correlation showed a high correlation coefficient (C.C= 0.947, P < 0.001).

The major cause of false negative cells is the manual scanning and human fatigue. The long scanning time, which was estimated to be on average 150 minutes, might be one of the reasons for the occurrence of false negatives due to the manual scanning. The second cause of false negatives is defective hybridization, which could be attributed to insufficient hybridization at the target site or fading of the fluorescent signals (Figure 4). The later is highlighted by the higher prolonged automatically adjusted exposure time for the Y probe channel (> 0.72 second) in contrast to an average of (<0.32 seconds) for the detected cells.

Next, we investigated if the percentage of cells with inadequate hybridization could be due to a drawback of the double spreading and Giemsa staining procedure.

We compared the hybridization efficiency of the Y probe on pure XY cells, without double spreading and Giemsa staining (see above), to that obtained from our FISH procedure on the pre-spread XY cells and we did not find any statistical significant difference of the percentage of non-hybridized (P=0.393) and poorly hybridized (P=0.179) cells of both groups. This led us to exclude the possibility of a procedure related effect on the hybridization efficiency.

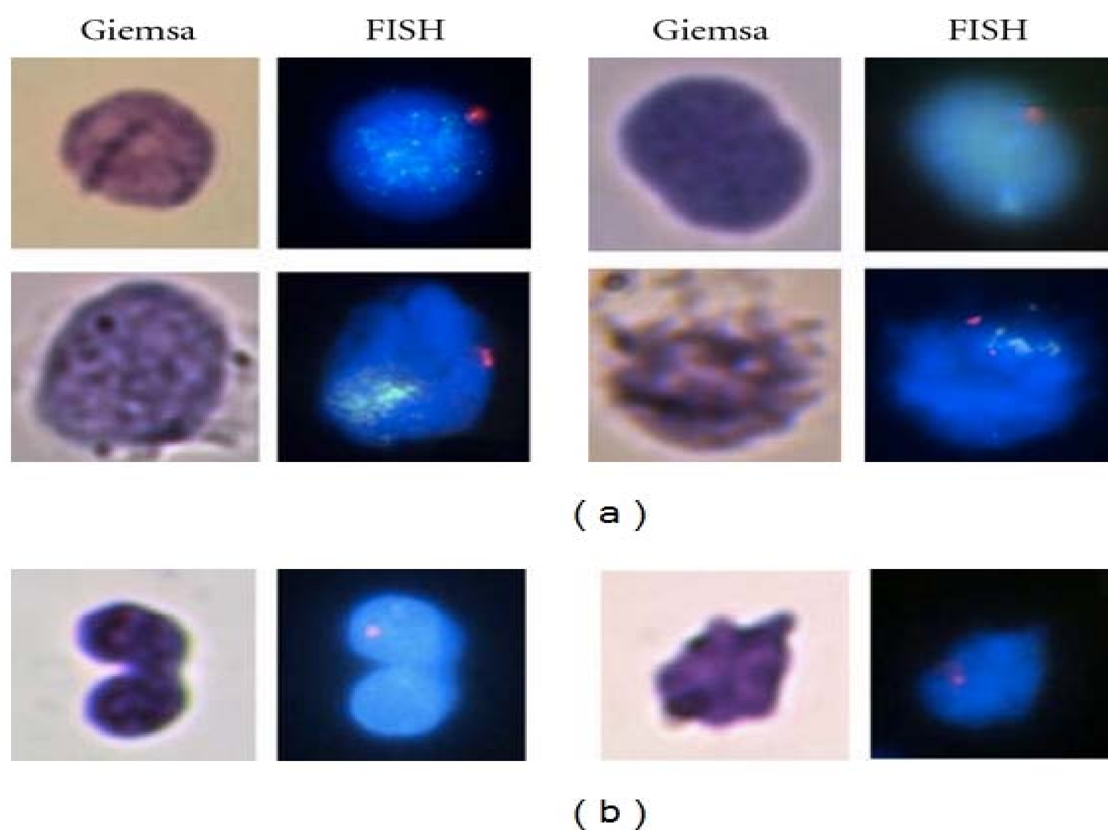

Figure 4 Giemsa and corresponding FISH photos of missed events due to inadequate hybridization (a) or non-hybridization (b).

Evaluation of hybridization of 23 missed events show that, 4 out of 148 cells (2.7%) were poorly hybridized and 2 cells (1.4%) were not hybridized at all for the Y chromosome and the hybridization was adequate in the remaining 17 cells (11.5%).

To evaluate extra-cells and exclude false positive events, we performed re-FISH on all detected cells, including the predefined XY cells and six additional cells. Evaluation of its reliability was measured on the detected predefined XY cells. Re-FISH gave an unambiguous reverse color pattern in 90.4% (113/125) of detected XY cells. Detected extra-cells did not show the expected reversed signal pattern after re-FISH hybridization except for three extra-cells (two in slide AMP-22 and one in slide SMP-51) as shown in table 2. Interestingly, these three cells were inside the pre-defined area of spreading, very close to other positive target cells in the corresponding slides. It appeared that they were missed in the original Giemsa stained target-counting step. These three events were included in the positive XY spread cells on the slides in the final tabulation for the sake of statistical analysis. Therefore, re-FISH is a highly reliable approach, which can help to exclude false positive events.

4. Discussion

In this study, we develop a robust protocol to validate the detection of rare events by a manual scanning and FISH technique. We found that manual scanning and FISH allows the detection of 1–10 targeted cells, among a total of 1.5×10^5 cells, with 99.9% of specificity, 84.5% sensitivity, and a positive predictive value of 97%. In addition, we found that this method is highly reliable and efficient with high Youden index of 0.85. Moreover, we determined the rate of false-negative and false-positive events and the inherent causes of their occurrence.

The experimental design, which involved the assessment of slides containing known numbers of predefined rare target cells, allowed, for the first time, the retrieval and evaluation of hybridization of false-negative or missed events. Our results indicated that the manual scanning process is responsible for 73.9% of false-negative events while the remaining 26.1% was due to the FISH technique.

The fatigue generated by the long time of scanning, on average 150 minutes per slide, and the screening of low numbers of small dots among thousands, might be some reasons of the occurrence of false negatives. These factors can be overcome by the

development and validation of automatic scanning to search for these kinds of rare events. The second cause of false-negative events was the FISH technique, responsible for missing 4% of the target cells and this percentage was not statistically different from the efficiency of the Y centromere probe. The diffuse and weak Y signal was mostly responsible for the FISH drawback. Diffused signals can be explained by chromatin extension forming chromatin fibres, which links two or more condensed domains of chromatin. These fibres usually show a very weak signal, which fades faster than a normal one [18]. The nature of defective signals resulting from over-decondensed chromatin points to the importance of pre-hybridization steps in the FISH technique. However, more effort should be oriented toward the reduction of false-negative events due to manual scanning, which can be overcome by the development of an automatic scanning system.

Interestingly, data collected in this study confirmed the reliability and accuracy of our previous methodology using manual scanning for the determination of the frequency of fetal cells in maternal blood. Using this methodology, we previously located a median of 2 to 6 fetal cells per milliliter of maternal blood in the second trimester of normal pregnancy between 18 and 22 weeks [9]. This number is increased by 3 to 5 times in cases of Down's syndrome. Similar increases were also detected in different types of aneuploidies [4,5].

According to our study, the number of detected fetal cells seemed to be underestimated by an average of 16% due to the occurrence of false-negative cells. Thus, these missing cells can be likely recovered by a robust automatic scanning, increasing the likelihood of the development of non-invasive prenatal diagnosis in future.

Our findings lay the groundwork for the validation of automatic scanning for the detection of fetal cells in maternal peripheral blood. Many innovative technologies have been developed to alleviate the burden of scanning large numbers of cells and allow rapid and precise detection of rare events using an automated slide-scanning device and image-analysis software [19,20].

A robust system allowing detection of one male fetal cell or one trisomic 21 cell among 10,000 to 100,000 maternal cells would be extremely useful. Such system would

obviate the need or at least significantly reduce the required level of enrichment of fetal cells and facilitate screening large number of slides, making prenatal diagnosis more easily achievable [18]. Evaluation of the efficiency of these automatic slide-scanning devices is mandatory before clinical implementation.

Different investigators have already tried to validate automatic scanning devices for the detection of fluorescent signals of rare cellular events [1,6,21,22]. When detection of extremely rare cellular events is required, an accurate evaluation is difficult to obtain. Some groups worked on real clinical samples and compared the results of automatic and manual scanning [19,21]. However, the accuracy of the manual scanning, which is considered the gold standard, in the detection of rare cellular events had never been validated. Other studies measured the detection efficiency by using prediluted artificial sample mixtures (spiked samples) [1,18,19,21-23]. Dilutions of target cells within a whole cell population are reliable within certain limits of dilution.

Nevertheless, in case of fetal cells, an average of 2 to 6cells/mL have been located by manual scanning of 20 to 30 slides with an average of 100,000 cells per slide [9]. In such situations, where the target cells represent an extremely low proportion with an average required dilution of more than $1{:}10^5$, the predilution strategy seems imprecise and could be considered as an approximation of the real situation. In summary, our protocol can be an accurate tool for the comparison of manual and automatic scanning and the development and validation of the latter for the detection or rare events such as fetal cells in the maternal circulation.

5. Conclusion

Our current investigation indicates that a small amount of circulating male fetal cells dispersed in thousands of female cells can be detected with high specificity and sensitivity using FISH and manual scanning. However, the FISH technique was responsible for missing of 4% of cells due to non-hybridization or inadequate signalling while 11.5% were missed as a drawback of the process of manual scanning. Even if the accuracy of manual scanning for signal counting is good, speed and reliability of manual scanning is dependent

on technical expertise. This methodology allowed us to determine the efficiency of detection of rare cell events by manual scanning. It establishes a standard for testing new detection strategies of rare event such as fetal cells in the maternal circulation using automatic scanning.

Acknowledgments

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Chapter II:

Development of a robust custom-made detection algorithm for detection of rare cellular events using an automated platform and validated its efficiency on slides with pre-defined numbers of rare events. We compared between manual and automatic scanning as well as between FISH and PRINS technique. We also tested this classifier for detection of fetal cells from maternal blood samples from normal and abnormal pregnancies.

Article 1:

Validation of automatic scanning of microscope slides in recovering rare cellular events: application for detection of fetal cells in maternal blood.

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My contribution in work:

I made the experimental design, carried out all of the experiments, analysed the results and wrote the first full version of the article.

Résumé

OBJECTIF

La détection d'évènements cellulaires rares est requise pour différentes applications dans le diagnostic en oncologie et en diagnostic prénatal non invasif. Quand les cellules cibles sont présentes avec une très faible fréquence, comme les cellules fœtales circulant dans le sang maternel, le balayage manuel en microscopie (BMan) devient très lourd, en temps et en technique, et peu approprié pour des applications cliniques. Comme alternative, nous avons optimisé un classificateur spécialement adapté pour la détection automatique des évènements cellulaires rares.

MÉTHODOLOGIE

En utilisant la plateforme automatisée de microscopie MetaSystems, nous avons développé un algorithme de détection et validé son efficacité de détection des cellules XY rares parmi une population de cellules XX. Les lames ont été préparées avec un nombre connu de cellules XY, hybridées suivant la technique d'hybridation *in situ* observée en fluorescence (FISH) ou en hybridation par synthèse *in situ* amorcée (PRINS). Le nombre de cellules fœtales a été évalué sur des échantillons de sang maternel hybridés par FISH.

RÉSULTATS

L'efficacité de détection de la technique FISH était de 88% (117/133) comparativement à 78% (53/68) avec la technique PRINS. Le balayage automatique a été plus efficace et constant que le balayage manuel même s'il requiert plus de temps pour le balayage. Les cellules fœtales ont été détectées dans des échantillons provenant de femmes enceintes de fœtus normaux et aneuploïdes.

CONCLUSION

Le balayage automatique semble plus efficace que le balayage manuel dans la détection des évènements cellulaires rares. Le balayage automatique combiné à la technique FISH est plus sensible que lorsque combiné à la technique PRINS. Cette étude valide la fiabilité du balayage automatique dans la détection des cellules fœtales hybridées en FISH à partir du sang maternel.

Abstract:

OBJECTIVE

Detection of rare fetal cells (FCs) in the maternal circulation could be used for non-invasive prenatal diagnosis. Considering that FCs in maternal blood are present in extremely low frequency, manual scanning is cumbersome, time-consuming and unsuitable for clinical applications. As an alternative, we optimized a custom-made classifier for automatic detection of FCs.

METHOD

Using MetaSystems' automated platform, we developed a robust detection algorithm and validated its efficiency on retrieval of rare XY cells in a pure population of XX cells. Slides were scanned for presence of predefined XY cells after fluorescence *in situ* hybridization (FISH) and primed *in* situ labeling (PRINS). Retrieval of FCs was also performed on samples from maternal blood.

RESULTS

The efficiency of detection of rare XY cells was 88% using FISH (117/133) in comparison to 78% (53/68) with PRINS. FC frequencies per 1 ml of maternal blood ranged from 3 to 6 FCs in normal pregnancies versus 13 to 21 FCs in Down syndrome pregnancies.

CONCLUSION

Automatic scanning was more efficient and consistent than manual scanning for detection of rare FCs and required considerably less operator time. Automatic scanning using FISH is more sensitive than that using PRINS. The study validates automatic scanning retrieval of FCs from maternal blood.

1. Introduction

Detection of rare cellular events within a large cell population carries great potential for the prospect of cancer management and non-invasive prenatal diagnosis (NIPD).[1-4] Routine prenatal diagnosis currently depends on collecting fetal samples through invasive procedures, which are associated with significant risk of pregnancy loss. Prenatal testing is, therefore, generally considered when the perceived risk for an abnormal pregnancy outweighs the procedure-related risk.[5] Development of a non-invasive diagnostic method can eliminate this risk and change the risk-benefit ratio, making it more likely for women to benefit from prenatal testing.

The presence of fetal cells (FCs) in maternal blood was first described more than a century ago and has since been confirmed by many investigators.[6-10] The use of these cells as an alternative source of fetal genetic material can provide a non-invasive, risk-free opportunity for detection of fetal chromosomal abnormalities. Although there is general agreement about the presence of FCs, their routine use is not yet feasible. Most researchers agree that the presence of FCs in maternal blood is a rare event with only one FC per 10^5-10^9 of maternal cells.[8,10,12] This makes their isolation difficult but not impossible. Manual screening was used for the retrieval of FCs from maternal blood after targeting a specific genetic marker exclusive to the FCs.[4,11,12] Considering their extremely low frequency in maternal blood, manual screening was cumbersome, time-consuming and unsuitable for clinical applications. Various purification procedures have been tried, but it has been difficult to use data obtained by these methods to generalize about absolute numbers of FCs in maternal blood. There is, therefore, a pressing need for systematic studies designed to evaluate enrichment procedures, time of entry of FCs into maternal blood, and their absolute concentrations at different periods of gestation. Our group and others applied a direct detection strategy, without prior enrichment to avoid losing fragile FCs during purification.[4,8,11,13] In this way, we were able to locate a median of four FCs per milliliter of maternal blood in euploid pregnancies by manual screening approach.[8] The accuracy and reliability of manual screening have been validated, but was dependent on operator expertise.[14] Therefore, automation will be required before widespread application in clinical

practice. A robust system allowing detection of rare FCs among thousands of maternal cells would be extremely useful. Such a system would remove the need or significantly reduce the required level of FC enrichment needed and facilitate screening large number of slides, making NIPD more easily achievable.[15]

In a previous study, we developed a strategy for spreading a predefined number of rare XY cells in a pure population of XX cells on slides and validated the efficiency of manual screening.[14] In the present study, we developed a robust custom-made detection algorithm for detection of rare cellular events using MetaSystems' automated platform and validated its efficiency on 90 slides with a pre-defined number of XY cells detected by fluorescence *in situ* hybridization (FISH) or by primed *in situ* labeling (PRINS) in comparison with manual screening. We have also tested this algorithm for detection of FCs from maternal blood samples from euploid and aneuploid pregnancies.

2. Materials and Methods

2.1 Sampling

Heparinized blood samples (20 mL) were obtained from eight healthy donors including two men and six non-pregnant women as well as from 12 cases of male pregnancies including six cases with normal fetal karyotype and six with trisomy 21 according to the protocol approved by our institutional research ethical committee after informed consent. Maternal blood was collected from women receiving prenatal care at our Centre 4 weeks after the amniocentesis and before any medications were given for termination of pregnancy. Blood was harvested, and small aliquots of donor XX and XY cells were prepared. For maternal blood, 3 mL of maternal blood was harvested and pooled in three microtubes (each represented 1 mL of maternal blood) as described in Krabchi *et al.*[8] Cells were fixed and stored in Carnoy solution (3:1, methanol:glacial acetic acid) at −20°C.

2.2 Preparation of slides with defined number of XY cells

Slides with defined numbers of XY cells, ranging from 0 to 8 cells per slide, were prepared from XX and XY cells as previously described.[14] Briefly, spreading of diluted fixed XY cells at one, two or three spots on each slide was carried out. Slides were encoded and stained with Giemsa, and XY cells were located, imaged, and scored. Slides were then subjected to subsequent spreading with XX cell suspension on top of XY spots. A total of 201 XY cells were distributed on 90 slides using sequential spreading technique. Slides with 100% XX cells were prepared as controls.

2.3 Spreading of maternal blood samples

Slides were prepared from one of the stored microtubes in a modified Thermotron environmental (CDS-5, Thermotron, Amsterdam, The Netherlands) at 25°C and 36% humidity. The number of slides ranged from 16 to 22 with an average of 1.5–2x10^5 cells per slide as previously described.[14]

2.4 Molecular detection: FISH and PRINS techniques

Dual-color FISH was performed using probes specific for chromosomes X and Y (CEP X: spectrum orange alpha-satellite; CEP Y: spectrum green satellite-III; Vysis/ABBOTT Diagnostics, Downers Grove, IL, USA) diluted in cDenHyb-1 (Insitus Biotechnologies, Albuquerque, NM, USA) as described before.[14] PRINS labeling reactions were performed as previously described.[16,17] The sequence of oligonucleotide primers used was as follows: Xc: GTTCAGCTCTGTGAGTGAAA[18] and YD599: TGGGCTGGAATGGAAAGGAATCGAAAC and YD600: TCCATTCGATTCCATTTTTTTCGAGAA.[19]

2.5 Automated microscopy

Automatic scoring was performed using Metafer, MetaSystems' automated imaging platform (Altlussheim, Germany). The system consists of a motorized Zeiss Axioplan2 microscope equipped with an appropriate filter set, a motorized slide stage, and a CCD camera. The Metafer software can detect multiple hybridization spots using specific color

channels. A binary image was obtained by thresholding the digitized images in a user-defined channel. Target cell size and shape are identified by the number of pixels to discard clusters and debris. The stage and image coordinates of retrieved cells are stored along with each cell's morphological features (area, shape, and dot count). The detected cells can be automatically relocated using the stored coordinates.

2.6 Target cells detection

A simple detection algorithm simulating manual detection of FCs in hematologic samples was used. The primary search was performed for Y signals using a spectrum green filter at 20x magnification. Suspected events were verified at 40x magnification for the presence of an X chromosome signal on a 4,6-diamidino-2-phenylindole (DAPI)-stained nucleus. Selected events were automatically imaged and recorded. The gallery was manually reviewed to reject obvious false events. Subsequently, the remaining cells were assessed under the microscope using the relocation option for final selection of potentially positive cells. Cells were considered if all the following criteria were met: (1) nuclei had two different fluorescent colored signals representing both X and Y chromosomes, (2) an intact nuclear border as indicated by DAPI staining (cells in direct contact were excluded), and (3) presence of fluorescent signals only through appropriate filters. Slides with pure XX cells and others spiked with a defined number of XY cells served as controls for optimization of the classifier. Knowing beforehand the numbers and locations of positive cells allowed for better characterization and fine-tuning of selection criteria and required magnification for optimum detection. After optimization, slides with defined numbers of target cells and maternal blood samples were scored.

2.7 Rehybridization and analysis of Re-FISH

A reverse-color FISH using opposite fluorochrome (X probe in green and Y probe in red) was carried out for recovered cells to exclude false positive cells. Detailed protocol for stripping and rehybridization for re-FISH can be found in Krabchi *et al.*[8] Slides with predefined XY cells were decoded, and both coordinates and shapes of captured events were compared with previously scored Giemsa-stained cells. Extra-detected cells were relocated

and examined for reverse hybridization pattern as previously described.[14] For maternal samples, all detected cells were relocated and examined for reverse hybridization pattern. Each target cell was imaged and compared with its corresponding FISH image for identification of true positive cells.

2.8 Statistical methods

The statistical analysis was performed using “proc reg” and “proc mixed” procedures of the Statistical Analysis System (SAS) software, version 9.1.3 (SAS Institute Inc., Cary, NC, USA). Spearman’s correlation was used to assess the process of the automatic scanning, and regression analysis curve was plotted to demonstrate detection efficiency of target cells. Index of Youden combines both the sensitivity and the specificity and is employed to capture the performance of diagnostic tests. It was used for assessment of methodology.

3. Results

We evaluated automatic retrieval of rare XY cells by interphase spot analysis using custom-made detection algorithm (Figure 1). Sixty slides of XX cells spiked with predefined numbers and locations of XY cells were hybridized by XY FISH probes and scanned blindly using our custom-made classifier. On the 60 slides, there were 133 XY cells among 90×10^5 XX cells distributed with an average of 1.5×10^5 XX cells per slide. The gallery size, number of fields, detected XY cells per slide, cellular locations, and images along with the time required for automatic scanning and operator revision were recorded.

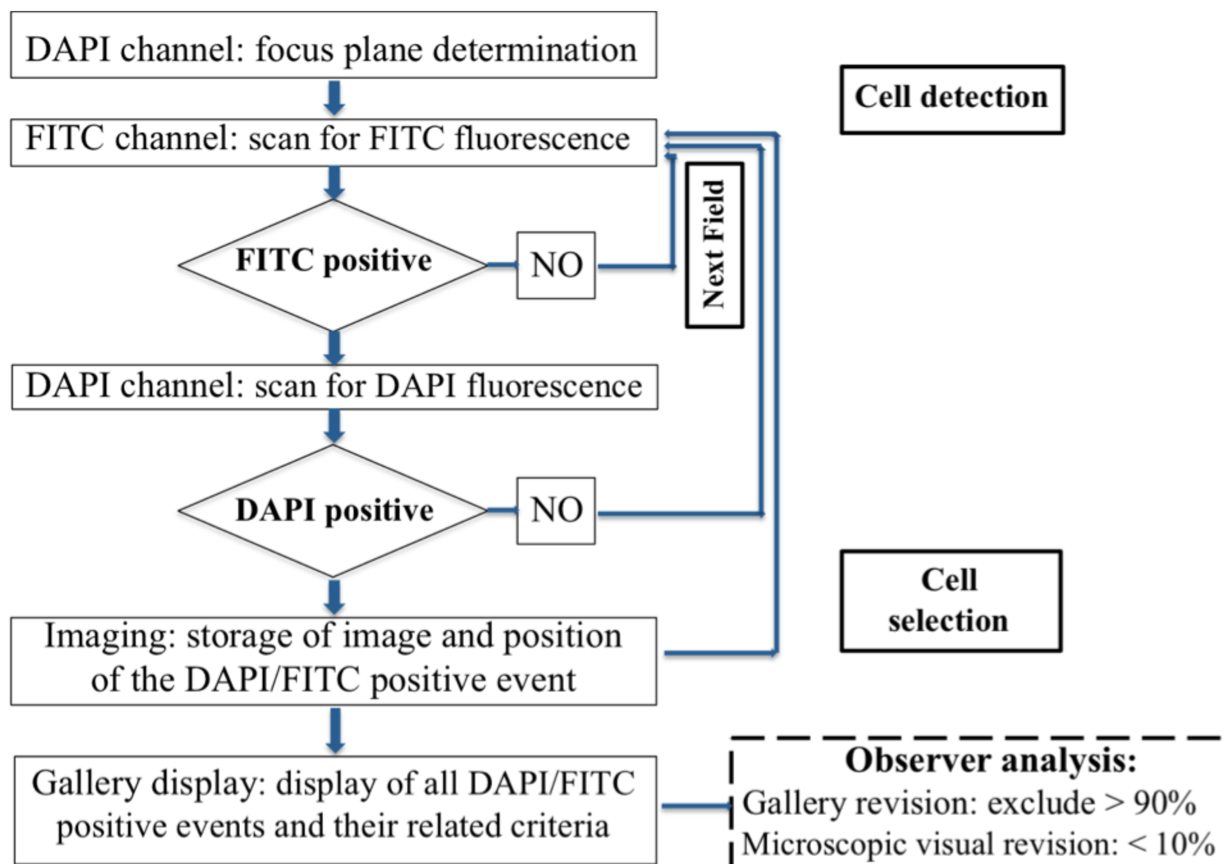

Figure 1 Schematic presentation of the custom-made detection algorithm used for detection of rare XY cells from pure population of XX cells.

Cells are located first in DAPI channel (blue) to determine the focus plane. Primary search is performed for Y-signal using spectrum green filter on 20X. Suspected events are verified at 40X for the presence of X chromosome signal on a DAPI-stained nucleus. If X chromosome signal (spectrum red) on DAPI stained background is found, picture is taken and image is stored. Otherwise, repeat the whole process in the next field and so on. After finishing the automatic scanning process the observer revise the gallery to exclude obvious false positive events (90%)t then the observer proceed to visual revision under the microscope using relocation option to verify the rest of the events (10%).

No significant correlation between scanning time (190–280 min; average of 239) and number of fields (1628–2320; average of 1987) has been found. Slide quality and level of noise were more important in reducing scanning time and size of generated galleries, which varied widely from 43 to 467 images with an average of 166 per slide. Most generated

images were false positives that can be easily rejected by reviewing the gallery; the operator only needed microscopic verification in less than 10% of the images. Most of background signals were excluded by bleed through signals that appeared in all filters.

The total operator scoring time ranged from 5 to 29 min, with an average of 15 min per slide. The slides were then decoded, and selected events were compared with Giemsa images. Comparison of locations and shapes brought up three possibilities as we have previously discussed[14] (Table 1).

Table 1 Interpretation of detected cellular events by automatic scanning according to the concordance of FISH or PRINS images with those previously taken with Giemsa.

Interpretation of Cellular event	FISH photo	Giemsa photo
Retrieved XY target (true positive cell)	Present	Present
Missed event	Absent	Present
Extra-cell detected	Present	Absent

Comparison of location and shape of captured events with previously recorded Giemsa images brought up three possibilities. The first one is when a captured fluorescent image matched with a Giemsa photo, in which, the cell is scored as recovered event. The second possibility is when no corresponding fluorescent image found for a Giemsa one, in which, the cell scored as a missed event. In this case, retrieval of the cell, using the coordinates and shape of the Giemsa image, and evaluation of its hybridization efficiency were performed. For the third scenario, a captured fluorescent image had no corresponding Giemsa one. The re-FISH was used to score the cell as true positive or false positive

The detection rate of true positive events was 117 out of 133 XY cells with an overall hybridization efficiency of 98.5% (Table 2). The sensitivity and specificity for detection of XY cells by FISH were 87.9% and 99.9% respectively, with a positive predictive value of 95.1%. Regression analysis showed a close concordance between numbers of detected cells and real numbers of XY cells (Figure 2) with a high Spearman's

correlation coefficient (C.C=0.963, *p*<0.001). The index of Youden of 0.88 confirmed the efficiency of automatic detection of rare cellular events hybridized by FISH.

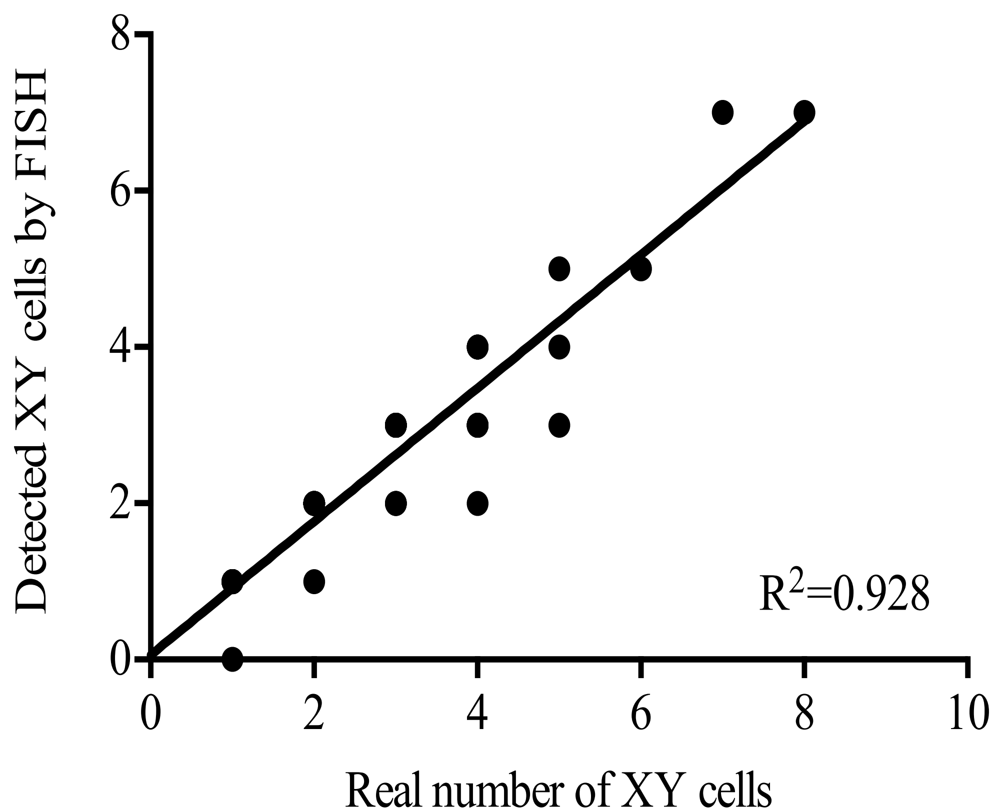

Figure 2 Regression analyses represent correlation between detected cells by FISH with automatic scanning and real number of XY cells on the slides.

Regression analysis plotted to demonstrate relation between numbers of detected cells by FISH technique and real numbers of XY cells and Spearman's correlation showed a high correlation coefficient (C.C= 0.963, P < 0.001).

Table 2 Results obtained by FISH technique.

Slide ID	Spread XY cells	Detected cells TP	Missed cells FN
AuSc-F01	2	2	0
AuSc-F02	3	3	0
AuSc-F03	1	1	0
AuSc-F04	8	7	1
AuSc-F05	5	5	0
AuSc-F06	1	1	0
AuSc-F07	2	2	0
AuSc-F08	1	1	0
AuSc-F09	3	3	0
AuSc-F10	3	2	1
AuSc-F11	4	2	2
AuSc-F12	4	3	1
AuSc-F13	5	4	1
AuSc-F14	6	5	1
AuSc-F15	7	7	0
AuSc-F16	1	1	0
AuSc-F17	2	2	0
AuSc-F18	1	1	0
AuSc-F19	3	3	0
AuSc-F20	4	4	0
AuSc-F21	1	1	0
AuSc-F22	3	3	0
AuSc-F23	1	1	0
AuSc-F24	2	2	0
AuSc-F25	2	2	0
AuSc-F26	3	3	0
AuSc-F27	2	1	1
AuSc-F28	0	0	0
AuSc-F29	4	4	0
AuSc-F30	0	0	0
AuSc-F31	2	2	0
AuSc-F32	0	0	0
AuSc-F33	2	2	0
AuSc-F34	1	1	0
AuSc-F35	0	0	0
AuSc-F36	5	3	2
AuSc-F37	3	3	0
AuSc-F38	1	1	0
AuSc-F39	0	0	0
AuSc-F40	1	0	1
AuSc-F41	0	0	0

AuSc-F42	2	2	0
AuSc-F43	0	0	0
AuSc-F44	1	1	0
AuSc-F45	1	1	0
AuSc-F46	0	0	0
AuSc-F47	3	2	1
AuSc-F48	0	0	0
AuSc-F49	1	1	0
AuSc-F50	4	3	1
AuSc-F51	2	2	0
AuSc-F52	4	3	1
AuSc-F53	3	3	0
AuSc-F54	0	0	0
AuSc-F55	1	1	0
AuSc-F56	1	1	0
AuSc-F57	4	4	0
AuSc-F58	0	0	0
AuSc-F59	3	2	1
AuSc-F60	4	3	1
Sum	133	117	16

TP: true positive, FN: false negative, ID: identification code. Spread XY cells represent the target rare cells on the slides within pure population of XX cells. Detected cells represent the true positive events detected by automatic scanning with FISH technique and missed cells represent the false negative events missed by the process of scanning.

Of the 16 missed events, two cells (1.5%) were not hybridized at all for Y chromosome, four cells (3%) were poorly hybridized (Figure 3), and the hybridization was adequate in the remaining 10 cells (7.6%). Statistically, this hybridization efficiency was not significantly different from the one obtained on pure XY cells after ordinary spreading. Of the 10 well-hybridized cells, three cells were detected by the scanning platform but rejected during manual revision of the gallery. Thus, automatic scanning was responsible for 43.7% of missed events (7/16), whereas the process of manual revision was responsible for 18.7% (3/16). FISH and technical procedures were responsible for 37.5% (6/16). Stripping and rehybridization were carried out on the slides with extra-detected events to exclude the remote possibility of being true positive missed by observer using Giemsa stain during slide preparation as previously described.[14] All additional detected cells failed to give the reverse

hybridization color pattern after rehybridization with opposite probe color (Supplementary Table S1).

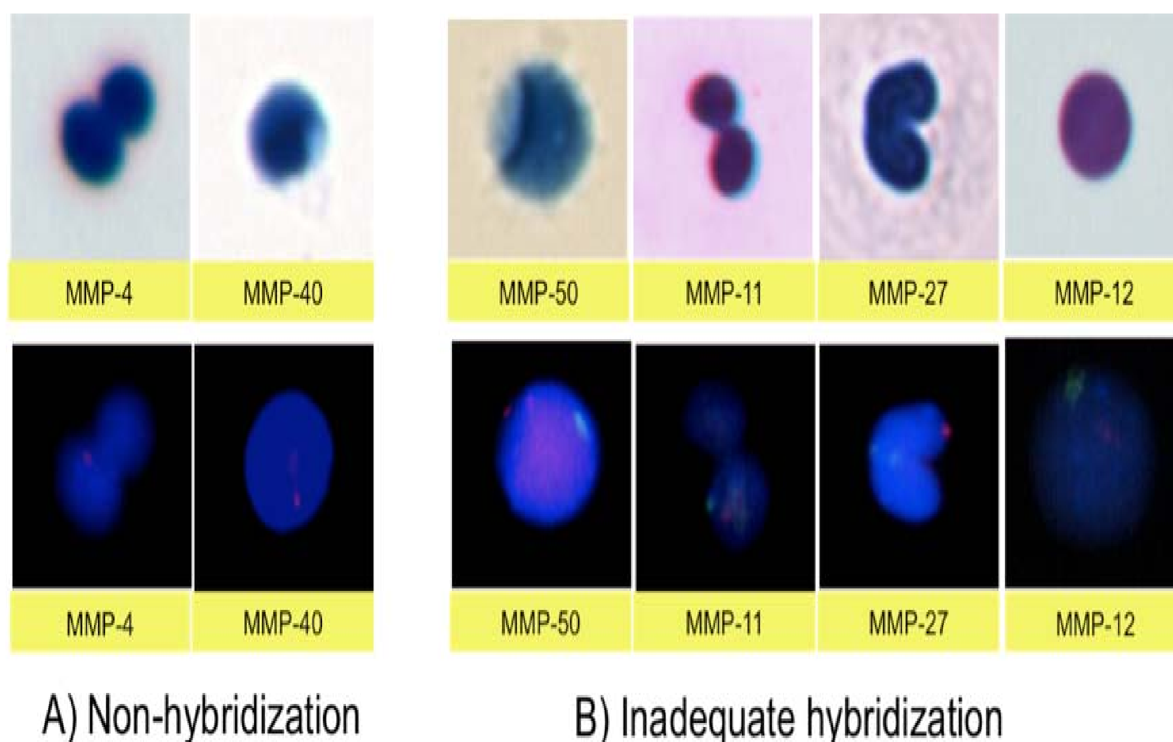

Figure 3 FISH and corresponding Giemsa images of missed events both due to non-hybridization (Panel A) or inadequate hybridization (Panel B).

Evaluation of hybridization of the 16 missed events show that, 4 out of 133 cells (3%) were poorly hybridized and 2 cells (1.5%) were not hybridized at all for the Y chromosome and the hybridization was adequate in the remaining 10 cells (7.5%).

As an alternative to FISH, 30 slides with an average of $1.5x10^5$ XX cells per slide were evaluated by PRINS in order to retrieve 68 XY cells. The detection rate of true positive events was 53 out of 68 XY cells with an overall hybridization efficiency of 96.5% (Table 3). Although having comparable specificity with FISH, PRINS has a lower sensitivity at 77.9%. Spearman's correlation showed a lower correlation coefficient of 0.882 between detected cells and real numbers of XY cells on the slides (Figure 4).

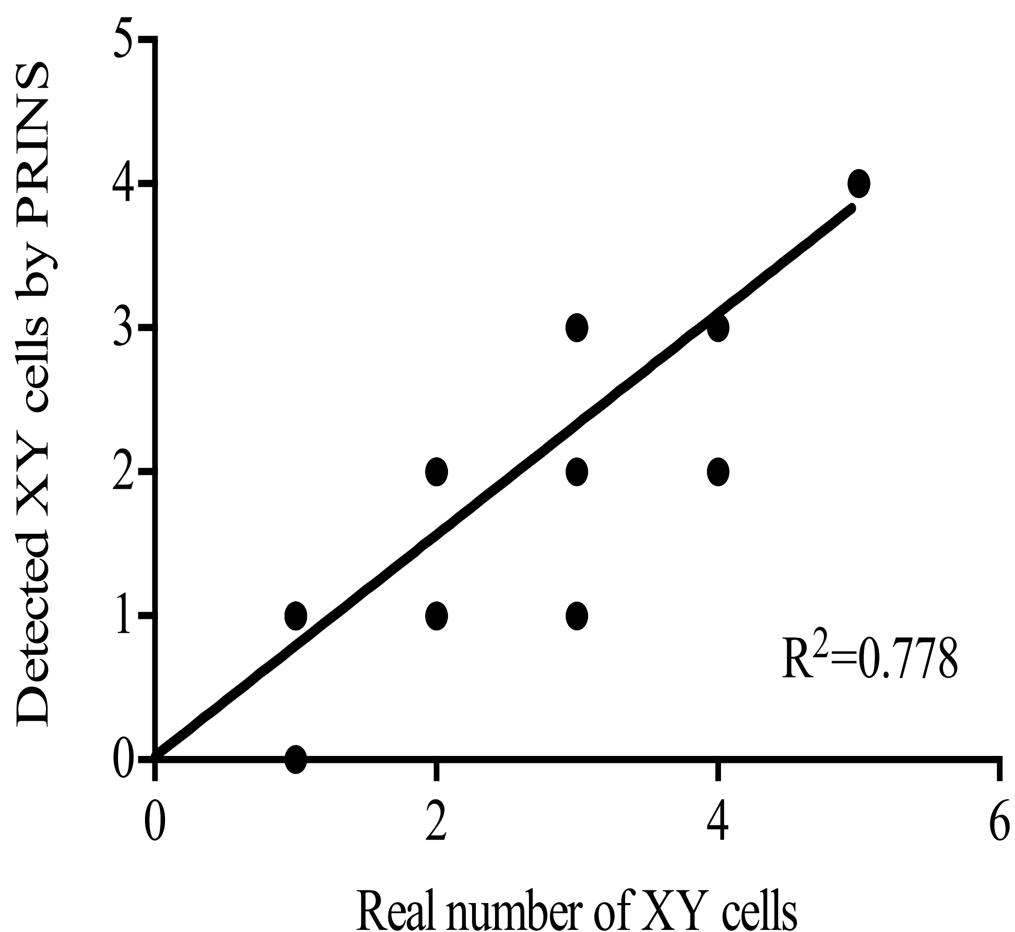

Figure 4 Regression analyses represent correlation between detected cells by PRINS with automatic scanning and real number of XY cells on the slides.

Regression analysis plotted to demonstrate relation between numbers of detected cells by PRINS technique and real numbers of XY cells and Spearman's correlation showed a lower correlation coefficient in comparison to FISH (C.C=0.882, P < 0.001).

Table 3 Results obtained by PRINS technique.

Slide ID	Spread XY cells	Detected cells TP	Missed cells FN
AuSc-P01	1	1	0
AuSc-P02	2	2	0
AuSc-P03	2	1	1
AuSc-P04	5	4	1
AuSc-P05	2	1	1
AuSc-P06	1	1	0
AuSc-P07	2	2	0
AuSc-P08	4	2	2
AuSc-P09	1	1	0
AuSc-P10	1	1	0
AuSc-P11	5	4	1
AuSc-P12	3	3	0
AuSc-P13	2	2	0
AuSc-P14	3	2	1
AuSc-P15	0	0	0
AuSc-P16	2	2	0
AuSc-P17	1	0	1
AuSc-P18	3	3	0
AuSc-P19	2	1	1
AuSc-P20	5	4	1
AuSc-P21	3	3	0
AuSc-P22	1	1	0
AuSc-P23	1	1	0
AuSc-P24	2	2	0
AuSc-P25	4	3	1
AuSc-P26	1	1	0
AuSc-P27	2	1	1
AuSc-P28	1	0	1
AuSc-P29	3	3	0
AuSc-P30	3	1	2
Sum	68	53	15

TP: true positive, FN: false negative, ID: identification code. Spread XY cells represent the target rare cells on the slides within pure population of XX cells. Detected cells represent the true positive events detected by automatic scanning with PRINS hybridization and missed cells represent the false negative events missed by the process of scanning.

After evaluating the classifier on artificial samples, 12 maternal blood samples from male pregnancy were processed: six cases with a normal fetal karyotype and six with trisomy 21. For normal pregnancies, four cases were sampled at the end of the first trimester between 11th and 13th weeks, whereas the other two were taken between the 18th and 20th weeks of gestation. All cases of trisomy 21 were sampled between the 16th and 20th weeks, and before interruption of pregnancy. The slides were processed by XY-FISH and automatically scanned. Generated events were revised, and selected events were evaluated by re-FISH to exclude false positive cells. True FCs were confirmed by re-FISH, and extra cells that failed to give a reverse signal pattern were excluded (Figure 5). Table 4 summarizes the results obtained from the analysis of maternal blood using the FISH technique.

Table 4 Results obtained from counting of Fetal cells in maternal blood by FISH.

Case number	**1**	**2**	**3**	**4**	**5**	**6**
Fetal karyotype	**46,XY (Normal male pregnancy)**					
Gestational age (weeks) Number of FISH slides	11w 20	12w 18	11w 17	13w 16	20w 19	19w 16
FCs confirmed (1 ml)	4	3	6	5	4	5
Ratio of FCs per slide (1 ml)	0.20	0.17	0.35	0.31	0.21	0.31
Case number	**7**	**8**	**9**	**10**	**11**	**12**
Fetal karyotype	**47,XY, +21 (Down syndrome male pregnancy)**					
Gestational age (weeks) Number of FISH slides	17w 18	20w 22	18w 17	19w 18	18w 20	17w 18
FCs confirmed (1 ml)	19	14	13	15	17	21
Ratio of FCs per slide (1 ml)	1.06	0.64	0.76	0.83	0.85	1.17

Table shows the Gestational age by weeks, Number of prepared FISH slides from one milliliter of maternal blood, number of fetal cells detected by automatic scanning and average number of fetal cell per slide of twelve cases of male pregnancies, six cases with normal fetal karyotype and six with trisomy 21.

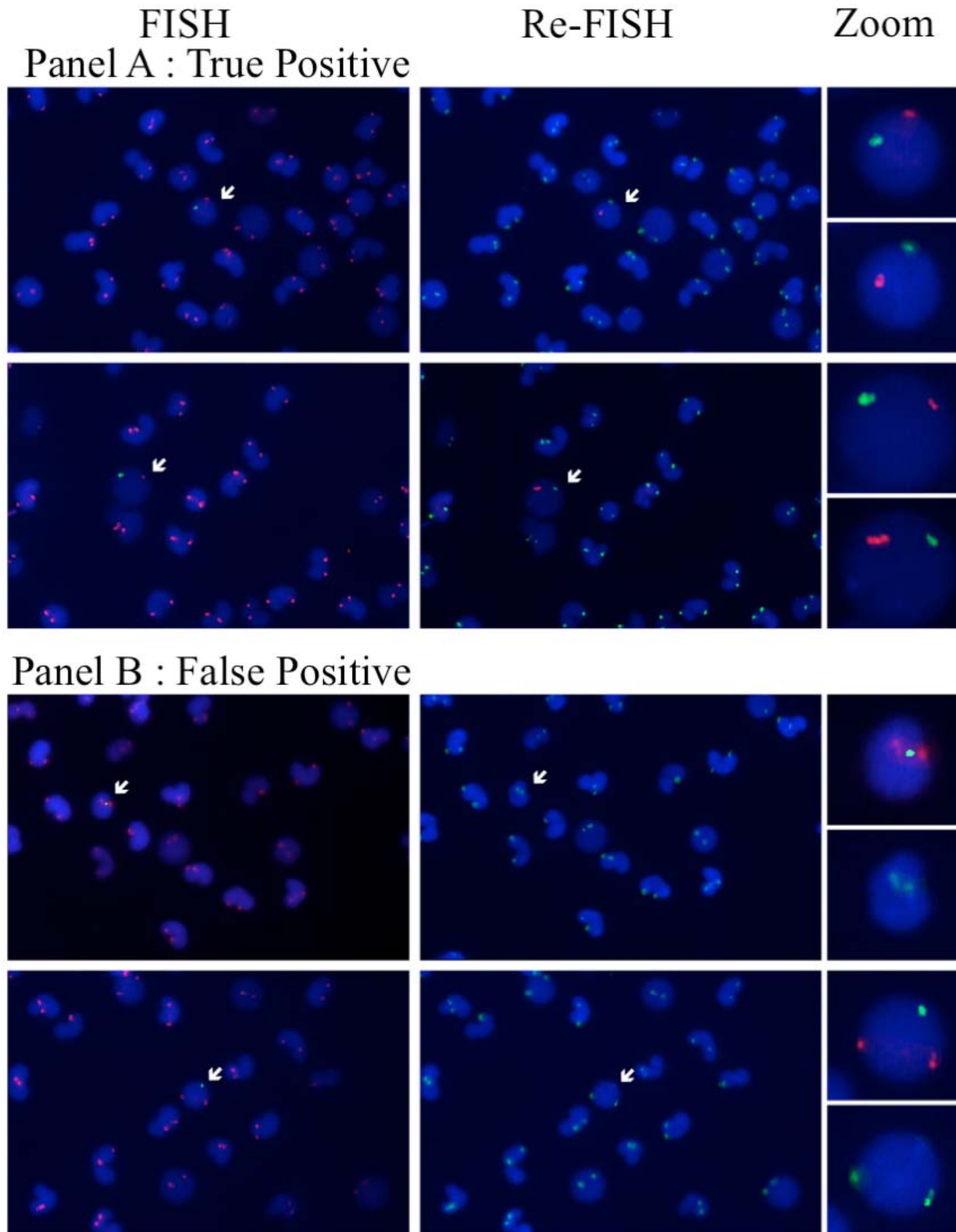

Figure 5 Assessment of selected events by re-FISH

Panel A: shows true fetal cells detected by FISH (Y probe in green and X probe in red) and confirmed by reverse color FISH (Y probe in red and X probe in green). Panel B: shows suspicious cells by FISH that failed to give reverse pattern (false positive).

The analysis of the number of FCs showed a significant difference between normal and aneuploid pregnancies ($p<0.001$), although we did not find a difference in the frequency of FCs between 1st and 2nd trimesters of normal pregnancies (Figure 6).

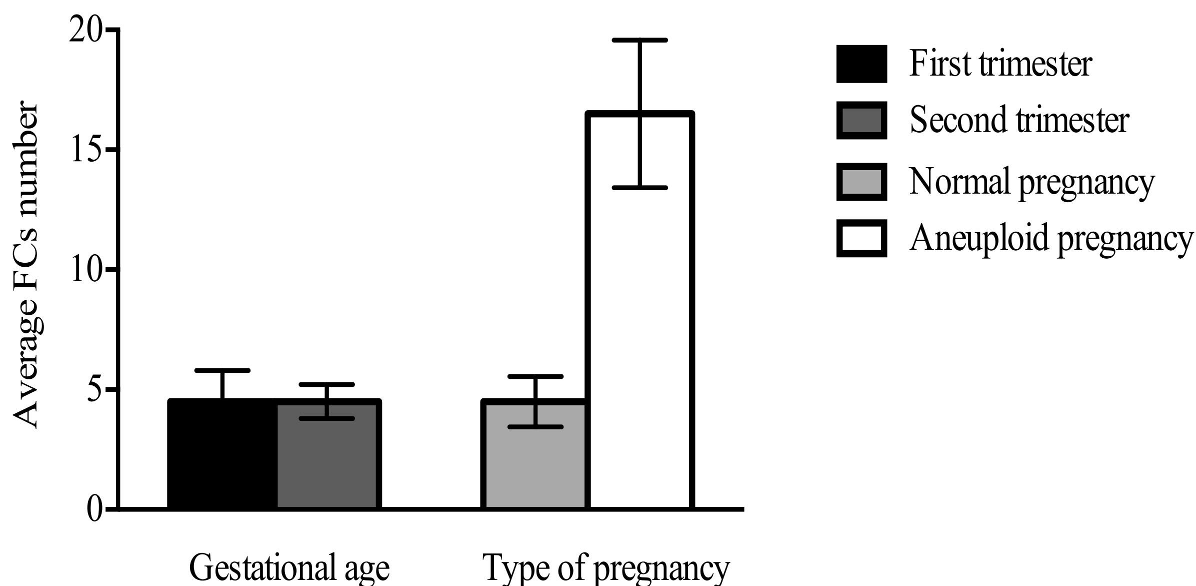

Figure 6 Comparison of average number of detected fetal cells in the first versus second trimester and in normal versus aneuploid pregnancies.

Analysis of number of fetal cells showed statistically significant difference between normal and aneuploid pregnancies ($P< 0.001$) while no difference was observed in the frequency of fetal cells between 1st and 2nd trimester.

4. Discussion

Researchers have documented the presence of FCs in maternal blood and have envisioned their use in NIPD.[1,8,20] FCs can be identified by molecular or immunological approaches. However, the key-limiting factor is their extremely low frequency in maternal blood, which makes manual detection labor-intensive and unsuitable for routine clinical application. This creates the need for automated systems that allow for reliable unbiased

detection of FCs from maternal blood. Automation will be able to enhance current research in NIPD and help in the evaluation of enrichment procedures in terms of both yield and purity.

Flow and imaging cytometry can be used for automatic detection, but only imaging cytometry can offer the ability to relocate detected cells using previously determined spatial locations on slides. This allows for the re-examination and further micromanipulation of such cells.[21,22] The automatic detection of fluorescent signals in interphase nuclei was first reported in 1997 using one-color FISH.[23] Subsequently, fully automated hybridization dot analyzers were developed for multi-color FISH specimens in both two and three dimensions.[24-26]

Although they proved to be efficient for routine clinical diagnostic tasks, their validation for rare cellular event detection has remained questionable. Evaluations using pre-diluted artificial sample mixtures[15,27-31] are reliable within certain limits of dilution. In the case of FCs, when target cells represent extremely low proportions, the pre-dilution strategy seems imprecise. Other strategies like comparing the efficiency of automatic scanning to manual detection[28,29] have also been proposed. However, manual screening, which is considered as the gold standard, has never been validated for the detection of a rare cellular event. Furthermore, the efficiencies of the platforms' built-in software for rare cellular event detection were usually unsatisfactory in terms of scanning time and sensitivity.

Therefore, we have previously developed a strategy for spreading predefined number of rare XY cells in a pure population of XX cells on slides and validated the efficiency of manual screening.[14] Here, we used the same strategy for the evaluation of automatic scanning using FISH and PRINS technique. Although Metafer/RC Detection built-in classifier can be used for automatic detection of rare cellular events, a semi-automated approach using a custom-made detection algorithm has been preferred for scoring FCs. The scanning platform was used as a tool to find potential target cells with final assessment being performed by experienced operator. An acceptable balance has been achieved between the required high sensitivity and the reduction of automatic scanning time.

Operator revision compensates for a possible lower specificity associated with this approach and highlights the importance of adapting the selection algorithm according to user's purpose and needs. This explains the difference in automatic scanning efficiency among various groups.[28,32,33]

Automatic scanning with FISH was 10% superior to automatic scanning with PRINS in the detection of FCs. The average scanning time was 239 minutes per slide, which is shorter than that which has been previously published by the same platform.[28] Automatic scanning has a better sensitivity (87.9%) than manual detection (84.5%) using FISH. Both methods have a high specificity of 99.9% but automatic scanning has a slightly lower positive predictive value of 95.1% versus 97% in manual detection. The index of Youden was 0.88 in automatic scanning compared with 0.85 in manual detection. Thus, automatic scanning is more sensitive than manual detection of rare XY cells using FISH technique. Although automatic scanning required a longer scanning time, the operator time was significantly reduced when compared to manual detection. Increasing the throughput of automatic scanning and using it overnight would potentially overcome this limitation.

The experimental design allowed for the retrieval and evaluation of missed events. Automatic scanning was responsible of 43.7% of missed events, whereas the process of manual revision was responsible for 18.7%. The FISH technique was responsible for the remaining 37.5%. The most common problem occurring during automatic scanning and leading to missing of true positive cells was the inadequacy in maintaining the plane of focus throughout the whole defined scanning area on the slide. The problem could arise from non-homogeneous mounting of the slides because applying a gentle homogenous pressure on the coverslips for 10 minutes or leaving the slides overnight at 4°C helped to reduce this problem.

Defective hybridization, which could be attributed to insufficient hybridization in the target site or the fading of the fluorescent signals, was second most common cause for missing target cells. This problem was highlighted by the prolonged average exposure time for the Y probe color channel in the missed events in contrast to that of detected cells. Diffused weak Y signals can be explained by chromatin overextension forming chromatin

fibers, which fades faster than usual.[15] This points to the importance of pre-hybridization steps in FISH preparations. Interestingly, a considerable proportion of cells were missed during manual revision of the generated gallery, although they were actually detected by automatic scanning. This emphasizes the operator expertise and importance of microscopic revision before excluding a suspect event.

After evaluating our custom-made classifier on artificial samples, it was tested for the detection of FCs from 12 maternal blood samples. Automatic scanning retrieved FCs from all cases of normal and aneuploid pregnancies. Interestingly, we did not find a significant difference in the frequency of FCs between the first and second trimesters of normal pregnancies. Most workers found a slight, but significant, increase in fetal nucleated red blood cells (NRBCs) between the first and second trimesters,[34-36] whereas there was a decreasing trend for the mean number of trophoblasts.[37] The peak number of trophoblasts was found to be between the 9th to 13th weeks of gestation.[38] As all first trimester samples were obtained between the 11th to 13th weeks, and we used a universal marker that detected both NRBCs and trophoblasts, the decreased number of NRBCs could have been compensated for by the increased number of trophoblasts. We think that this period at the end of the first trimester may present an opportunity for an early NIPD, although this should be verified on large series of samples.

The current results also confirmed the increased frequency of the FCs in aneuploid pregnancies when compared with normal pregnancies ($p<0.001$). It is possible that fetuses compromised by genetic disorders have more fetal NRBCs secondary to the chronic stimulation of hypoxic bone marrow. Alternatively, a placental defect in an aneuploid pregnancy could have resulted in greater FC trafficking. Interestingly, our findings correspond with previous results of Krabchi *et al*., on the frequency of FCs in maternal blood using manual screening.[8,11] However, the number of FCs seemed to be underestimated by at least 16% because of the inherent false negative rate associated with manual detection.[14]

A robust automatic scanning can greatly increase the likelihood of development of NIPD. The combination of sex-independent immunohistochemical labeling of one type of

FCs, automation, and microdissection of the FCs would also be extremely valuable to overcome the rarity of FCs in maternal blood, because sample purity would not be of utmost importance and maternal contamination would not interfere with automatic scanning. Furthermore, amplification and analysis of a few recovered FCs using arrays or DNA sequencing could provide a genome-wide picture not only for the diagnosis of major fetal chromosomal aneuploidies but also for single gene disorders.

Conclusion

Our current investigation indicates that small amounts of circulating male FCs dispersed in thousands of female cells can be detected with high specificity and sensitivity using automatic scanning. Automatic scanning is more sensitive than manual screening in the detection of rare cellular events. The detection efficiency of automatic scanning with FISH technique was superior to PRINS. Data have confirmed the reliability and accuracy of automatic scanning in the detection of FCs in maternal blood. Automatic scanning with an interactive result review requires considerably less operator time than manual scanning. In this study, the throughput was limited to an average of six slides per day per automatic scanner, but current advances in scanning technology have the potential to increase the throughput by two to five times. In summary, automatic scanning alleviates the burden of scanning large numbers of slides and allows rapid and precise detection of rare cellular events.

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Chapter III:

Evaluation of the impact of enrichment of fetal cells from maternal blood by density gradient centrifugation which was used as an initial step of enrichment in the vast majority of enrichment protocols published to date and development of an alternative version of the procedure that reduce fetal cell loss.

Article 1:

Evaluation of the impact of density gradient centrifugation on fetal cell loss during enrichment from maternal peripheral blood

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My contribution in work:

I made the experimental design, carried out all of the experiments, analysed the results and wrote the first full version of the article.

Résumé

OBJECTIF

La séparation par centrifugation sur gradient de densité est habituellement utilisée comme une étape initiale des protocoles d'enrichissement pour la purification des cellules fœtales provenant du sang maternel. Plusieurs protocoles ont été utilisés mais aucune approche n'a été assez efficace pour fournir un diagnostic prénatal non invasif (DPNI). Les procédures et méthodes sont difficiles à comparer à cause de la non-uniformité des protocoles entre les différents groupes. La récupération des cellules fœtales est mise en péril par leur perte durant le processus d'enrichissement. Toutes les pertes de cellules fœtales doivent être minimisées à cause de l'effet multiplicatif des processus d'enrichissement à plusieurs étapes. L'objectif principal de cette étude était d'évaluer la perte de cellules fœtales causée par la centrifugation sur gradient de densité.

MÉTHODES

Les cellules fœtales ont été quantifiées dans le sang périphérique de femmes enceintes de grossesses normales et aneuploïdes avant et après enrichissement par centrifugation sur gradient de densité utilisant l'Histopaque® 1,119 g/ml.

RÉSULTATS

La centrifugation sur gradient de densité cause une perte majeure de 60-80% des précieuses cellules fœtales, ce qui peut compliquer les étapes subséquentes du processus d'enrichissement. La perte de cellules fœtales peut être significativement minimisée par l'élimination des manipulations agressives comme la centrifugation.

CONCLUSION

Les données obtenues soulèvent des questions concernant le choix d'une étape de centrifugation sur gradient de densité dans l'enrichissement des cellules fœtales rares et appuient plutôt les versions non-agressives présentées ici ou la priorisation d'autres méthodes d'enrichissement.

Abstract

OBJECTIVE

Physical separation by density gradient centrifugation (DGC) is usually used as an initial step of multi-step enrichment protocols for purification of fetal cells (FCs) from maternal blood. Many protocols were designed but no single approach was efficient enough to provide non-invasive prenatal diagnosis (NIPD). Procedures and methods were difficult to compare due to the non-uniformity of protocols among different groups. Recovery of FCs is jeopardized by their loss during the process of enrichment. Any loss of FCs must be minimized because of the multiplicative effect of each step of the enrichment process. The main objective of this study was to evaluate FC loss caused by DGC.

METHODS

FCs were quantified in peripheral blood samples obtained from both euploid and aneuploid pregnancies before and after enrichment by buoyant DGC using Histopaque 1.119 g/ml.

RESULTS

DGC results in major loss of 60-80% of rare FCs, which may further complicate subsequent enrichment procedures. Eliminating aggressive manipulations can significantly minimize FC loss.

CONCLUSION

Data obtained raise questions about the appropriateness of the DGC step for the enrichment of rare FCs and argues for the use of the alternative non-aggressive version of the procedure presented here or prioritizing other methods of enrichments.

1. Introduction

A long sought goal of prenatal diagnosis has been the replacement of current invasive procedures of fetal sampling by non-invasive methods with virtually no risk to mother, fetus and pregnancy. Data generated in different laboratories led to the conclusion that fetal material (fetal cells and more recently cell-free fetal DNA) are, undeniably, present in maternal blood.[1-5] Investigators envisaged that they could provide an alternative source of fetal genetic material for non-invasive prenatal diagnosis (NIPD). Although cell-free fetal DNA was successfully used with expensive technologies like massive parallel and deep sequencing to diagnose fetal aneuploidies[6-9] and has largely eclipsed the use of purified fetal cells (FCs) in NIPD. FCs can potentially provide pure fetal genetic material and their use can consequently be easier, affordable and clinically acceptable approach for NIPD.

Considering the extreme low frequency of FCs in maternal blood and relative abundance of contaminating maternal cells,[10-12] various purification and enrichment procedures have been employed. By far the simplest was the buoyant density gradient centrifugation (DGC), which exploits the differing densities of nucleated FCs likely to be present and which separates and enriches a specific FC type from a heterogeneous cell population.[13] Since FCs, after DGC, were still dispersed between large amounts of maternal cells, further enrichment was required. Numerous methods of cell separation have been developed and various FC markers have been used in the isolation of FCs from maternal blood. Most published studies described the use of fluorescent-activated cell sorting (FACS),[14,15] magnetic-activated cell sorting (MACS),[16,17] selective maternal cell lyses[18], charge flow separation[17] and lectin base method.[19]

Over the past two decades, investigators have devised and pursued different strategies that depend on combinations of two or more successive steps of enrichments to provide efficient isolation of FCs from maternal blood. Although a number of reports describe successful enrichment of FCs, a preferred protocol has not been established.[3,15,18,20,21] All of these multi-step procedures required many manipulations in which a considerable number of FCs could be lost. Comparison of these strategies has been the subject of several reviews but comparative analysis has been limited by the fact that the

samples were being processed across the entire protocol rather than determining the efficiency and impact of each single step alone.[22-25] The numbers of FCs obtained were also too small to reflect the efficiency of an enrichment system and the initial number of target cells was never determined. Moreover, the application of enrichment protocols across varying gestational ages and the differences of the specificity of FC markers used in both enrichment and detection further complicates this analysis. It would have been more appropriate to evaluate each step alone to devise the most efficient protocol for the enrichment of FCs from maternal blood.

The main objective of this study is to evaluate FC loss during the process of DGC as the most common initial step of enrichment used in the vast majority of designed protocols published to date.[18-20,26-34] A technical obstacle, which had to be overcome, was how to know the exact number of FCs in a specific volume of maternal blood. Establishment of the total number of FCs in the maternal blood sample before and after enrichment is critical before optimising any procedure. Earlier estimates of the frequency of fetal to maternal cells have been based primarily on the DNA analysis of Y chromosome sequences using polymerase chain reaction (PCR) in male pregnancies.[11,28] These experiments gave a wide estimated range but no absolute number had been determined. In our group, Krabchi *et al.*[35-37] implied a direct detection strategy, without prior enrichment, and have offered conclusive data of the absolute number of FCs per milliliter of maternal blood using a cumbersome manual scanning approach.

We recently developed a robust detection algorithm for detection of rare cellular events using fluorescence-based automated microscopy and used the sequential spreading slide approach in the comparison of manual and automated scanning of 60 slides with pre-defined number and location of rare male cells in a pure population of female cells using fluorescence *in situ* hybridization (XY-FISH)[38,39]. We validated the manual approach of Krabchi *et al.*[35-37] and further refined its efficiency by automation. Consequently, we tested this method for the detection of FCs from the maternal blood of both normal pregnancies and Down syndrome aneuploid pregnancies.

In this study, we applied this methodology in evaluating the loss of FCs during enrichment by DGC and explored its etiology. We also presented a modified method of density gradient, which reduces FC loss to be considered in future prospective enrichment protocols.

2. Materials and Methods

2.1 Sample preparation

Peripheral maternal blood samples (10 mL) were obtained in sodium-heparinized Vacutainers (Becton Dickinson VACUTAINER Systems, Franklin Lakes, NJ, USA) from 12 pregnant women attending the High-Risk Pregnancy Clinic after obtaining written informed consent that had been approved by our institutional ethical research committee. In each case, the blood sample was divided into two portions of 1 mL and 5 ml for direct and indirect harvest respectively. White blood cells (WBCs) were counted by manual hemocytometer.

2.2 Direct harvest

For each case, 1 ml whole blood were dispensed into 250 µL aliquots in 4 tubes, washed with Hank's balanced salt solution (HBSS) or phosphate-buffered saline solution (PBS), and harvested by standard cytogenetic techniques. The cell suspensions were pooled in one microtube (equivalent of 1 mL of maternal blood) as described in Krabchi *et al*.[35-37]

2.3 Indirect harvest after density gradient

For each case, 5 mL of blood was diluted 1:1 with PBS. Each 5 mL of diluted blood was layered over 3 ml of Histopaque 1.119 g/mL (Sigma Chemical Co., St Louis, MO, USA). One tube was centrifuged at 400 g for 30 min at room temperature (RT) to eliminate non-nucleated erythrocytes and all cells at the gradient interface were recovered. The remaining plasma was aspirated, centrifuged and any pellet obtained was added to the enriched fraction. The other tube was left undisturbed for 5-6 hours and then the enriched fraction at the gradient interface was recovered in exactly the same way. The enriched fraction recovered at the gradient interface and the pellet, if any, of each tube was washed as

described before and resuspended in 1 mL PBS. Cells were enumerated by hemocytometer, and then harvested by standard cytogenetic techniques. Each cell suspension was pooled in one microtube (equivalent of 2.5 mL of maternal blood).

2.4 Spreading of MB

For each case, three sets of slides were prepared as described before[39] from the stored microtubes. One represented the direct harvest of 1 mL of maternal blood while the other two represented the cell yield obtained after density gradient of 2.5 mL maternal blood with and without centrifugation. The number of the slides per set ranged from 16 to 22 with an average number of $2.0x10^5$ cell per slide as previously described.[38,39]

2.5 FISH procedure

Slides were first aged overnight at 37°C and, then, immersed in 2xSSC at 37°C for 30 minutes. Slides were dehydrated through a series of ethanol baths (70%, 80%, 100%). Dual-color FISH was performed using probes specific for chromosomes X and Y (CEP X: spectrum orange alpha-satellite and CEP Y: spectrum green satellite-III; Vysis/ABBOTT Diagnostics, Downers Grove, Illinois, USA) diluted 1:100 and 1:300 respectively in cDenHyb-1 (Insitus Biotechnologies, Albuquerque, NM) as previously described.[38,39] The slides and the probes were co-denatured at 75°C for 5 minutes before being sealed with rubber cement and placed in a humid chamber for hybridization at 37°C for 16 hours. Coverslips were then carefully removed and the slides were washed with a solution of 0.4xSSC/0.3% NP-40 at 72°C for 2 minutes. A second wash was performed in a solution of 2xSSC/0.1% NP-40 at room temperature for 3 minutes. After a final wash with distilled water, the slides were mounted in DAPI II (0,1 M Tris pH 8.0, 90% glycerol, 1 mg/mL *p*-phenylenediamine, 0,01% 4,6-diamidino-2-phenylindole (DAPI).

2.6 Automated microscopy and cell detection

Automated scoring was performed as described before[39] using our optimized custom-made classifier of MetaCyte software (Metafer3/Metafer4 scanning platform; MetaSystems, Altlussheim, Germany).

2.7 Re-hybridization and analysis

A reverse-color FISH (re-FISH) was done for all slides with recovered cells as described in Krabchi *et al.*[35-37] to verify the identity of detected cells. Briefly, coverslips were removed by dipping the slides in a pre-warmed 2xSSC bath at 37°C for 10 minutes. Stripping of FISH probes were performed by denaturing the slides with 70% formamide/2xSSC at 73°C for 2 min 30 sec. Slides were then dehydrated in ethanol and air-dried. Finally, slides were processed through a second round of FISH procedure using the opposite fluorochrome labeling (X probe in green and Y probe in red). Slides were loaded and detected cells were relocated and examined under the microscope. Each cell is compared with its corresponding captured image for identification of true positive cells, which show reverse pattern of FISH signals. Target cells were imaged and registered as previously described.[38,39]

2.8 Statistical analysis

Multivariate ANNOVA using General Linear Model and Bonferroni correction of multiple comparisons using IPM statistical package of social science (IPM SPSS Statistics, version 20.0).

3. Results

Density gradient centrifugation (DGC) has been invaluable in the isolation of FCs from maternal peripheral blood. DGC is performed in order to collect the mononuclear cell layer potentially enriched with FCs while removing contaminating granulocytes and mature red blood cells, mostly of maternal origin. An understanding of the DGC step is essential for refining the procedure and for obtaining significant insight into devising an optimal protocol of enrichment that could provide sufficient number of FCs and lead to successful NIPD.

The original purpose of this work was to evaluate the efficiency of DGC and to make a comparison between the different gradients. In preliminary experiments, we found that the yield of FCs in the enriched fraction was less than expected relative to the original number of FCs. These findings prompted us to evaluate the FCs in the rejected fractions of

few samples (samples 1-4 in Table 1). Surprisingly, the cumulative values of the two fractions were lower than the original number of FCs by an average of 16.6% (range: 0-25%). Therefore, the objective of the study was changed to explore the impact rather than the efficiency of DGC on FC loss during enrichment from maternal blood.

To address this issue, we evaluated twelve cases of pregnancies involving male fetuses comprised of six cases with normal fetal karyotype and six with trisomy 21. The normal maternal blood samples were obtained from women attending prenatal care while the cases of trisomy 21 were taken from those referred for amniocentesis after genetic counseling for high-risk pregnancy due to abnormal marker screen or suspicious sonographic findings. For the cases of normal pregnancies, four cases were sampled at the end of the first trimester between 11th and 13th weeks while the other two were taken between 18th and 20th weeks of gestation before any invasive procedures. All of the cases of trisomy 21 were sampled between 16th and 20th week before confirmation of fetal karyotype by amniocentesis. Samples from the trisomy 21 pregnancies were collected two to four weeks after the amniocentesis and before any medications were given for termination of pregnancy. All blood samples were processed immediately after collection.

We quantified FCs of maternal samples by automatic scanning using the Y-chromosome as universal marker both before and after enrichment by density gradient using Histopaque 1.119 g/ml with and without centrifugation. FCs were considered positive if all of the following criteria were met: 1) the nuclei had two different fluorescent hybridization signals, through appropriate filters, representing X and Y chromosomes, 2) the nuclei had an intact nuclear border as indicated by DAPI staining (cells in direct contact were excluded), and 3) the recovered nuclei gave a reverse pattern of fluorescent signals by Re-FISH (Figure 1).

We also counted the total number of cells (TCs) by hemocytometer before and after enrichment by both methods. Table 1 displays all results obtained from six normal pregnancies in the 1st and 2nd trimester while Table 2 displays results of six aneuploid pregnancies with trisomy 21. All results were normalized to 10 mL maternal blood aliquots to permit comparative analysis.

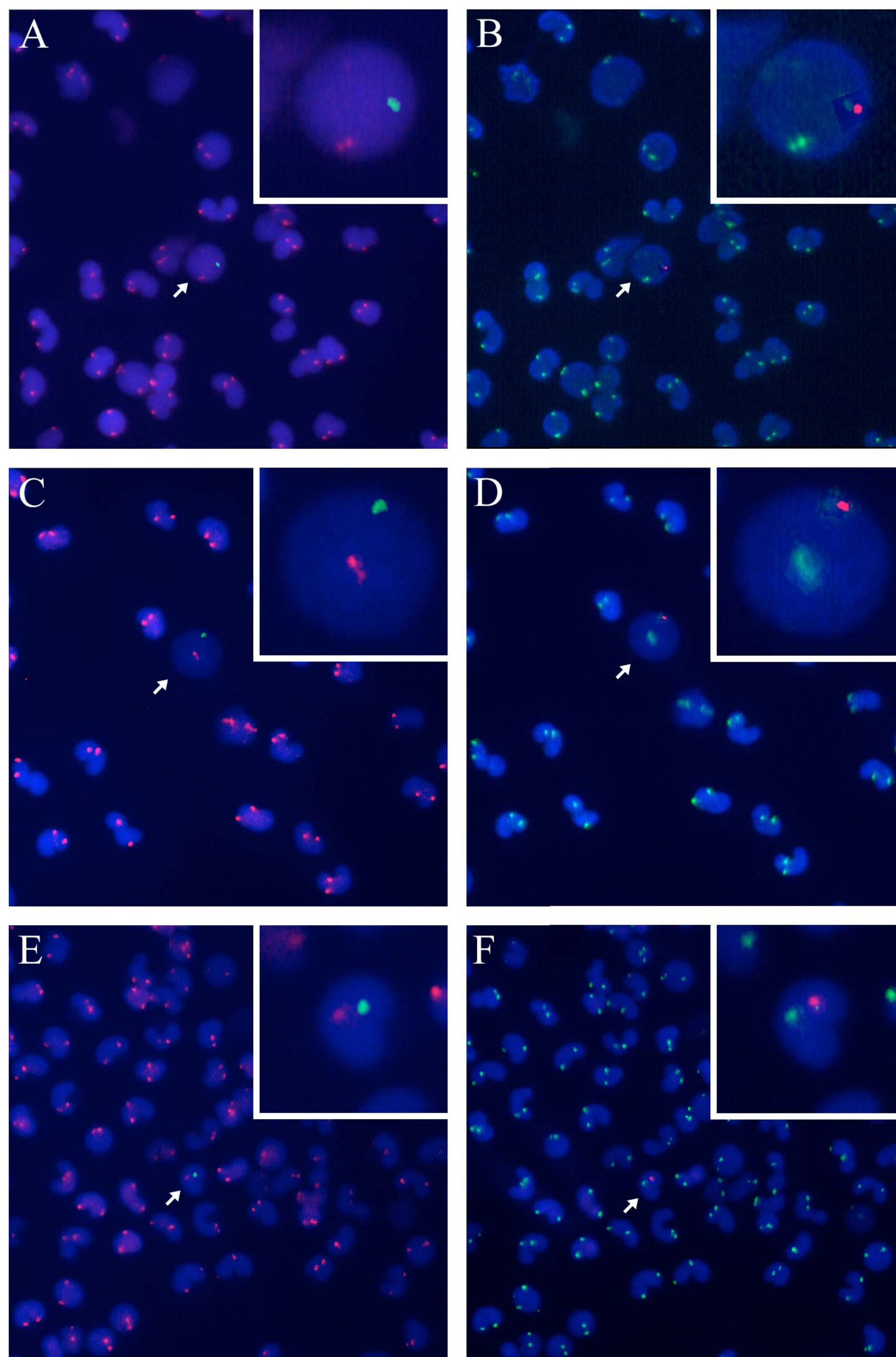

Figure 1 – Fetal cells detected by FISH (images A, C and E) and confirmed by reverse color FISH (images B, D and F) respectively.

Fetal cell detection was performed by FISH technique (Y probe in green and X probe in red) on DAPI stained nuclei with intact nuclear border as shown in images (A, C and E) and confirmed by reverse color FISH (Y probe in red and X probe in green) as shown in images (B, D and F) respectively.

Table 1 Results obtained from normal pregnancy cases (cases 1-6) normalized to 10 ml maternal blood.

Case number	**1**	**2**	**3**	**4**	**5**	**6**
Fetal karyotype	**46,XY (Normal male pregnancy)**					
Gestational age (weeks)	11w	12w	11w	13w	20w	19w
Maternal peripheral blood:						
Total cells (10 ml) x 10^6	45.4	54.2	67.6	44.8	64.2	72.4
FCs confirmed (10ml)	40	30	60	50	40	50
Histopaque 1.119 yield with centrifugation at 400 g for 30 min:						
Recovered cells (10 ml) x 10^6	20.5	28.7	30.0	20.5	28.4	30.9
Cells Depletion factor	2.21	1.89	2.25	2.19	2.26	2.34
Cell Loss (%)	54.8%	47.1%	55.6%	54.3%	55.8%	57.3%
Recovered FCs (10ml)	10	10	20	10	10	20
FCs depletion factor	4	3	3	5	4	2.5
FCs Loss (%)	75.0%	66.7%	66.7%	80.0%	75.0%	60.0%
FCs in rejected fraction (10 ml)	20	20	30	30	N/A	N/A
Histopaque 1.119 yield by gravity effect at 1 g for 5-6 hours:						
Recovered cells (10 ml) x 10^6	21.3	24.9	28.0	24.6	27.1	34.3
Cells Depletion factor	2.13	2.18	2.41	1.82	2.37	2.11
Cell Loss (%)	53.1%	54.1%	58.5%	45.1%	57.8%	52.6%
Recovered FCs (10ml)	20	10	20	20	20	20
FCs depletion factor	2	3	3	2.5	2	2.5
FCs Loss (%)	50.0%	66.7%	66.7%	60.0%	50.0%	60.0%

FCs: fetal cells. N/A: not acquired. Table shows the Gestational age by weeks, Total number of cells before and after enrichment by density gradient centrifugation with and without centrifugation, number of fetal cells recovered by scanning before and after enrichment by both methods and calculated fraction (percentage) and depletion factor of total and fetal cell loss by both methods in six cases of euploid pregnancies with normal fetal karyotype. All results were normalized to 10 ml aliquots of maternal blood to permit analysis

Table 2 Results obtained from aneuploid pregnancy cases (cases 7-12) normalized to 10 ml maternal blood.

Case number	**7**	**8**	**9**	**10**	**11**	**12**
Fetal karyotype	47,XY, +21 (Down syndrome male pregnancy)					
Gestational age (weeks)	17w	20w	18w	19w	18w	17w
Maternal peripheral blood:						
Total cells (10 ml) x 10^6	53.8	75.4	62.4	48.6	80.2	42.6
FCs confirmed (10ml)	190	140	130	150	170	210
Histopaque 1.119 yield with centrifugation 400 g for 30 min:						
Recovered cells (10 ml) x 10^6	28.8	34.7	27.4	19.1	35.5	21.8
Cells Depletion factor	1.87	2.17	2.28	2.55	2.26	1.95
Cell Loss (%)	46.5%	53.9%	56.1%	60.8%	55.8%	48.7%
Recovered FCs (10ml)	40	30	50	30	40	60
FCs depletion factor	4.8	4.7	2.6	5.0	4.3	3.5
FCs Loss (%)	78.9%	78.6%	61.5%	80.0%	76.5%	71.4%
FCs in rejected fraction (10 ml)	N/A	N/A	N/A	N/A	N/A	N/A
Histopaque 1.119 yield by gravity effect 1 g for 5-6 hours:						
Recovered cells (10 ml) x 10^6	26.6	32.2	25.9	21.8	31.8	24.5
Cells Depletion factor	2.02	2.34	2.41	2.23	2.52	1.74
Cell Loss (%)	50.5%	57.3%	58.5%	55.2%	60.3%	42.5%
Recovered FCs (10ml)	60	50	60	50	50	90
FCs depletion factor	3.2	2.8	2.2	3.0	3.4	2.3
FCs Loss (%)	68.4%	64.3%	53.8%	66.7%	70.6%	57.1%

FCs: fetal cells. N/A: not acquired. Table shows the Gestational age by weeks, Total number of cells before and after enrichment by density gradient with and without centrifugation, number of fetal cells recovered by scanning before and after enrichment by both methods and calculated fraction (percentage) and depletion factor of total and fetal cell loss by both methods in six cases of aneuploid pregnancies with trisomy 21. All results were normalized to 10 ml aliquots of maternal blood to permit analysis

The recovered cells as well as the calculated fraction and depletion factor of TC and FC loss by both methods of density gradient were compared to their original values in the maternal blood. The mean number of TCs and FCs were 59.3 ±12.9 x 10^6 (range: 42.6-80.2) and 105 ±66.4 (30-210) in maternal blood samples. The recovered TCs and FCs were 27.2 ±5.5 x 10^6 (19.1-35.5) and 27.5 ±17.1 (10-60) after DGC at 400 g for 30 min, and 26.9 ±4.1 x 10^6 (21.3-34.3) and 39.2 ±24.3 (10-90) after DGC at 1 g for 5-6 h, respectively. The number of TCs and FCs were reduced by a factor of 2.2 ±0.20 and 3.9 ±0.9 after DGC at

400 g, and 2.19 ±0.24 and 2.7 ±0.5 after DGC at 1 g, respectively. DGC at 400 g results in an average loss of 73 ±7% (ranges: 60-80%) of target FCs in comparison to 61 ±7% (50-70%) using DGC at 1 g.

The corresponding values of TCs and FCs were also obtained for each subgroup separately including 1st and 2nd trimester of normal pregnancies and 2nd trimester for cases of trisomy 21. ANOVA test was used in the analysis of multiple variants with Bonferroni correction. Both methods have insignificant reduction of TC loss (p=0.875) while extraction by DGC at 1 g resulted in a significant reduction of FC loss (p=0.002) with significant improvement of enrichment factor (p=0.005) as shown in Figure 2.

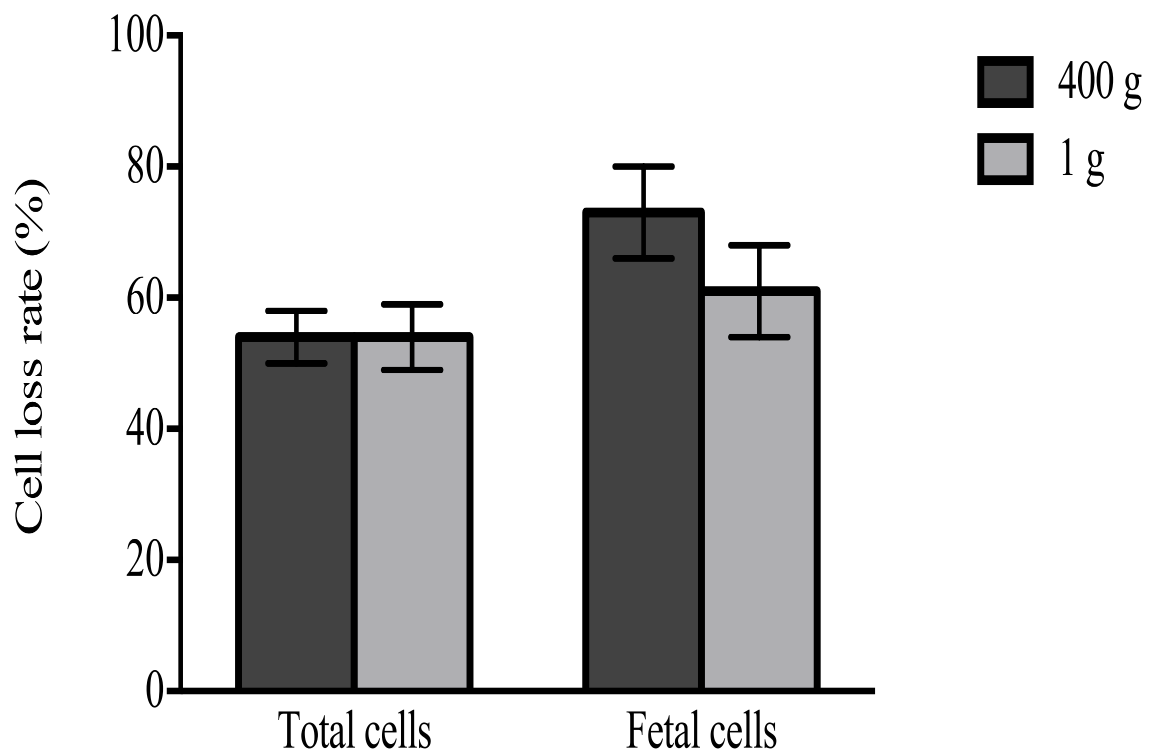

Figure 2 - Histogram comparing average and standard deviation (SD) of total and fetal cell loss.

Statistical analysis showed significant reduction of FC loss at 1 g in comparison to 400 g (P=0.002) without significant difference at the level of TC loss (P=0.875)

4. Discussion

DGC is used as a preliminary step of enrichment of FC from maternal blood in large number of papers published to date. The trend to use DGC in FC enrichment started in 1990 using Histopaque 1.077 g/ml, in a model mixture of adult blood spiked with fetal cord blood.[13] Three years later the same authors reported 25-fold enrichment with triple gradient. In fact, this represented the efficacy of the entire protocol including FACS rather than just the first enrichment step alone.[15] Since then, many groups have used single, double or triple gradient as part of their protocols.[19,31,32,34] Few comparative studies have been done. Although non-uniformity of the published protocols, there was general agreement that increased density resulted in improved FC recovery. It also resulted in an increased maternal cell contamination and subsequent sorting time.[40-42]

The experiments performed during this study were not designed to determine the efficiency but rather to evaluate the impact of enrichment by DGC on FC loss. The very low number of FCs in maternal blood does indeed demand the greatest accuracy and rigorous attention to details. It might be worth pointing out that considerable amount of cell loss during an enrichment step might not be relevant when the cells of interest are abundant and a single enrichment method is used, but is quite dramatic when the target cells are present in minimal amounts and multi-step enrichment procedures are required.

To address this issue, a proper understanding of the DGC procedure is required. When a heterogeneous cell population is centrifuged in a density gradient whose density at the bottom is greater than the density of the different cells present in the sample, the cells will float to a position in the gradient where the density of the solution is identical to that of the cells.[43] The density and volume distribution profiles of different variety of peripheral blood and bone marrow cells are available in the literature but there is still controversy regarding that of FCs, presumably because of their extreme low frequency in maternal blood, which has prevented reliable density curves from being drawn.[44] Therefore, the study of FCs densities has been guided by the aforementioned *in-vitro* model and by using adult blood mixed with cord blood to overcome this limitation.[13]

Most workers focus on nucleated red blood cells (NRBCs) and to a lesser extent on the trophoblasts as best candidates to be used in NIPD. The optimum gradient, which minimizes target cell loss but also limits contamination by maternal cells, is unclear although a general agreement was reached that the yield of FCs after DGC tends to be directly related to the used density but on the expense of the purity of the final sample.[40-42]

The NRBCs probably have bimodal distribution in both the light and the heavy portion of the gradient.[43] Furthermore, NRBCs are in a state of dynamic change and cells progressively become smaller with a lower cytoplasmic to nuclear ratio with maturation.[45] This variability causes them to sediment over a wide density range with more than 80% overlapping with maternal cells at a given density. Most NRBCs settled between 1.077 and 1.110 g/mL,[16,20] whereas the trophoblasts were found to settle mainly between 1.051 and 1.064 g/mL.[46,47] Narrowing the 'density window' was found to reduce the final yield but increase the cell purity.

Since almost all nucleated cells present in human peripheral blood and bone marrow are lighter than 1.090 g/ml,[44] the use of a very high density cut off, such as 1.119 g/ml, should allow the recovery of all FCs present in maternal blood, but without any enrichment.[48] This is because all nucleated cells are equally retrieved.[48] This is the rational behind using this density gradient for the design of experiments performed during this investigation to determine the impact of DGC on FCs.

In this study, we provide evidence that the application of DGC for isolation of rare FCs from maternal blood is associated with a major loss of 60-80% of target FCs with an average of 73% in both normal and aneuploid pregnancies. Theoretically, loss of FCs at this high density cut off is usually due to entrapment of FCs between mature red blood cells that aggregate at the bottom of the tube when they come in contact with the gradient solution. Another possible explanation is that part of the cells could be lost as a consequence of the cytotoxicity of the density gradient medium. These latter findings are consistent with those of Choolani and his group[45] who exclusively studied the buoyant densities of first-trimester fetal nucleated erythroblasts enriched from fetal blood samples. They observed that, even with the most commonly used gradients for FC enrichment from maternal samples such as

Ficoll 1.077 g/mL and 1.119 g/ml, 91.4% and 68.3% of NRBCs settle down, respectively, in the erythrocyte pellet at the first-step DGC process. Interestingly, they found also that the median recovery of fetal NRBCs from mixtures in maternal blood was superior with Percoll 1.118 g/mL with 64.1% recovery as compared to 35.3% using Ficoll 1.119 g/mL which is probably due to a difference in the density medium cytotoxicity rather than to the gradient itself.[45]

We also hypothesized that a considerable number of FCs could be lost by aggressive manipulation during the DGC procedure. In order to explore this possibility with our samples and because centrifugation is not absolutely necessary so that most cells, given enough time, will eventually settle down by gravity at 1 g, we replicated each experiment on the same gradient by replacing vigorous centrifugation with 5-6 h of vertical incubation. Such a simple modification in the procedure significantly reduced the average FC loss by 12% so that the final range was between 50-70% and without significant reduction of TC yield. We think that the process of DGC preferentially affects rare FCs more than the cells of maternal origin. Interestingly, it has been shown that a significant number of FCs undergo apoptosis and thus may become fragile and liable to rupture with time.[49-51] We believe that not only time but also aggressive manipulations affect these rare cells. This could be a major drawback of many multi-step enrichment protocols employed in FC purification. It is worth pointing out that the low number of cells retrieved using a three-step enrichment protocol could be due to the application of an inappropriate step resulting in a major FC loss or due to too many steps being involved in the enrichment process. Besides, the enrichment procedures are time-consuming which has an additive effect on the loss of the fragile FCs.

We also tested the hypothesis that the process of DGC preferentially affects the FCs of aneuploid pregnancies. We did not find a significant difference of the percentage of FC loss either between that of first and second trimester samples of normal pregnancies or between normal and aneuploid pregnancies. This argues for the fact that the FC loss is merely related to the enrichment procedure rather than to the status of the FCs. Additionally, it is possible that the bone marrow is stressed to produce more NRBCs in aneuploidies but these cells are physiologically the same as those found in a normal pregnancy.

5. Conclusion

Results obtained in this study have many implications in the context of NIPD. Mechanical separation by DGC has received great attention in the purification of FCs from maternal blood, despite the fact that this procedure is associated with a significant loss of 60-80% of rare FCs, which could probably complicate subsequent enrichment procedures. Since DGC is usually followed by other methods of enrichments, further loss would be expected and, thus, the eventual number of FCs available could be insufficient for genetic diagnosis.

In addition, DGC results in only a slight enrichment of fetal NRBCs due to the broad density distribution profile of FCs. Putting all of this together, this raises questions about the appropriateness of DGC for the enrichment of rare events. On the other hand, the efficiency of the density gradient on FC enrichment could be improved by testing different gradients, densities and conditions. Alternatively, the elimination of this step or its replacement by an immune-sorting strategy would enhance recovery of rare FCs and cut down the number of enrichment steps and, consequently, reduce FC loss.

Furthermore, automation and micromanipulation would be of great help, as purity in that case would not be of utmost importance for clinical applicability and contamination with maternal cells would not interfere with automatic scanning. However, such automated diagnostic techniques would need to be prospectively evaluated in proper clinical trials before being offered as a diagnostic service.

In the light of results obtained in this study, our initial conception about the frequency of the FCs in maternal blood has been changed. We now believe that the real number of FCs is probably much higher than what has been published. The manipulations used in cytogenetic harvesting of cells are likely more aggressive than that of DGC. However, this does not affect the result of this study as the same type of manipulation is used to assess the frequency of the FCs before and after their enrichment by DGC.

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Chapter IV:

The purpose of this study was to assess the feasibility of using single fetal cells to determine fetal sex and major chromosomal abnormalities by quantitative fluorescence-polymerase chain reaction (QF-PCR) as a proof of conception of the feasibility of fetal cells in non-invasive prenatal diagnosis.

Article 1:

Rapid aneuploidy detection of chromosomes 13, 18, 21, X and Y using QF-PCR with few microdissected fetal cells.

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This article is in press in the *Journal of Fetal Diagnosis and Therapy* (Emad et al., 2014a).

My contribution in work:

I made the experimental design, carried out most of the experiments, analysed the results and wrote the first full version of the article.

Résumé

OBJECTIF

L'analyse d'évènements cellulaires rares peut apporter de nouvelles approches dans le diagnostic en oncologie et en diagnostic prénatal. La petite quantité d'ADN disponible dans des cellules qui sont rares comme les cellules fœtales circulant dans le sang maternel, est un facteur majeur limitant pour leur utilisation dans des applications cliniques. Les méthodes traditionnelles d'amplification du génome complet à partir d'un très petit nombre de cellules (SC-WGA) et leur analyse adéquate ont représenté un immense défi jusqu'à récemment. L'objectif de cette étude a été d'évaluer la faisabilité de la technique de PCR quantitatif en fluorescence (QF-PCR) à déterminer les anomalies chromosomiques à partir de seulement quelques cellules fœtales.

MÉTHODOLOGIE

Des cellules en culture provenant de 24 échantillons de liquide amniotique ont été utilisées pour une extraction standard d'ADN et à partir de cinq (5) cellules fœtales récupérées par microdissection. L'amplification du génome complet a été effectuée à partir de l'ADN extrait des cellules micro-disséquées. L'amplification par PCR des séquences courtes répétées en tandem spécifiques pour les chromosomes 13, 18, 21 ainsi que pour les chromosomes sexuels a été effectuée sur les échantillons amplifiés et extraits de façon standard. Le dosage des allèles et le sexage ont été analysés quantitativement après la séparation des fragments par électrophorèse capillaire.

RÉSULTATS

L'analyse des microsatellites amplifiés par QF-PCR a montré une forte corrélation du nombre de chromosomes entre les ADN extraits ou amplifiés quand cinq cellules et plus sont utilisées comme matériel de départ. Les résultats concordent avec les analyses cytogénétiques conventionnelles.

CONCLUSION

Il est possible d'obtenir une bonne couverture génomique à partir du SC-WGA, ce qui permet d'utiliser cette technique dans des applications où une quantité infime d'ADN est

disponible. Dans un contexte clinique, cette amplification associée à la technique QF-PCR peut représenter une méthode fiable, précise, rapide et peu coûteuse de détection des anomalies chromosomiques majeures chez le fœtus.

Abstract

OBJECTIVE

Analysis of DNA from small numbers of cells, such as fetal cells in maternal blood, is a major limiting factor for their use in clinical applications. Traditional methods of single-cells whole genome amplification (SCs-WGA) and accurate analysis have been challenging to date. Our purpose was to assess the feasibility of using a few fetal cells to determine fetal sex and major chromosomal abnormalities by quantitative fluorescent-polymerase chain reaction (QF-PCR).

METHODS

Cultured cells from 26 amniotic fluid samples were used for standard DNA extraction and recovery of five fetal cells by laser-capture microdissection. SCs-WGA was performed using the DNA from microdissected cells. PCR amplification of short-tandem repeats specific for chromosomes 13, 18, 21, X and Y chromosomes was performed on extracted and amplified DNA. Allele dosage and sexing were quantitatively analyzed following separation by capillary electrophoresis.

RESULTS

Microsatellite QF-PCR analysis showed high concordance in chromosomal copy number between extracted and amplified DNA when 5 or more cells were used. Results were in concordance with that of conventional cytogenetic analysis.

CONCLUSION

Satisfactory genomic coverage can be obtained from SCs-WGA. Clinically, SCs-WGA coupled with QF-PCR can provide a reliable, accurate, rapid and cost-effective method for detection of major fetal chromosome abnormalities.

1. Introduction

The detection and molecular characterization of rare cellular events was first suggested more than one century ago [1], but has only recently become a clinical reality. It is now clear that these cells can provide novel approaches for cancer management, preimplantation genetic and non-invasive prenatal diagnosis (NIPD) [2-6].

Current prenatal diagnosis depends on invasive procedures and is associated with small but finite risk of pregnancy loss. The use of circulating fetal cells in maternal blood as an alternative source of fetal genetic material can provide a non-invasive, risk-free opportunity for prenatal diagnosis [7-9].

On a parallel context, occult shed tumor cells can travel through the blood to distant sites. Detection, monitoring, and molecular analysis of these cells will provide a powerful approach for detection and targeting of metastatic cells. It will also help in addressing relevant topics such as early detection of recurrence and minimal residual disease [10-12].

Molecular characterization of rare cellular events is also essential in pre-implantation genetic diagnosis (PGD), which combines assisted reproductive technologies with genetic analysis of single cells, enabling the screening of embryos prior to their maternal transfer to avoid transfer of embryos affected with a specific genetic disease [3,13]. Furthermore, forensic medicine benefits from advanced single-cell technologies, as often only tiny amounts of material are available for analyses [14].

Many methods have been proposed for the detection of rare cellular events including flow cytometry and immunofluorescence microscopy [15-18]. Interphase fluorescent *in situ* hybridization (FISH) is a commonly used single-cell approach that allows visualization of chromosomal regions in the nucleus. This technology critically depends on the selection of probes and does not provide a screen of the entire genome within individual cells. In addition, the percentage of chromosomal anomalies correctly identified by FISH could be as low as 65% [19,20].

As an alternative to FISH, molecular characterization can be done using single cell based amplification approach to obtain satisfactory genomic coverage followed by an appropriate molecular diagnostic technique. Single cells-based whole genome amplification (SCs-WGA) has the goal of generating enough quantities of genomic DNA from small amounts of starter material with minimal representational bias. The protocols usually include an optimized cell lysis protocol incorporated into the fragmentation step in addition to the universal primers for whole genome amplification. The term “single cells whole genome amplification” SCs-WGA is used for protocols developed for entire genome amplification from few numbers of cells and not necessary one single cell. Further downstream, this enables robust molecular characterization and detection of chromosomal abnormalities [21,22].

Although different types of chromosomal abnormalities have been successfully identified by array-based comparative genomic hybridization (aCGH) following WGA of a small number of cells, the current aCGH protocol is a costly and time-consuming process that does not fit easily into all clinical schedules, particularly if specimens are required to be shipped to a reference laboratory [23-27]. Looking for an alternative approach that is clinically practical and has the potential to detect chromosomal abnormalities and single gene disorders, the present study focuses on evaluating the fidelity of WGA using DNA obtained from a small number of fetal cells for rapid and cost-effective prenatal diagnosis by multiplex QF-PCR [28]. This study, however, will not include characterization of single gene disorders although this is potentially possible.

We recently developed a robust detection algorithm for rare cellular events using fluorescence-based automated microscopy [29] and validated its efficiency in the detection of rare fetal cells in maternal blood (unpublished data). In this study, we provide a proof-of-concept of the feasibility of using a few fetal cells in NIPD. A small number of unfixed amniotic fetal cells obtained by laser microdissection were used for the detection of fetal sex and major fetal chromosomal aneuploidies using GenomePlex SCs-WGA technology (Sigma, Oakville, Ontario, Canada). This technology is based upon the generation of library molecules flanked by universal priming sites from non-enzymatic random fragmentation of

limited amounts of genomic DNA. This was followed by amplification using universal oligonucleotide primers through limited number of cycles to generate sufficient quantities of representative DNA suitable for varieties of downstream reactions. We then validated the DNA fidelity on QF-PCR using multiplex fragment analysis. The results were compared with that of extracted unamplified DNA and standard conventional cytogenetic analysis.

2. Materials and Methods

2.1 Sample preparation

The Ethics Research Committee of the CHUS (Centre Hospitalier Universitaire de Sherbrooke) approved the protocol used in this study. The amniotic fluid was obtained after informed consent from 26 patients by amniocentesis in the second trimester between the 16th to the 20th week of gestation. Immediately after sampling, the amniotic fluid samples were cultured then harvested by standard techniques to obtain the fetal karyotype. Cells derived from the initial culture were centrifuged at 700 g for 10 min at 20°C and washed three times in PBS before being transferred to a membrane-coated slide for microdissection. The spreading was done in a modified Thermotron environmental control chamber (CDS-5) (Thermotron, Amsterdam, the Netherlands) at 25°C and 40% humidity. Slides were dried within the Thermotron chamber, encoded and used immediately or stored at −20°C. In all cases, ordinary DNA extraction and cytogenetic analysis were done according to standard protocols. Slides were encoded and cells dissected blindly.

2.2 Laser capturing microdissection

Laser capture microdissection (LCM) was performed using a microscope equipped with a motorized scanning stage and a CCD camera. Membrane-coated slides were screened using a phase-contrast microscope for identifying cells of interest by morphology. The laser beam was adjusted to cut a circle in the membrane bearing the cell of interest with an adequate safety distance. The desired sample was collected using a LCM Eppendorf tube with a special collection cap covered with an adhesive layer. The dissected material was lifted directly into the cap and the tube was carefully removed and closed. The contents of the tube were used immediately or stored at −20°C.

2.3 Whole genome amplification

For WGA, genomic DNA samples were amplified using the GenomePlex SCs-WGA kit (Sigma) according to the manufacturer's protocol. After amplification, excess primers and dNTPs were removed using GenElute PCR Clean-Up Kit (Sigma) resulting in an average volume of 50 µl of DNA. Five µl of the PCR product could be checked on a 1.5% agarose gel. Reamplification was an optional step that could have been done if needed. The samples were stored at −20°C until further processing.

2.4 QF-PCR analysis

QF-PCR was carried out using a Multiplex PCR Master Mix (Qiagen, Toronto, Ontario, Canada) and a Primer Mix 10X designed for the identification of aneuploidy of chromosomes 13, 18, 21, X and Y (table 1). The standard Primer Mix contains 17 polymorphic markers (four for chromosomes 13, 18 and 21; five for sex chromosomes including the SRY gene). Specific multiplexes for each chromosome (13, 18, 21 and X/Y) were also designed to confirm detection of aneuploidies in the samples (table 2). Forward primers were labeled with Well-Red D2, Well-Red D3 or Well-Red D4 (Beckman Coulter, Mississauga, Ontario, Canada). Amplified fragments were separated by capillary electrophoresis using the GenomeLab GeXP (Beckman Coulter) as described by the manufacturer. Results were finally analyzed using the GeneMarker software version 1.97 (SoftGenetics, State College, PA). The ratio between the peak heights was calculated for each marker. We assigned ratios between 0.8 and 1.4 as a normal peaks and values greater than 1.8 or less than 0.65 as abnormal. Normal heterozygous subjects are expected to show two peaks (1:1 peak ratio) while trisomies are visualized either as three peaks with ratios between 0.8 and 1.4 (triallelic) or two peaks with a ratio greater than 1.8 or less than 0.65 (2:1 peak ratio). A single homozygous peak as well as multiple small peaks were considered uninformative. A minimum of two informative markers is required for confident diagnosis.

Results obtained by QF-PCR of WGA-DNA from each specimen were analyzed, then decoded and compared with that obtained by standard DNA extraction as well as the fetal karyotype obtained by cytogenetic analysis of amniotic cells from the same sample.

Table 1 Polymorphic markers used in the Primer Mix 10X

Marker name	Location	Heterozygosity	Allele size range (bp)	Primer sequences (5->3; forward, reverse)
AMXY	Xp22.1-22.31 Yp11.2	-	106-112	D2-CCCTGGGCTCTGTAAAGAATAGTG ATCAGAGCTTAAACTGGGAAGCTG
D13S252	13q12.2	0.64	280-308	D2-GCAGATGTACTGTTTTCCTACCAA AGATGGTATATTGTGGGACCTTGT
D13S305	13q12.2-q14.1	0.75	430-465	D3-GCCTGTTTGAGGACCTGTCGTTA TGGTTATAGAGCAGTTAAGGCAC
.D13S628	13q31.1	0.70	324-369	D4-TAACATTCATTGTCCCTTACAGAT GCAAGGCTATCTAACGATAATTCA
D13S634	13q21.33	0.85	385-440	D4-GGCAGATTCAATAGGATAAATAGA GTAACCCCTCAGGTTCTCAAGTCT
D18S1371	18q12.3	0.87	130-158	D3-CTCTCTTCATCCACCATTGG GCTGTCAGAGACCTGTGTTG
D18S386	18q22.1	0.89	330-440	D2-TGAGTCAGGAGAATCACTTGGAAC CTCTTCCATGAAGTAGCTAAGCAG
D18S391	18p11.31	0.75	182-220	D4-GGACTTACCACAGGCAATGTGACT TAGACTTCACTATTCCCATCTGAG
D18S535	18q12.3	0.82	455-500	D4-CAGCAAACTTCATGTGACAAAAGC CAATGGTAACCTACTATTTACGTC
D21S1411	21q22.3	0.93	256-340	D3-ATAGGTAGATACATAAATATGATGA TATTAATGTGTGTCCTTCCAGGC
D21S1435	21q21.2	0.75	170-210	D2-CCCTCTCAATTGTTTGTCTACC ACAAAAGGAAAGCAAGAGATTTCA
D21S1437	21q21.1	0.78	112-126	D4-ATGTACATGTGTCTGGGAAGG TTCTCTACATATTTACTGCCAACA
D21S226	21q21.3	0.54	440-470	D2-GCAAATTTGTGGATGGGATTAACAG AAGCTAAATGTCTGTAGTTATTCT
DXYS218	Xp22.32 Yp11.3	0.65	218-254	D2-TGTGTTTGGGTTTCCTCTGT CGAAACTCCGTCTCAAAATA
HPRT	Xq26.2	0.75	263-299	D4-ATGCCACAGATAATACACATCCCC CTCTCCAGAATAGTTAGATGTAGG
SRY	Yp11.2	-	470	D3-GAATATTCCCGCTCTCCGGA GCTGGTGCTCCATTCTTGAG
X22	Xq28 Yq	0.91	194-238	D3-TCTGTTTAATGAGAGTTGGAAAGAAA ATTGTTGCTACTTGAGACTTGGTG

The table shows the polymorphic markers used in the Primer Mix 10X by QF-PCR including: the marker name, location, degree of heterozygosity, allele size and primer sequence for each marker.

Table 2 - Polymorphic markers used in the chromosome-specific primer mix

Marker name	Location	Heterozygosity	Allele size range (bp)	Primer sequences (5->3; forward, reverse)
D13S258	13q21	0.89	230-267	D3-ACCTGCCAAATTTTACCAGG GACAGAGAGAGGGAATAAACC
D13S317	13q22-13q31	0.79	157-201	D2-ACAGAAGTCTGGGATGTGGA GCCCAAAAAGACAGACAGAA
D18S1002	18q11.2	0.80	340-370	D3-GTTTGATGGGAGGAAGCTATCTAT GTGAAGTAGCGGAAGGCTGTAAT
D18S847	18q21.1	0.76	208-229	D3-TTAAATTTGACTCTGAGAGTTCTCC CAGATGGCCTGTAGTGGAAC
D18S499	18q21.32-21.33	0.72	260-300	D4-AGATTACCCAGAAATGAGATCAG GCTCCATAAGCCAAATAGAGTCAA
D21S1270	21q22.11	0.87	285-340	D4-CTATCCCACTGTATTATTCAGGGC TGAGTCTCCAGGTTGCAGGTGACA
DXS981	Xq13.1	0.86	230-260	D3-CTCCTTGTGGCCTTCCTTAAATG TTCTCTCCACTTTTCAGAGTCA
DXS6803	Xq21.21	0.68	111-180	D2-GAAATGTGCTTTGACAGGAA CCTCAAAACAAAAAGGAACAT
DXS6854	Xq25	0.73	90-125	D4-AGCACTTCTCCTACAACCCTC CAGCCTGGGCAGTAGAGA CT
DYS448	Yq11.2	0.69	350-380	D2-CAAGGATCCAAATAAAGAACAGAGA GGTTATTTCTTGATTCCCTGTG

The table shows the polymorphic markers used in the chromosome-specific primer mix by QF-PCR including: the marker name, location, degree of heterozygosity, allele size and primer sequence for each marker.

3. Results

We provide below a detailed protocol from cell preparation to single-cell isolation by laser microdissection and WGA to generate templates for QF-PCR analysis (figure 1). Initially, we tested WGA products from 1, 5, 10 and 20 cells and their diluted human genomic DNA-equivalent templates by agarose gel electrophoresis. All samples gave distinct patterns on an agarose gel (figure 2), which resulted in successful WGA in all titration experiments. The smears ranged from 100 to 1,500 bp with a mean size of 400 bp.

Negative controls did not produce any smear. Although we saw amplification on the agarose from a single cell, satisfactory genomic coverage by QF-PCR was only obtained in 33.3% of cases in contrast to more than 90% when five or more cells were used as templates on repeated trials. Indeed, lower amounts of DNA (<5 cells) will generate amplification products, but results obtained in downstream applications may not be faithful to the result that would have been obtained from non-amplified DNA. The DNA yield after WGA from 5 cells obtained by laser-capture microdissection was in the range of 4 to 5 μg whereas the DNA yield after reamplification of a primary PCR product was in the range of about 6 to 8 μg. The DNA yield increased exponentially with increased numbers of cells used as a template, reaching 11 to 12 μg when 20 cells or their equivalent diluted gDNA were used. These amplification products were also suitable for conventional and array CGH (data not shown). Although we obtained some satisfactory results using conventional CGH, especially when an amplified product was used as a reference, the process was very cumbersome and time consuming. The objective of this study was to develop a better – and clinically practical – approach to avoid the inherent inconveniences of array CGH.

We tested the feasibility of using the multiplex QF-PCR and its compatibility with WGA products from controlled number of cultured amniotic fetal cells (5 cells) obtained by laser-capture microdissection (figure 3). Amniocytes were obtained after approximately 2-3 weeks of culture with up to 3 passages and amount of viable cells >80%. In the current study, we used cultured amniocytes identified by morphology for the detection of fetal sex and major chromosomal abnormalities but there is no reason why this protocol would not work with uncultured cells or with cells identified by antibody staining or immunohistochemistry, although this was not done here.

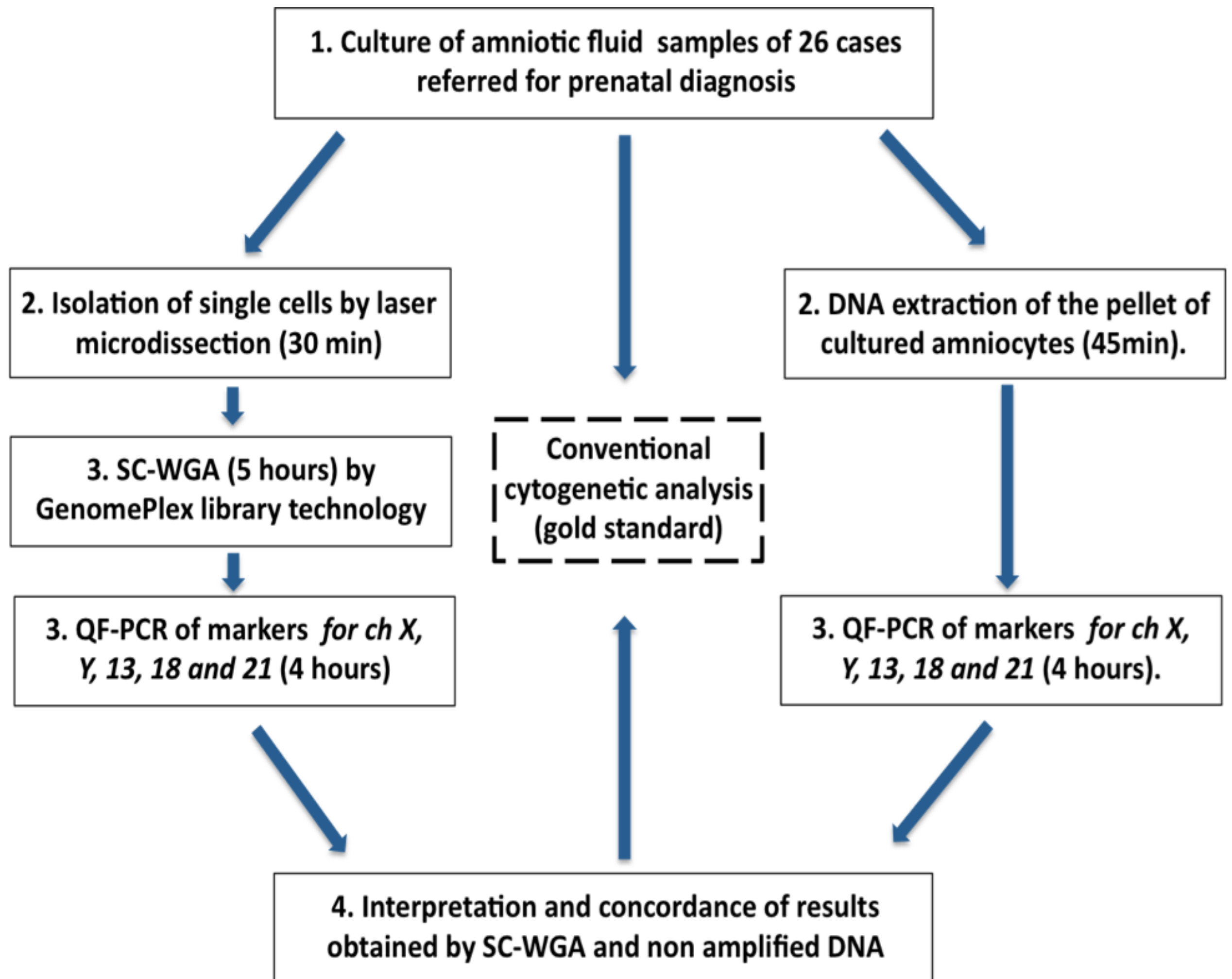

Figure 1 Flow chart of the entire methodology.

Protocol include cell preparation, single-cell isolation by laser microdissection, WGA to generate templates for QF-PCR analysis and comparison of results obtained by GenomePlex SCs-WGA and non-amplified DNA with conventional cytogenetic analysis.

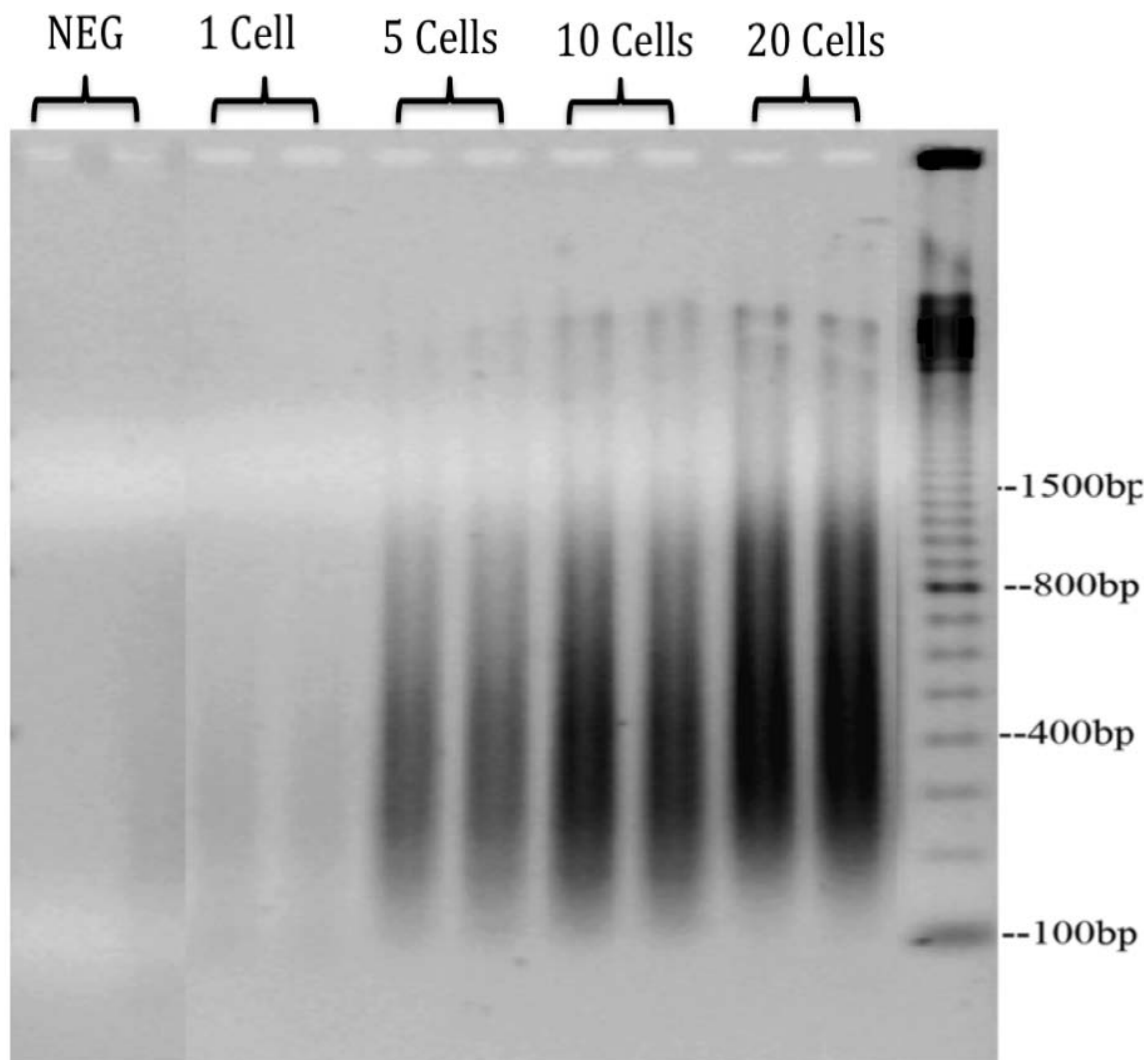

Figure 2 Comparison of DNA yield obtained by whole genome amplification of serial numbers of single cells.

Whole genome amplification was performed on 1, 5, 10 and 20 isolated cells in comparison with a negative control in duplicate experiments using the GenomePlex SCs-WGA. The obtained DNA fragments were separated by electrophoresis on 1.5% agarose, coloured with ethidium bromide, exposed to UV and photographed.

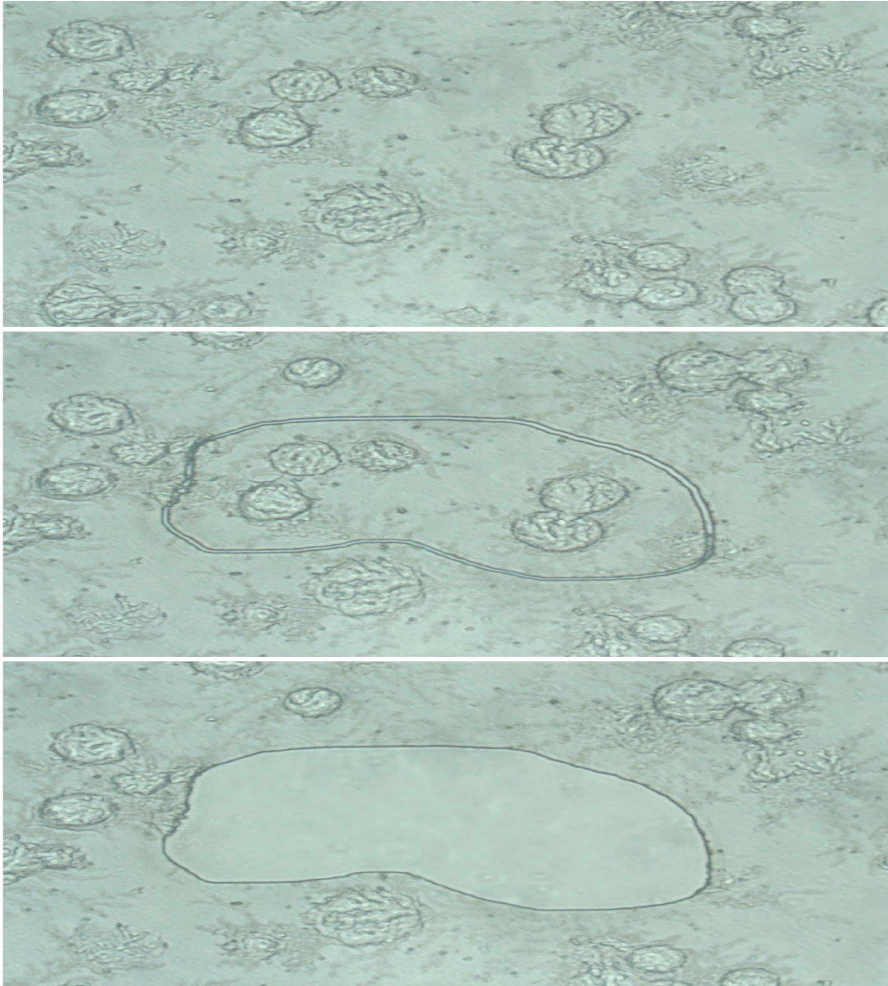

Figure 3. The process of Laser-capture microdissection of single cells on the slides.

The figure shows the process of laser-capture microdissection: Selection of single cells (5 cells) before microdissection (higher panel), laser microdissection (middle panel) and after the recuperation of microdissected cells (lower panel).

The experimental design involved evaluation of 26 samples obtained for this study. Among these samples, only 2 failed to generate sufficient WGA DNA materials for subsequent QF-PCR analysis apparently due to the bad quality of the original cellular DNA on the slides on repeated trials. A second attempt of SCs-WGA has been needed for 5 cases to generate efficient DNA amplification and successful QF-PCR analysis. Chromosomal anomalies were detected in 8 out of 24 samples comprising six trisomies and two triploidies (table 3).

Table 3 Results obtained by QF-PCR of amplified and extracted DNA in comparison to standard chromosomal analysis of fetal Karyotype.

Patient ID	*Fetal Sex*	*Fetal Karyotype*	*QF-PCR standard extracted DNA*	*QF-PCR SCs-WGA*
1	Female	T21	OK	OK
2	Male	Normal	OK	OK
3	Female	Normal	OK	OK
4	Female	Normal	OK	OK
5	Male	Normal	OK	OK
6	Male	Normal	OK	OK
7	Male	Normal	OK	OK
8	Male	T21	OK	OK
9	Male	T21	OK	OK
10	Female	Normal	OK	OK
11	Female	Normal	OK	Failed
12	Male	Normal	OK	OK
13	Female	Normal	OK	OK
14	Male	Normal	OK	OK
15	Male	Normal	OK	OK
16	Male	T21	OK	OK
17	Female	Normal	OK	OK
18	Female	Normal	OK	Failed
19	Male	T13	OK	OK
20	Male	Normal	OK	OK
21	Female	Normal	OK	OK
22	Female	Normal	OK	OK
23	Female	Triploidy	OK	OK
24	Female	Triploidy	OK	OK
25	Male	Normal	OK	OK
26	Male	T21	OK	OK

The table shows comparison of the QF-PCR results obtained from whole genome amplification of 5 microdissected cells and that obtained by standard DNA extraction of fetal cells from amniotic fluid culture in comparison to the results of standard analysis of fetal karyotype for fetal sex and major chromosomal aneuploidies of the 26 patient samples.

The normal cases consisted of 9 cases with a normal male karyotype (46,XY) and 9 with a normal female karyotype (46,XX). The trisomies involve 5 cases of chromosome 21 and one of chromosome 13. Four trisomy samples showed a male pattern with trisomy 21 (figure 4) while one showed a female pattern with trisomy 21 and the other one showed a male pattern with trisomy 13. Two female samples showed a triploid pattern (figure 5).

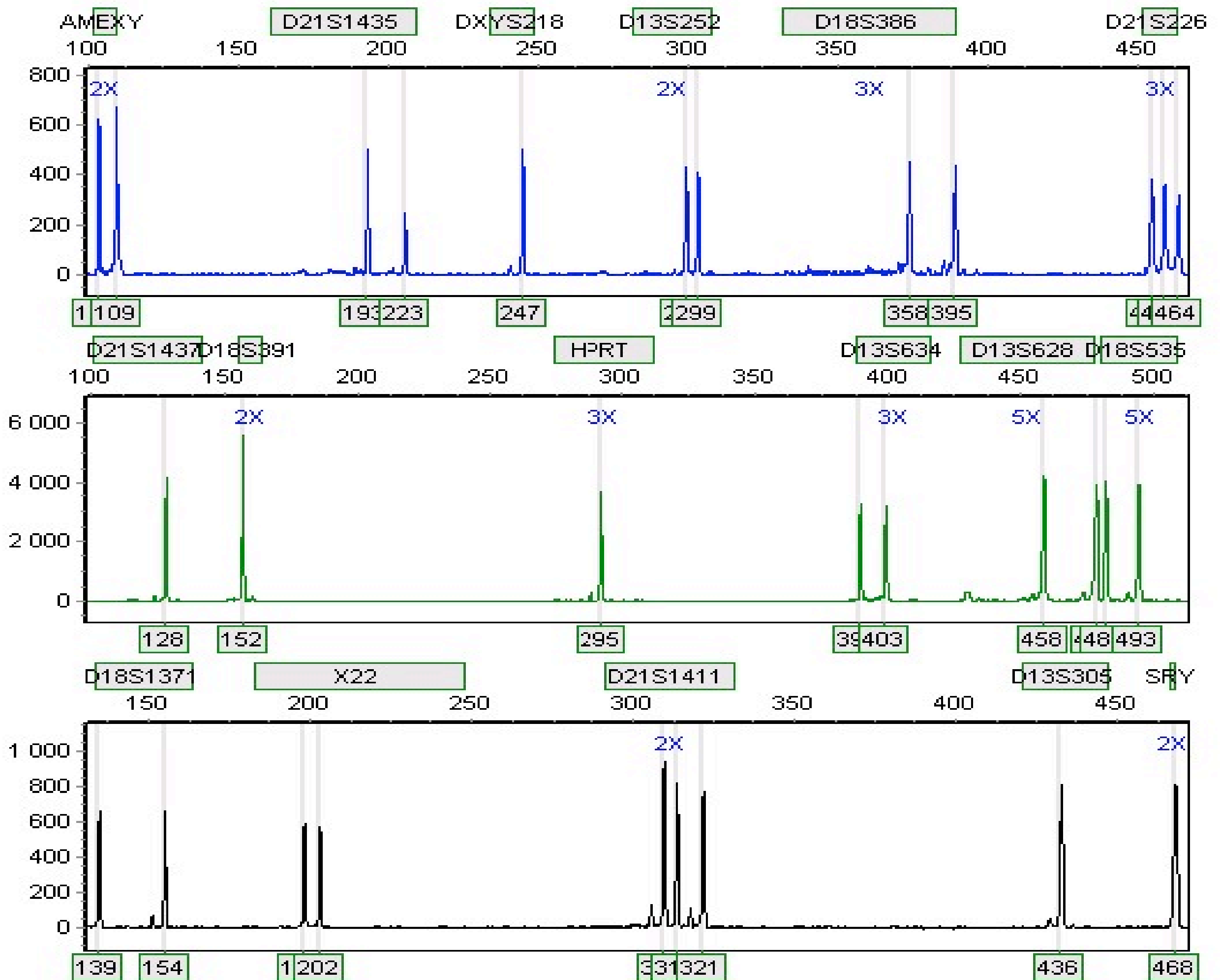

Figure 4. The QF-PCR profile of a male trisomy 21 from amplified DNA from 5 microdissected cells.

The figure show the analysis of whole genome amplified DNA from 5 microdissected cells by QF-PCR of a case of male trisomy 21: D21S226 and D21S1411 markers show triallelic trisomic peaks, D21S1435 shows diallelic trisomic peaks whereas the D21S1437 marker shows a single peak. The fetal sex was confirmed by the presence of a peak for SRY gene.

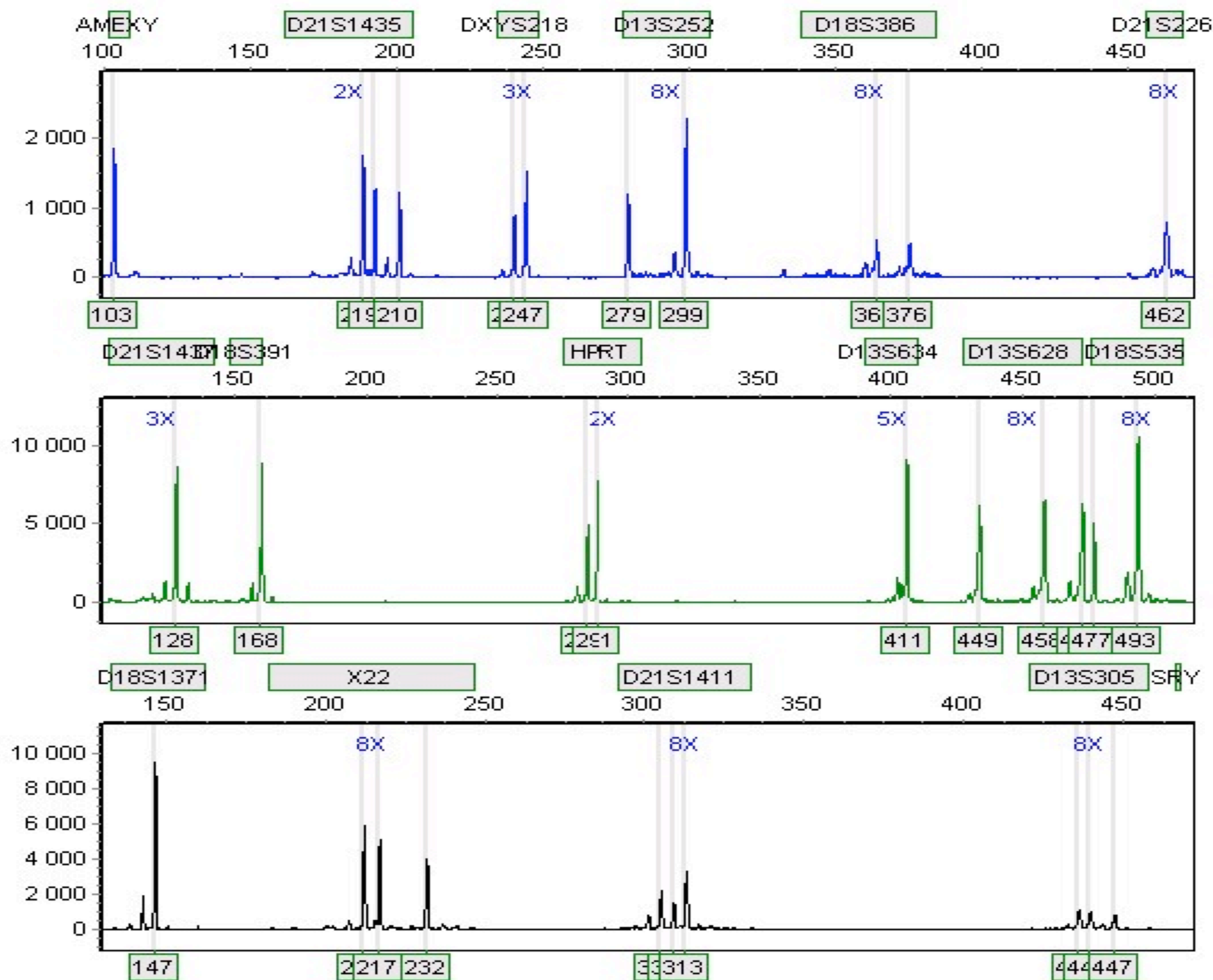

Figure 5 The QF-PCR profile of a female triploidy case from amplified DNA from 5 microdissected cells.

The figure show the analysis of whole genome amplified DNA from 5 microdissected cells by QF-PCR of a case of triploid female fetus: D21S1435 and D12S1411 markers for chromosome 21 show triallelic peaks, D13S305 and D13S628 markers for chromosome 13 present triallelic peaks whereas D13S252 shows a diallelic trisomic peak. For sexual chromosomes, absence of peak for SRY with triallelic peaks for X22 and diallelic trisomic peak for DXYS218 and HPRT confirm the female sex.

All but 2 samples were successfully diagnosed by QF-PCR and the results were available within 48 hours, and were in concordance with QF-PCR results of extracted unamplified DNA as well as the traditional karyotyping of cultured amniotic cells (table 4). Fetal sexing was correctly reported for all the female samples with absence of the unique sequence of SRY gene. In some samples with a lower DNA quality, the detection of a single sequence was difficult and therefore, 5 of normal male samples were further confirmed with the DYS448 which is a polymorphic marker with up to 20 repeats. Aneuploidies were confirmed by the replication of QF-PCR tests as well as by using back-up markers to increase the level of confidence. There were no reported sex chromosome aneuploidies or trisomy 18 within this panel. However, in the initial stage of validation of the STRs markers used in this study, we successfully confirmed the diagnosis of 2 sex chromosome aneuploidies (data not shown).

Table 4 Cumulative results obtained from QF-PCR analysis of amplified DNA from single cells for determination of fetal sex and major chromosomal aneuploidies.

Karyotype	*Sex*	*Cases (n)*	*Confirmed (n)*	*Failed (n)*
Normal	Female	9	7	2
Normal	Male	9	9	-
T13	Male	1	1	-
T21	Female	1	1	-
T21	Male	4	4	-
Triploidy	Female	2	2	-

The table shows the cumulative results obtained from whole genome amplification of 5 microdissected cells by QF-PCR to correctly identify the fetal sex and major chromosomal aneuploidies for prenatal diagnosis

4. Discussion

Clinical practice is largely dependent on what has been made available by improvements in technology and by the availability of its delivery to large segments of the population. Although a variety of applications in research and clinical diagnostics critically depends on reliable and unbiased methodologies to amplify and analyse DNA from small numbers or even single cells; the scoop of this article will focus on their implications in the field of prenatal diagnosis.

Prenatal diagnosis is a well-established part of health care that enables timely interpretation of the fetal medical condition, thus giving the parents the choice to either continue or interrupt the pregnancy. Given the current state of medical technology, routine prenatal diagnosis requires the use of invasive obstetric techniques, with probes or needles being inserted into the uterus, for the collection of fetal cells and, therefore, involves a finite risk of the iatrogenic abortion [7,30]. In these cases, fetal cells obtained by amniocentesis or chorionic villus sampling are cultured and a full karyotype analysis is performed. This requires significant labor costs, considerable technical expertise, and, sets a limit to the number of analyses, which an experienced technician can safely handle. In addition, the time needed to culture fetal cells and complete the analysis ranges from 10 to 21 days, which is generally considered to be a psychological burden and results in late terminations after pathological diagnosis [31,32].

In the early 1990s, FISH and, more recently, QF-PCR entered the field of prenatal diagnosis to allow rapid prenatal diagnosis within 24–48 h [33,34]. However, the results were – and still are – considered preliminary while awaiting the results of the gold standard, which is a full karyotype. QF-PCR has some advantages over FISH [35] in that QF-PCR can be done with fewer cells and, since the analysis can easily be automated, many samples can be processed at the same time with only 30 minutes of operator time. QF-PCR can also detect maternal cell contamination, which cannot be disclosed by FISH in cases of female fetuses. Based on these considerations, QF-PCR is increasingly being considered and proposed as a complementary investigation or even approved in some jurisdictions as an alternative to conventional cytogenetic analysis and considered as a stand-alone test in routine prenatal diagnosis as outlined in the Joint of the Society of Obstetricians and Gynaecologists of Canada and College of Medical Geneticists (SOGC-CCMG) Clinical Practice Guideline [36-38]. Although these techniques hasten the process of prenatal diagnosis, they did not overcome the risk associated with invasive sampling of fetal tissues, which limits offering prenatal diagnosis to all pregnant women and shorten its application to those at high risk, as estimated by increased maternal age, abnormal biochemical markers and ultrasonographic findings [39,40].

NIPD is a long sought goal and could be the best alternative to overcome these limitations. It is now well known that fetal material (cells, nucleic acids) can be detected in blood of pregnant women. The presence of fetal cells in maternal blood has been well documented and consequently envisioned to enable NIPD [29,41-44]. In our group, Krabchi et al. [45] have offered conclusive data that fetal cells are present in the maternal blood of all pregnant women and their number ranges between 2 and 6 fetal cells per ml in normal pregnancies. Although the general agreement about the presence of fetal cells in the maternal blood, the scarce amount of DNA contained in these rare cells is a major problem that poses a challenge for their use in clinical practice.

One of the most exciting developments in single cell analysis has been the evolution of protocols designed to amplify the entire genome from single cells. SCs-WGA provides a supply of sample DNA that can be further reassessed, allowing confirmation of diagnosis using different methods. Several PCR-based protocols for WGA have been established and their applications evaluated. These include: (1) primer extension pre-amplification (PEP), a Taq DNA polymerase PCR-based reaction that utilizes 15 base oligonucleotide primers of random sequence to initiate DNA synthesis [46-48]; (2) degenerate oligonucleotide-primed PCR (DOP-PCR) which depends on priming from short sequences specified by the 3′ end of the oligonucleotides during the initial low temperature cycles of the PCR reaction [49-51]; and, (3) multiple displacement amplification (MDA), an isothermal genome amplification using Phi29 DNA polymerase [52].

A significant drawback of WGA techniques is that amplification of repetitive DNA sequences, such as STR, is error prone if performed on WGA products. In some studies over 50% of amplified fragments differed from their expected size, presumably due to the uniformly low temperatures needed for WGA that could allow slippage of the DNA chain during product generation. In addition, these protocols have their limitations, including limited yield, strong biases and low genome coverage, defined as gene representation [22,53-57]. Conversely, GenomePlex WGA, described in this protocol, is a proprietary amplification technology based on non-enzymatic random fragmentation of genomic DNA. The protocol involves conversion of the genome into an in vitro molecular library of DNA

fragments, followed by incubation at various temperatures to add adaptor sequences with specific PCR priming sites to both ends of every fragment. This library is replicated using a linear, isothermal amplification in the initial stages, followed by a limited round of geometric amplification. The fragment library can then be amplified several thousand-fold to generate milligram quantities of DNA [58].

In contrast to traditional WGA techniques, GenomePlex amplification technology is generally less affected by DNA quality and is more applicable to DNA extracted from various sources. The amplified DNA thus produced is suitable for a wide range of downstream genetic assays and therefore has the potential for use not only in academic research, but also in forensic and diagnostic laboratories [59-61]. QF-PCR of WGA products was able to successfully detect fetal sex and major chromosomal abnormalities in more than 92% of the tested samples. Only two cases failed to show the normal female pattern due to inadequate quality of the original DNA from cultured cells, which subsequently decreased the quality of results obtained in downstream application of QF-PCR. We may hypothesize that results would have been better from fresh amniotic fluid samples, as previously reported with array CGH experiments from cultured and uncultured amniotic fluid due to difficult control of DNA quality of cultured cells [62,63]. In these cases, however, it is essential to use increased quantities of starting DNA to guarantee a satisfactory genomic coverage of final product.

Although array CGH has been proposed as a genome-wide assessment approach of chromosomal abnormalities after WGA of few number of cells, array CGH traditionally requires DNA from larger number of cells in addition to the high cost and complicated equipment [64]. Furthermore, laser capture microdissected cells often yield limited quantities of genomic DNA, making array CGH analysis challenging. Another disadvantage of an array CGH system is the period required for DNA labeling, hybridization and analysis of results, especially with the many incidental findings of unknown clinical significance, which creates an ethical dilemma and raises the maternal anxiety [65]. Further research is required to shorten this time and to resolve these problems.

Cell-free fetal DNA is more and more used with massively parallel sequencing [66-68] or targeted deep sequencing [69,70] to test for aneuploidy or single-gene disorders. Even if this test has very low false-positive and false-negative rates, it is still consider as a screening test and its application in clinics could be cumbersome and considerably expensive. In comparison with these techniques, QF-PCR is very simple, rapid and cost-effective approach, and thus more suitable for integration in the clinical applications. It also has the advantage of providing a quick response, which relieves maternal anxiety. QF-PCR, being less expensive and almost entirely automated, enables more women to undergo prenatal diagnosis without a significant increase in health expenditure [37,38,71,72]. The use of innovative technology like automatic cellular scanning of slides for the detection of fetal cells from maternal peripheral blood, which we have recently optimized and used for detection and quantification of fetal cells in the maternal blood in euploid and aneuploid pregnancies will help to avoid the traditional cumbersome and time consuming manual detection of rare fetal cells from maternal blood [73].

This study shows that the utilization of 5 isolated cultured amnoicytes can provide an accurate and reliable NIPD. Satisfactory genome coverage can be obtained from WGA in more than 90% of cases. Our findings provide a proof for the feasibility of NIPD from very small numbers of fetal cells and suggest that detection, amplification and analysis of as few as 5 fetal cells that could be obtained from 2-3 ml of maternal peripheral blood [45] can be quite enough to provide an accurate NIPD. The few number of fetal cells required together with the use of automatic scanning for the detection of fetal cells [73] and SCs-WGA will help to overcome the problem of rarity of these cells in the maternal blood and make their use in NIPD much more easily achievable. Although this method is not a completely mature by now due to absence of perfect antigen that can recognize 100% of fetal cells, relentless efforts continue and should lead to the development of this antigen in the near future [74,75]. However, this protocol will be still feasible with the available markers that recognize specific types of fetal cells as the fetal trophoblasts or normoblasts but with likely larger amount of maternal blood as there is no general agreement about their exact frequencies per ml of maternal blood.

Limitations of our protocol are that single-cell isolation requires expensive equipment and precautions. As the handling of few cells will always remain a very delicate task, the application of this protocol will require some experience.

5. Conclusion

With multiplex QF-PCR, WGA from as few as five fetal cells can accurately and reliably used for detection of fetal sex and major chromosomal abnormalities and most likely single gene disorders as well. Our findings lay the groundwork for the use of very low levels of fetal cells in maternal blood for NIPD.

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CHAPTER V: DISCUSSION

DISCUSSION

Development of NIPD has the potential to reduce, or even eliminate, the risk of miscarriage associated with invasive diagnostic procedures like amniocentesis and CVS. Both procedures are also time consuming, costly and face problems of culture failure and culture artefact. Serious maternal complications such as abdominal cramps, vaginal bleeding and amniotic fluid leakage may occur in up to 3% of cases (Finegan et al., 1990). In addition, CVS may have a discrepancy between culture cytogenetic results and the actual fetal karyotype due to confined placental mosaicism (Slunga-Tallberg and Knuutila, 1995) or the phenomenon of trisomy rescue and natural selection against aneuploidy (Ledbetter and Engel, 1995). The frequency of procedure-related losses can be reduced by serum marker screening, which identify women with an increased risk of bearing abnormal fetuses. However, serum screening is a statistical method that identifies only 60-70% of fetuses with Down syndrome and has a 5% false positive rate (Phillips et al., 1992).

The demonstration that FCs (Walknowska et al., 1969) and fetal DNA (Lo et al., 1990) can be obtained from MB opened the possibility of obtaining fetal genetic material for prenatal diagnosis without the need for invasive testing. Fetal DNA can be retrieved from maternal plasma but is traditionally limited to the diagnosis of a few paternally inherited single gene differences in which the fetal genes mutations are genetically different from the corresponding sequences in the mother. In contrast, the analysis of intact FCs permits the diagnosis of any fetal aneuploidy or monogenic disorder (Zamerowski et al., 2001). Currently, the research in this area not only focuses on the understanding of their biological role and effect on the mother but also how to isolate and use these nucleated FCs in NIPD. However, their rarity in MB has hindered the development of a non-invasive test. Their number can be still increased by various enrichment techniques. Therefore, NIPD using a cell-based strategy can be divided into three phases: (i) enrichment of FCs from MB; (ii) identification of enriched cells; and, (iii) making the genetic diagnosis.

Through the last several decades, considerable efforts have been made to gain access to FCs from MB because this would prepare the ground for NIPD with virtually no risk to

the mother or the pregnancy. Yet, no conclusive progress has been made in this field of research in spite of decades of work. In order to implement FCs in clinical applications, a significant amount of work has to be devoted to the development of an accurate and reliable method of identification of FCs. Improvement of enrichment procedures which have to be more effective and not so labour-intensive as current ones, optimization of selection and analysis of FCs in order to provide unequivocal identification of chromosomal aneuploidies and single gene disorders from a small number of FCs. It will also be important to determine more precisely at what stage of pregnancy FCs can be reliably detected.

In summary, a thorough assessment of the available protocols and introduction of innovative technologies are required before implementation of these rare FCs in NIPD. The data presented in this thesis summarize on one hand the information collected from literature that is relevant for a comprehension of the subject. In addition to the results of our work in the assessment and development of accurate and reliable identification of FCs (Article I, Chapter I), quantification of the FCs in MB at the time of the pregnancy (Article I, Chapter II), comparison of the efficacy of different identification techniques, evaluation and improvement of enrichment procedures (Article I, Chapter III) and, finally, the optimization of genetic analysis for identification of fetal aneuploidies and major chromosomal abnormalities from single FCs (Article I, Chapter IV). The main objectives were to assess the current available techniques and development of future trends for NIPD using FCs in MB

1. Development of a strategy for the evaluation of detection of rare cellular events

Detection of rare cellular events is required for different applications. Cancer initially arises as an organ-confined lesion from which occult cells travel through the blood to anatomically distant sites to develop metastatic disease, which is a major cause of cancer-related death in patients with solid tumors. Detection, monitoring, and molecular analysis of these cells can provide a powerful approach for cancer management such as in the detection of recurrence and minimal residual disease (Szatanek et al., 2008; Cristofanilli et al., 2007). At the same time, the detection of intact FCs in the MB at the time of pregnancy can provide an opportunity for collecting fetal genetic material for NIPD without the need for invasive

procedures. Interestingly, in both approaches, the disseminated cells are present in the circulation in extremely low concentrations, estimated to be in the range of one target cell in the background of 10^6–10^7 normal blood cells (Pantel and Otte, 2001; Hamada et al., 1993).

Various protocols with either manual or, more recently, automatic scanning, can be used for retrieval of these rare cellular events from peripheral blood after molecular or immunological identification of specific genetic markers, exclusive of target cells, by various techniques (Ntouroupi et al., 2008; Johnson et al., 2007; Krabchi et al., 2006c; Krabchi et al., 2001). Evaluation of the efficacy of a particular protocol is mandatory before its clinical application. In general, assessment of the efficacy of detection of rare cellular events is always problematic, as is an accurate evaluation is difficult to obtain with currently available methodologies.

Moreover, the establishment of the original number of rare events is critical for optimizing any enrichment procedure. Knowing the number of rare cells before and after enrichment is mandatory to measure the efficacy and to evaluate the number of cells lost or destroyed during enrichment. It is worth pointing out that increasing the efficacy of detection can even reduce the level of enrichment required, making NIPD much more easily achievable (Yan et al., 2000).

In the context of detection of FCs from MB, optimisation of FC selection was made using model mixture experiments whereby adult non-pregnant blood was spiked with certain amounts of FCs (Yan et al., 2000; Bohmer et al., 1999). This dilution strategy is reliable within certain limits but it is almost impossible to obtain an accurate evaluation in cases of extreme rare events, such as FCs in MB, in which an average of 2 to 6 cells have been located by manual scanning of 20 to 30 slides with an average of 200,000 cells per slide (Krabchi et al., 2001). In such situations, the dilution strategy seems imprecise and could be just considered as an approximation of the real situation as it is not possible to tell for certain the exact original number of the FCs present in each slide.

Other groups worked directly on maternal samples and compared the efficacy of automatic to manual scanning (Johnson et al., 2007; Kilpatrick et al., 2004; Kraeft et al.,

2004). However, the accuracy of the manual scanning, which is taken as the gold standard in the detection of rare cellular events, has never been validated. Therefore, there was a pressing need to develop a strategy for the accurate evaluation of the detection of rare cellular events.

We used sequential spreading approach to put a pre-determined number of rare cellular events (< 10 cells) of a particular type on the predefined areas on a slide in the first round of spreading. The target cells were then stained with Giemsa and photos of Giemsa-stained cells were taken and registered with their coordinates on the slide. On the second round, we spread an average of 2.0×10^5 cells of different types on top of the whole spreading area of the slide, simulating the average concentration of cells on the slides prepared from MB. For instance, Giemsa-stained cells are those of uniform differential karyotype such as male cells to which are added female cells that are not GIemsa-stained. The slides were then coded and blindly scanned (Article I, Chapter I). This strategy permits the precise evaluation rare cellular events detection. Furthermore, it allows the retrieval of the missed events as well as the evaluation of their hybridization signals after scanning to dissect the etiology of their missing and determine the impact of the identification technique used in their retrieval.

This strategy could generally be applied in the evaluation of detection of rare cellular events and has many potential applications in different fields such as cancer and NIPD. In the context of detection of FCs from MB, we used a few XY-cells as rare events in a pure population of XX-cells; however, there is no reason why it should not work with any type of rare cellular events as long as there is a specific marker exclusive for the target cells of interest.

In the context of NIPD, it can be used in the evaluation of manual and automatic scanning as well as in the comparison between the detection efficacy of different markers and identification techniques. Moreover, this strategy could be used in the optimization and comparison of different enrichment procedures. To our knowledge, this is the most precise approach available to date to evaluate the efficacy of the detection of rare cellular events.

2. Optimization and measuring the efficacy of manual scanning

The number of FCs is very low compared to that of maternal cells. Accurate identification of circulating FCs is essential for their reliable use in prenatal diagnosis. The identification of these cells by targeting specific genetic markers exclusive for the FCs by molecular cytogenetic techniques like FISH and PRINS have been used before by many groups for estimating their number in the MB (Orsetti et al., 1998b; Goldberg, 1997; Hamada et al., 1993). Using this approach, our group previously established an estimate of the absolute number of FCs in normal and aneuploid pregnancies (Krabchi et al., 2006b; Krabchi et al., 2006c; Krabchi et al., 2001). Although these studies yielded important information concerning the number of circulating FCs in MB, the results are possibly skewed by the fact that the methodology of detection of these rare events by manual scanning with either of these techniques has never been evaluated.

In the process of testing and optimizing the methodology to devise an efficient protocol for identification and selection of FCs from MB, the evaluation of the efficacy of manual scanning is mandatory. On one hand, the evaluation of the efficacy of any protocol is required before its clinical application. On the other hand, the evaluation of the efficacy of manual scanning will be a prerequisite for the validation of automatic scanning. Although manual scanning is very cumbersome and time consuming, the sensitivity of the technique in the detection of rare cellular events is far more important than any other consideration. In other words, automatic scanning should have a better or at least comparable sensitivity to manual scanning in the detection of rare events before switching to automation.

We used the previously mentioned strategy to evaluate the efficacy of manual scanning in the detection of rare cellular events hybridized using FISH (Article I, Chapter I). The experimental design involved the assessment of 60 slides containing known number of XY cells (< 10 cells) in pre-defined areas among pure population of XX cells blindly by two independent observers. The protocol allowed for the evaluation of the efficacy of detection of manual scanning by knowing the exact number and coordinates of XY cells on the slides. For the first time, the protocol also allowed for the retrieval and evaluation of hybridization of missed events which, consequently, helps to determine the efficacy of FISH technique in

recovering rare cells and differentiate between cells missed due to defective hybridization from those missed by the process of manual scanning itself.

In general, the accuracy of manual scanning for detection of rare events was quite good with an average of 84.5% (125/148) sensitivity. The FISH technique was responsible for missing 4% (6/148) either due to non-hybridization in 1.3% (2/148) or inadequate signalling in 2.7% (7/148) while 11.5% (14/148) of cells were missed as a lack in the process of the manual scanning itself (figure 1). However, the range of 74.5% to 90% in the detection efficacy and 2.2 to 2.6 hours in the average scanning time between two observers suggested a considerable range of inter-individual variations, meaning that laboratories with more senior personnel may provide more dependable results than those with less experienced staff (Article I, Chapter I). Interestingly, these limitations could be circumvented using automatic scanning

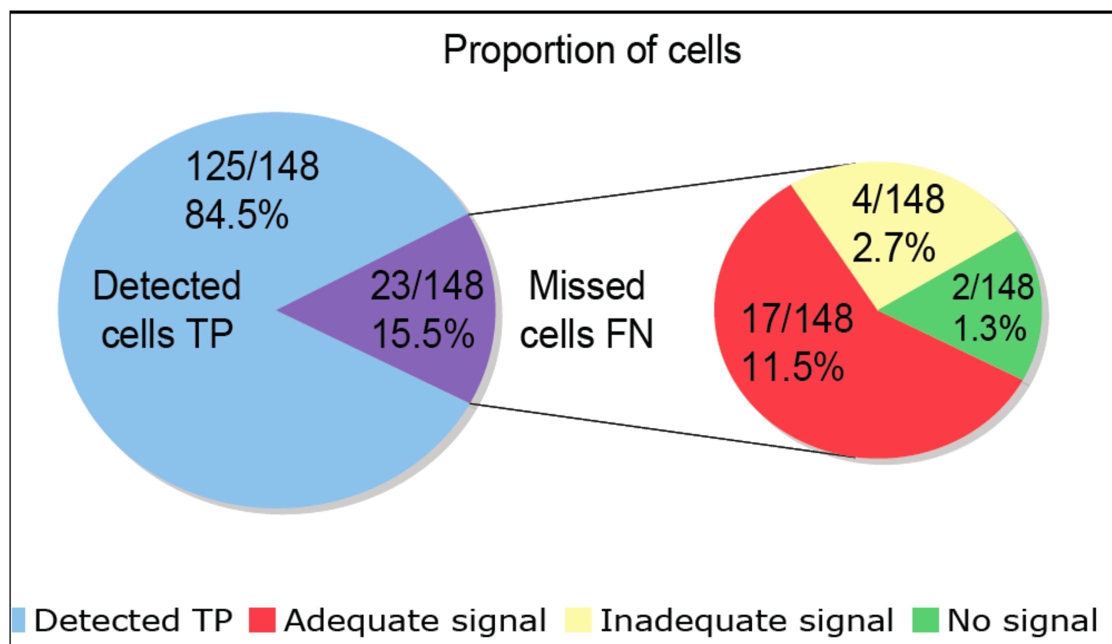

Figure 1 Summary of the results of manual scanning using FISH technique for detection of rare cellular events.

TP: true positive, FN: false negative. The figure shows the absolute number and percentage of detected and missed target cells on slides by manual scanning with evaluation of the hybridization efficiency of the missed events.

3. Optimization and measuring the efficiency of automatic scanning

In the context of locating FCs from MB by manual scanning, one milliliter of maternal peripheral blood is concentrated in a cellular suspension of 300 µL. A spreading of 15 µL/slide corresponds to an average of 20 slides per case (range: 16 to 24 slides) with an average number of 2.0 x 10^5 cells per slide as previously described (Article I, Chapter I). The duration of the observation of each slide is approximately 150 minutes by manual scanning. An average of 4-6 cells/ml has been located by scanning an average of 20 slides in normal pregnancies (Emad et al., 2012; Krabchi et al., 2001). The analysis of all slides of one patient thus accounts for 6 to 7 days of fulltime work. Therefore, manual scanning is very cumbersome, time consuming and not suitable for clinical applications. There is, therefore, an urgent need for automation in the detection of FCs. Automated microscopy has the potential to make the procedure practical on a large scale because: (i) it reduces cost and relieves tedious human work; (ii) it can automatically determine statistically significant results; and, (iii) it can support expanded testing without requirements for additional personnel or space. Automation is not only required for the eventual clinical application of NIPD using FC in MB, but is also critical for research progress to be made in this field

Therefore, many innovative technologies have been developed to alleviate the burden of scanning large numbers of cells and allow for rapid and precise detection of rare cellular events (Ntouroupi et al., 2008; Seppo et al., 2008; Johnson et al., 2007; Kilpatrick et al., 2004; Mehes et al., 2001). Automated approaches have transcended the investigational and developmental stage and systematic application of these approaches to the detection of FCs from MB is ongoing. Both flow and image cytometry can detect cells, but only image cytometry can provide re-location of detected cells using previously determined spatial locations on slides. This allows for re-examination and further manipulation of these cells. In image scanning devices, cells are located in one colour channel (e.g. blue for DAPI staining), and dots are counted in user-selected channels. A count of the number of dots of each colour in the nucleus provides chromosomal enumeration. For each slide, scanning results are reported as a distribution of `dots-per-cell' for each colour. Although they proved efficient for routine clinical tasks, their application in the detection of rare events is limited

due to difficult evaluation with currently available methodologies (Johnson et al., 2007; Kilpatrick et al., 2004). Accurate evaluation is required for both optimization of the selection criteria and the development of an efficacious algorithm for the identification of FCs from MB.

3.1.Development of custom-made detection algorithm

The software that controls the automated microscope generally includes functions for spatial and photometric calibration, automatic focusing, image scanning and digitization, background subtraction and colour compensation, nuclei segmentation, location and measurement, and FISH dot counting. Broadly, there are two types of operational systems in automatic scanning devices, the closed systems like Ikonisys (Ikonisys Inc., New Haven, Connecticut) and the opened systems like MetaSystems (MetaSystems Inc., Altlussheim, Germany). In closed systems, the company optimizes the selection criteria for a specific purpose and users are only allowed to apply an integrated classifier for their application; whereas, in the open systems, users are allowed to develop their own detection algorithm and fine tune the selection criteria in a process called microscopy training to optimize their own classifier according to their specific application (Merchant and Castleman, 2002). We tried both systems for the detection of FCs from MB but the open platform of MetaSystems was more accurate and reliable..

Automatic scoring was performed using MetaSystems image cytometry platform (Altlussheim, Germany). Although Metafer/RC Detection mode integrated in the system software can be used for automatic detection of rare cellular events, a semi-automated approach using a custom-made detection algorithm was preferred. An automated classification algorithm was implemented to detect FCs by employing a series of relatively simple routine patterns of recognition as shown in figure 1 (Article I, Chapter II). We used our experience in manual scanning to devise a simple but efficacious protocol simulating the manual detection of FCs in MB. The scanning platform was used to find potential target cells with a final assessment being performed by an experienced operator. Cells's location and primary selection was performed using spectrum green for the Y-signal at 20X magnification. Suspected events were verified at 40X magnification for the presence of X

chromosome signal on a DAPI-stained nucleus. Slides with pure XX cells and others spiked with defined number of XY-cells served as controls for optimization of microscope training process. Knowing beforehand the numbers and locations of positive cells allowed for better characterization and fine-tuning of selection criteria and required magnification for optimum detection (Article I, Chapter I).

3.2.Measuring the efficacy of automatic scanning

We used the previously mentioned strategy to evaluate the efficiency of automatic scanning in the detection of rare cellular events hybridized by FISH (Article I, Chapter I). The experimental design involved the assessment of 60 slides containing pre-defined XY cells (< 10 cells) among a pure population of XX cells blindly with an average of 2.0×10^5 cells per slide. Selected events were imaged and recorded. The gallery was manually sorted to reject false events. Remaining cells were, then, visually assessed under the microscope for final selection of positive events. Selected events were compared with pictures and coordinates of real XY cells on the slides to evaluate the detection efficiency of automatic scanning. Missed events were retrieved and evaluated for hybridization using FISH technique to differentiate between cells missed due to defective hybridization from those missed as a drawback of automatic scanning.

The sensitivity of automatic scanning in the detection of rare events was 87.9% (117/133) in comparison to 84.5% (125/148) with manual observation. Spearman's correlation showed a high correlation coefficient (C.C= 0.96, $P < 0.001$, N=60) in comparison to correlation coefficient of 0.94 in manual scanning. Index of Youden of 0.88 confirmed the superiority of automatic scanning over manual detection with an index of 0.85 in the detection of rare cellular events. Evaluation of FISH technique was very comparable to that obtained by manual scanning. FISH hybridization was responsible for missing of 4.5 % (6/1133) either due to non-hybridization in 1.5% (2/133) or inadequate signalling in 3% (4/133) while 7.5% (10/1133) of cells were missed as a drawback of the process of automatic scanning in which three cells were actually detected by scanning but rejected during observer revision (figure 2). Although automatic scanning requires longer time (an average of 239 minutes) the operator time was significantly reduced, with an

average of 15 minutes compared to 150 minutes per slide for manual scanning. Furthermore, automatic scanning is not operator dependent and consequently, more consistent than manual scoring. In addition it can be used all day long with the same efficacy (Article I, Chapter II).

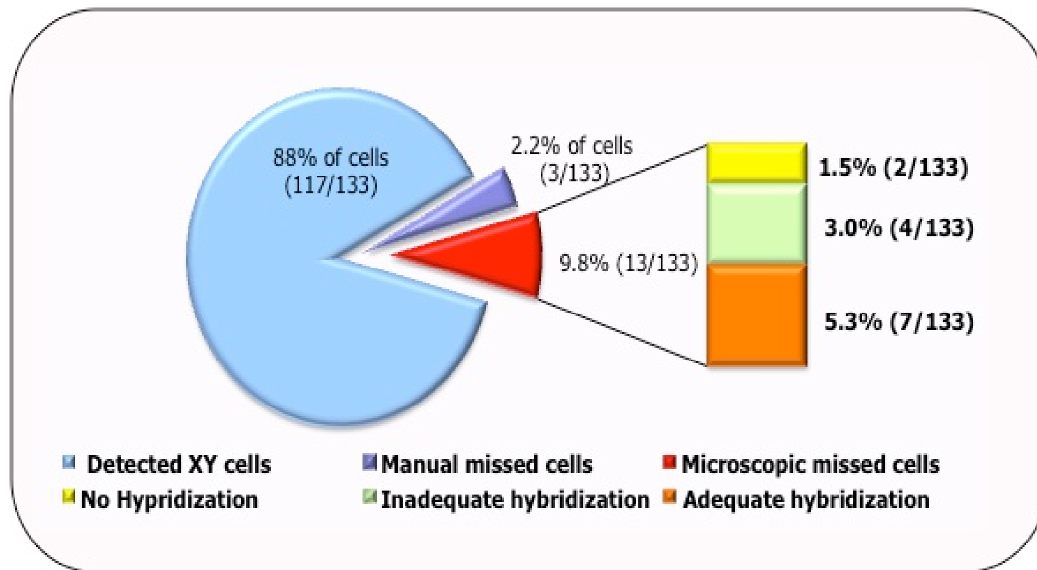

Figure 2 Summary of the results of automatic scanning using FISH for detection of rare cellular events.

TP: true positive, FN: false negative. The figure shows the absolute number and percentage of detected and missed XY cells on slides by automatic scanning using FISH technique with evaluation of the hybridization efficacy of the missed events.

The accuracy of prenatal diagnosis using FCs depends upon the specificity of their identification from MB. Validation of manual scanning for detection of rare cellular events paved the way for the automatic detection of FCs from MB. Automation not only helps in realization of NIPD, but is also essential for research progress in this field. We have applied automatic detection of FCs in the evaluation and comparison of different identification and enrichment approaches.

4. Identification and quantification of fetal cells from maternal blood

Accurate establishment of the frequency of FCs in the MB is a basic parameter to determine the feasibility of using FCs in NIPD and critical before optimising any

enrichment procedures. It is also known that gestational age influences FC trafficking and thus, affects frequency of FCs in MB; however, an ideal gestational age for FC sampling has not yet been established (Kuo, 1998; Hamada et al., 1993).

In spite of many attempts to estimate their number and evaluate changes in their frequency with gestational age, there is still much controversy concerning these aspects. The numbers obtained vary widely, ranging from 1 to 150 FCs per ml of MB as well as the trend of change in their frequency with gestational age (explained in detail in the introduction section) (Lim et al., 2001; Rodriguez de Alba et al., 2001; Kuo, 1998; Shulman et al., 1998; Sohda et al., 1997; Hamada et al., 1993). This reflects variability in experimental conditions of detection, identification and isolation of FCs, timing of recovery, enrichment procedures and type of target cells.

Studies which count specific types of FCs, based on staining or morphological criteria probably overestimated the total number of cells because no FC recognition system is completely specific (Lim et al., 2001; Troeger et al., 1999a; Wachtel et al., 1998; Sohda et al., 1997). On the other hand, less generous predictions but more precise values were given by studies which have been based on DNA analysis of the Y chromosome sequences as a universal marker that recognise all FCs using polymerase chain reaction (PCR) in male pregnancies (Kuo, 1998; Bianchi et al., 1997). However, PCR experiments gave a wide estimate range but no absolute number has been determined.

Recent studies have indicated that FCs can be detected directly from the MB without prior enrichment to avoid losing fragile FCs. This was done using techniques such as FISH and PRINS. In our group, Krabchi et al (Krabchi et al., 2006b; Krabchi et al., 2006c; Krabchi et al., 2001) implied a direct detection strategy, without prior enrichment, and have offered conclusive data concerning the absolute number of FCs in one ml of MB in the second trimester using cumbersome manual scanning approach. Results were probably skewed by the efficacy of manual scanning in the detection of rare events. We validated the manual approach of Krabchi (Article I, Chapter I) and further refined its efficacy by automation. Consequently, we tested this classifier for the detection of FCs from MB on twelve cases of both euploid and aneuploid pregnancies with Down syndrome. Detected

events were evaluated by re-FISH to exclude false positive cells. True FCs confirmed by re-FISH were tabulated whereas; extra-cells, which failed to give reverse signal pattern, were excluded (Figure 5, Chapter II).

4.1.Comparison of fetal cells in the first and second trimester

For cases of normal pregnancies, six cases were processed four cases have been sampled at the end of the first trimester while the other two have been taken between 18th and 20th week of gestation. Analysis of the number of confirmed FCs did not show a difference in the frequency of FCs between 1st and 2nd trimester of normal pregnancies.

Most groups found an increase in fetal NRBCs between the first and second trimesters (Rodriguez de Alba et al., 2001; Ganshirt et al., 1998; Ganshirt-Ahlert et al., 1993) while there is a decreasing trend of the trophoblasts (Lim et al., 2001) from their peak at 9th to 13th weeks of gestation (Taniguchi R, 2001). As all our first trimester samples were obtained between 11th to 13th weeks and we used a universal marker for detection of both NRBCs and trophoblasts, the low number of NRBCs could have been compensated by a high number of trophoblasts. Although this should be confirmed on a larger series of samples, we think that this window period at the end of the 1st trimester may present an opportunity for an early NIPD.

4.2. Comparison of fetal cells in normal and aneuploid pregnancy

For cases of aneuploid pregnancies, six cases with trisomy 21, sampled in the second trimester, were processed. Analysis of the number of confirmed FCs did show a significant difference in the frequency of FCs between normal and aneuploid pregnancies ($P < 0.001$) (Figure 6, Chapter II).

Interestingly, our findings matched with previous findings of Krabchi et al., and others using cumbersome manual scanning (Krabchi et al., 2006b; Krabchi et al., 2006c; Krabchi et al., 2001). However, their number of FCs seemed to be underestimated by at least 16%, apparently, due to the inherent false negative rate associated with manual detection (Article I, Chapter I).

5. Optimization and comparison of efficiencies of different techniques

FCs could be identified by immunological and molecular techniques. Earlier studies used immunological approaches to target particular types of FCs, however, most detected cells were found to be of maternal origin (Lim et al., 2001; Troeger et al., 1999a; Wachtel et al., 1998; Sohda et al., 1997). Recent studies have employed molecular cytogenetic techniques for more precise identification of FCs, and, once located, the cells can be further identified by micromanipulation (Reading et al., 1995; Hamada et al., 1993).

Accuracy in prenatal diagnosis using FCs depends upon the specificity of their identification. Therefore, one of the main objectives of our group was to develop and optimize methods of molecular cytogenetics in order to increase their sensitivities for the detection of rare events.

Working on FISH and PRINS techniques for years in our laboratory enabled us to develop a great expertise in the use of these techniques to detect rare cells with a concentration lower than 0.01% (Yan et al., 2000) (Krabchi et al., 2006a; Yan et al., 2000). FISH technique is more popular and is used by many groups all over the world but is much more expensive than PRINS. Enthusiasm to extend molecular genetic diagnostic options, for techniques like PRINS, was mainly to reduce the cost of any potential diagnostic test using FCs, making NIPD more likely acceptable. Krabchi et al., have previously used both techniques in the identification of FCs from MB and stated that they have comparable efficacies (Krabchi et al., 2006b; Krabchi et al., 2006c; Krabchi et al., 2001).

In this context, we committed ourselves to two main objectives: (i) reducing the cost of FISH technique without affecting its sensitivity for detection of rare events; and, (ii) evaluating and comparing the two techniques in the detection of rare cellular events.

5.1. Validation of FISH probe dilution with commercial buffer

The experimental design involved the evaluation of the hybridization efficiency of two FISH probes; CEP X: spectrum orange alpha-satellite and CEP Y: spectrum green satellite-III; (Vysis/ABBOTT Diagnostics, Downers Grove, Illinois, USA) on 3000 cells of

harvested blood samples of female and male donors, respectively, by two independent observers on three replicates of experiments (1000 cells per each experiment). The same experimental design was used to evaluate the hybridization efficacy of 5 serial dilutions of each probe (1/50, 1/100, 1/200, 1/300, 1/400) with a commercially available FISH hybridization buffer cDenHyb-1 (Insitus Biotechnologies, Albuquerque, NM). Exact “t” and ANOVA test showed statistically significant differences using dilutions of 1/200 and 1/400 for CEP X and CEP Y probes respectively).

Therefore, we used CEP X and CEP diluted 1:100 and 1:300 respectively in cDenHyb-1 buffer in the validation of detection of rare cellular evens by manual and automatic scanning as well as detection and quantification of FCs from MB. The same dilutions were also used in the comparison of the efficiency of FISH and PRINS techniques. This allowed for marked reduction of the cost of FISH technique without affecting the sensitivity of the probes in the detection of rare events. This would make NIPD using FCs more advantageous and cost-efficacious, in comparison even with the routine fetal karyotype of cultured FCs obtained by invasive methods in routine prenatal diagnosis.

5.2. Evaluation and comparison of the efficacy of FISH and PRINS techniques

We used the previously mentioned strategy to spread 90 slides with pre-defined XY cells as target cells, less than 10 cells per slide, among a pure population of XX cells with an average of 2.0×10^5 cells per slide. We compared the efficiency of automatic scanning in the detection of rare cellular events hybridized by FISH and PRINS technique. Automatic scanning with FISH was 10% greater in sensitivity than PRINS when detecting rare cellular events. The overall hybridization efficiency was 96.5% in PRINS when compared to 98.5% with FISH. Spearman’s correlation showed a lower correlation coefficient of 0.882 between detected cells and real numbers of XY cells on the slides in PRINS when compared to 0.96 with FISH (Article I, Chapter II). Results indicated that FISH technique, even with the dilution buffer, is significantly superior to PRINS in detection of rare cellular events. Taking this valuable information into consideration in devising future protocols for the detection and isolation of FCs from MB will improve the efficiencies and reduce the cost of these protocols making NIPD more likely achievable.

6. Evaluation and improvement of enrichment protocols

A prenatal test utilizing FCs from maternal circulation would necessitate the recovery of most FCs, while eliminating as many maternal cells as possible. Therefore, various purification and enrichment procedures have to be applied. However, the isolation of FCs from the maternal circulation presents considerable challenges, given their limited numbers, even with purification, thousands of cells have to be examined to locate one target cell. Numerous groups applied different multi-step protocols using combinations of two or more enrichment procedures to enrich FCs from MB, mostly without testing their efficacies in an *in* vitro model (Kitagawa et al., 2002; Zhao et al., 2002; de Graaf et al., 1999; Ganshirt-Ahlert et al., 1993). The in vivo enrichment efficacy reflects that of the entire protocol rather than single individual procedure and non-uniformity of the procedures used among different protocols. In addition the exact number of FCs circulating in MB cannot be known with certainty to evaluate each protocol. Knowing the number of FCs before and after enrichment is mandatory to measure the efficacy and to evaluate the number of cells lost or destroyed during enrichment.

Furthermore, the studies also differed from each other in some ways, making direct comparisons unreliable, as for example: (i) application of enrichment protocols across varying gestational ages; (ii) usage of non-specific FC markers for enrichment and identification; (iii) absence of subsequent analysis with FISH to prove the fetal origin of detected cells; and, (iv) samples obtained either from two different groups of women or pre and post invasive procedure. Therefore, currently there is no accurate assessment of the efficacy of individual procedures and no standardized model is available to evaluate the *in vivo* efficacy of FC enrichment protocols

It is worth to mention here also that the fragility of the FCs in MB limits attempts of FC enrichment. The least aggressive method of processing of the samples would be thus most adequate. In fact, it is proven that the FCs in MB are cellular elements not only rare, but also vulnerable. It was observed that some of FCs initiate apoptosis at the time of their passage to maternal circulation. The analysis by TUNNEL technique (*Terminal* dUTP Nick *End Labelling*) allowed for the detection of fragments of DNA of FCs origin (Sekizawa et

al., 2000) and showed that a significant number of FCs undergo apoptosis within short time, less than three hours, of their presence in MB. In fact, it is one of the proposed mechanisms by which FCs would be eliminated from MB (Kolialexi et al., 2004). This conception must be taken into consideration in the development of non-invasive prenatal diagnosis using FCs and maximum precautions should be taken to avoid aggressive manipulations in the recovery of FCs from MB.

One of the objectives of our work was to develop an efficacious method that could be used to evaluate FC enrichment *in vivo* in a standardized fashion through knowing the exact number of FCs before and after enrichment. We applied this concept to the evaluation of the process density gradient centrifugation as the most common initial step in the vast majority of FC enrichment protocols published to date (Al-Mufti et al., 2003; Kitagawa et al., 2002; Vona et al., 2002; Parano et al., 2001; Rodriguez de Alba et al., 2001; de Graaf et al., 1999; Ganshirt et al., 1998; Kuo, 1998; Oosterwijk et al., 1998).

The experimental design involved the quantification of total and FCs in twelve cases of normal and aneuploid pregnancies both before and after enrichment using Histopaque 1.119. Considering the risks inherent to the brittleness of FCs and in order to avoid subjecting them to the further stress of a mechanical nature, we replicated the experiment with and without the routine 30 minute centrifugation. .

We provided evidence that the application of density gradient centrifugation for isolation of rare FCs from MB is associated with a major loss of 60-80% of rare FCs, which would greatly limit the recovery efficacy of further purification steps and, thus, the eventual number of FCs available for analysis could be insufficient for making a genetic diagnosis. Furthermore, the elimination of aggressive centrifugation and allowing the cells to sediment by gravity reduced the average FC loss by 12%, probably due to fragility of FCs (Article I, Chapter III). Therefore, the elimination of this step or its replacement by the non-aggressive version of the procedure in future protocols would enhance the recovery of rare FCs and reduce the number of enrichment steps and consequently reduce FC loss. Furthermore, automation and micromanipulation would be of great help, as purity would not be of utmost importance and maternal contamination would not interfere with automatic scanning.

7. Optimization of genetic analysis from few fetal cells

One of the main strategies that has emerged to exploit the FCs in NIPD was the genetic analysis of a few cells but, in fact, this strategy is mandatory for many different applications. The analysis of rare cellular events and their molecular characterization can provide novel approaches for cancer management, pre-implantation, genetic and forensic medicine (Maheswaran and Haber, 2010; Wei et al., 2007; Thornhill and Snow, 2002). Nevertheless, the scarce amount of DNA obtained from these rare cells poses a challenge for clinical applications.

One of the most exciting developments in single cell analysis has been the evolution of protocols designed to amplify the entire genome from a single cell, which, could provide a supply of DNA sample that can be further reassessed, allowing confirmation of diagnosis using different methods (Dean et al., 2002; Xu et al., 1993; Telenius et al., 1992).

Array-CGH has been proposed as a genome-wide assessment and was successfully used for molecular characterization of chromosomal abnormalities from single cells after whole genome amplification. However, the current protocol is costly, time-consuming and does not seem to fit into the clinical schedule (Fiegler et al., 2007; Hu et al., 2007). Novel strategies such as the use of short tandem repeats (Pertl et al., 2000) and differentially methylated sequences (Poon et al., 2002) have been explored but as yet seem unlikely to be applicable for NIPD of the common aneuploidies or monogenic disorders.

Looking for an alternative approach that is clinically practical and has the potential to detect chromosomal abnormalities and single gene disorders, we focused on evaluating the fidelity of DNA from a few FCs in terms of detection of fetal sex and major chromosomal abnormalities using rapid and cost effective multiplex QF-PCR (Article I, Chapter IV). We provided a detailed protocol ranging from sample preparation, laser capture microdissection, and a few cell whole genome amplification, and then analysis of short tandem repeats using QF-PCR to detect fetal sex and major chromosomal aneuploidies.

The experimental design involved the evaluation of 26 cases of normal and aneuploid pregnancies from single FCs of amniotic fluid. High concordance, >90%, in chromosomal copy number between extracted and amplified DNA was obtained when five or more cells were used as templates. These results support the feasibility of using rare FCs in NIPD.

Clinically, few cell-whole genome amplifications coupled with QF-PCR can provide a reliable, accurate and rapid method for prenatal diagnosis of major fetal aneuploidies. This protocol is advantageous in comparison to routine prenatal diagnosis with either analysis of fetal karyotype or even expresses FISH of FCs obtained by invasive methods. Routine fetal karyotype requires significant labor costs, considerable technical expertise, and, in addition, the time needed to culture FCs and complete the analysis, which ranges from 10 to 21 days (Simoni et al., 1983; Niazi et al., 1981). Express FISH can reduce the time but is still costly with a low throughput and inapplicable for diagnosis of single gene disorders.

QF-PCR analysis can easily be automated; many samples can be processed at the same run within 30 minutes. QF-PCR can also detect maternal cell contamination, which cannot be disclosed by FISH in cases of female fetuses (Langlois and Duncan, 2011; Leung et al., 2004; Leung et al., 2003).

On the other hand, this protocol is superior to other scenarios of NIPD using comparative genomic hybridization. When compared to genomic hybridization, QF-PCR is simple, rapid and cost-effective approach, and thus more suitable for integration in clinic. It also has the advantage of providing a quick response, which relieves maternal anxiety. QF-PCR, being less expensive and almost entirely automated, permitting more women to undergo non-invasive prenatal diagnosis without a significant increase in health expenditure (Grimshaw et al., 2003; Wells et al., 1999).

8. Perspective

Through the last two decades, considerable efforts have been made to gain access to the FCs in the MB. Most of the efforts were directed toward the development of strategies for identification, isolation, enrichment and analysis of FCs from MB. Evaluation and comparison of these strategies has been difficult in the absence of an appropriate *in vitro* model. At the same time, the *in vivo* efficacy only reflected that of the entire protocol rather than individual strategies within the protocol. Many fetal markers and techniques have been tested in absence of proper ways to evaluate them. The literature is burdened with tens of procedures and methods but no single optimal protocol using FCs has been approved for clinical practice.

We believe that everything is possible but one has to find the right way of doing it. FC usage in NIPD depends to a great extent on the application of successive strategies of identification, enrichment and analysis of rare events. The development and optimization of these main strategies to devise an optimal protocol would likely lead to a successful NIPD. For the first time we have shown in our work an optimized and appropriate *in vitro* model for the accurate assessment of the detection of rare cellular events (Article I, Chapter I). We assumed at that time that this model could have several potential applications not only in the FC project but also in other applications in which detection and analysis of rare events is essential. We have already used this model in the evaluation and validation of manual and automatic scanning which is a comparison between different detection techniques (Article I, Chapter II) and in the evaluation of density gradient centrifugation, which is one of the most important and commonly used enrichment procedures (Article I, Chapter III). We have also demonstrated proof of concept for the feasibility of using rare FCs in NIPD through micromanipulation and whole genome amplification followed by multiplex QF-PCR analysis (Article I, Chapter IV).

However, many interesting approaches and FC markers need to be properly evaluated. For example, the recognition of FCs by targeting different fetal cellular markers and their selection by fluorescent systems (FACS) or magnetic beads (MACS) were the subjects of many investigations. The techniques of cellular sorting seem very promising

since handling of only few FCs was already carried out successfully. However, the best approach to use still needs comparison and evaluation of different strategies (D'Souza et al., 2007; Wada and Kitagawa, 2004; Zhao et al., 2002; Troeger et al., 1999b; DeMaria et al., 1996; Johansen et al., 1995).

Another very important question, which remains until now partially unanswered, is the exact frequencies of FCs at various stages of the pregnancy. It is important to determine the frequency of FCs at different stages of pregnancy to determine the optimal time for retrieving FCs from MB. Although considerable efforts have been done, no consensus has been reached (Lim et al., 2001; Rodriguez de Alba et al., 2001; Kuo, 1998; Shulman et al., 1998; Sohda et al., 1997; Hamada et al., 1993). Our preliminary data indicated that there might be a brief window of time at the end of the 1st and the beginning of the 2nd trimester, which may present an opportunity not only for non-invasive but also for an early NIPD. However, these findings are very preliminary and should be verified using large series of samples. Ideally, the experimental design should involve periodic analysis of samples of the peripheral blood from a control group of women during their pregnancies using an interval of two to three weeks apart.

Many fetal cellular markers have been identified. Testing, optimization, and comparison of different fetal markers would also be of utmost important to devise a sex-independent approach for the identification of FCs. Immunological and genetic markers can be broadly used to identify FCs. Many immunologic markers have been investigated but apparently no single one was entirely specific to FC (Lim et al., 2001; Troeger et al., 1999a; Wachtel et al., 1998; Sohda et al., 1997). Genetic markers are likely more sensitive and specific. We have already used Y chromosome in our studies and it was very specific. The Y chromosome is considered a universal marker for FCs in male pregnancies. There is no comparable universal marker for female pregnancies. To circumvent this problem of a universal sex marker, it is possible to target specific fetal genes that are expressed only in the fetal period by the trophoblastic or the erythroblastic cells. This would permit the detection of FCs regardless of fetal sex. PRINS technique can detect m-RNA *in situ* on intact FCs (Bains et al., 1993; Mogensen et al., 1991). Genes of interest would include

gamma Hb, HPL, HCG and HLA-G. The use of modern technology will also help in the identification of target genes by comparing the expression of fetal and maternal cells. Interestingly, Brinch et al., found five candidate genes whose protein are located on the outer surface of the cell membrane of FCs and not expressed by the maternal cells (Brinch et al., 2012). In a more recent work, the same Danish group proposed extravillous trophoblasts as a potential target for NIPD due to their unique expression of both mesodermal and ectodermal markers (Hatt et al., 2013).

Although the identification of FCs is still not completely accomplished due to the absence of a perfect antigen that can recognize 100% of fetal cells, relentless efforts continue and should lead to the identification of this antigen in the near future. After the identification of FCs, suspected chromosomal aneuploidies could be tested by FISH technique or microdissected and analysed by QF-PCR as mentioned before. Furthermore, determination of the source of FCs (either placental or haemopoietic) is also of considerable interest, as this may lead to the development of new tools permitting the efficacious isolation of FCs from MB samples.

According to current protocol, it is necessary to prepare approximately 16-24 slides per one ml of MB to retrieve two to six FCs in normal pregnancies. One of the most exciting ideas is to optimize an enrichment method to allow concentrating the FCs contained in 5 ml of MB on one or two slides. Based on the number of fetal cells per ml of MB, there would be around 10-20 cells per slide. This will be very helpful to relieve the manual burden of preparing and scanning large numbers of slides on one hand, and in reducing significantly the cost of FISH probes on the other hand. Unrepentant progressive improvement of enrichment strategies, will certainly lead to a successful NIPD using FCs.

CHAPTER VI: CONCLUSION

CONCLUSION

Offering prenatal diagnosis to all pregnant women is limited by the risk of iatrogenic abortion associated with sampling of FCs by invasive methods (Finegan et al., 1990). The demonstration of the presence of FCs in maternal circulation during pregnancy offers NIPD opportunities. The risky invasive methods could then be replaced by non-invasive simple samples from peripheral venous blood without slightest risk for the ongoing pregnancy. There is no doubt that FCs do exist in MB and that they can be used for analysis of fetal aneuploidies and inherited Mendelian genetic disorders, but until now clinical application has not been established. Prenatal diagnosis using FCs from MB depends upon three main strategies ranging from identification, enrichment and genetic analysis of FCs. Devising of optimal protocols for these strategies will pave the way for the development of NIPD in clinical practice.

Scientists agree that the presence of FCs in MB is a rare event, which makes their isolation difficult but not impossible (Bianchi et al., 2002; Ariga et al., 2001; Krabchi et al., 2001). Their frequency can even be increased by various techniques of enrichment, which are still under development for the majority of protocols.. Even after enrichments, thousands of cells have to be examined to locate one FC. Most groups located FCs by a cumbersome time-consuming manual scanning approach and used techniques such as FISH or PRINS for the detection of these rare cells without evaluating the detection efficacy of either of these techniques in an appropriate *in vitro* model (Krabchi et al., 2006c; Krabchi et al., 2001; Orsetti et al., 1998b; Hamada et al., 1993).

Furthermore, innovative technologies have been developed to relieve the burden of manual scanning but their validation for detection of rare events in clinical practice remained problematic (Johnson et al., 2007; Kilpatrick et al., 2004; Hennerbichler et al., 2002). Accurate evaluation is not only required for validation but also for optimization of different procedures and strategies used for FC selection, isolation, identification and enrichment from MB in order to devise the optimal protocols which could lead to successful NIPD.

In our work, considerable efforts have been made to design an appropriate *in vitro* model for accurate assessment of the detection of rare cellular events. This model has been used in the evaluation of manual scanning (Article I, Chapter I) as well as in the optimization and validation of automatic scanning (Article I, Chapter II). Not only does automation help in realization of NIPD, but it is also essential for research progress in this field.

The same strategy has been also used in the evaluation and comparison of FISH and PRINS techniques after their optimization for the detection of rare cellular events. Results indicated that FISH technique, even when its probe mixed with the dilution buffer, is superior to PRINS technique. This permits a marked reduction of the cost of FISH technique without affecting its ability to detect rare cells (Article I, Chapter II). Automatic scanning has been used to quantify FCs in both euploid and aneuploid pregnancies. Establishment of the exact number of FCs in MB is essential in assessing the feasibility of FCs for NIPD and for the evaluation of FC enrichment procedures. Furthermore, automation and micromanipulation could reduce the level of required enrichment, since purity would not be of utmost importance and maternal contamination won't interfere with automatic scanning. Interestingly, it has been shown that FCs can be detected in greater frequencies in aneuploid pregnancies. A considerable number of aneuploidies cases were studied in this work and these findings were confirmed.

Automatic scanning has been also used in quantification of FCs before and after enrichment by density gradient centrifugation, which is the most common initial step of most enrichment protocols published to date (Article I, Chapter III). We provided evidence that application of this step is associated with a major loss (60-80%) of FCs, which would probably jeopardize subsequent steps of the enrichment protocol. Consequently, the eventual number of FCs available for final analysis may not be sufficient for NIPD. Furthermore, the elimination of aggressive centrifugation significantly reduced FC loss by about 12%. Therefore, elimination of this step or its replacement by the non-aggressive version of the procedure would enhance recovery and cut down the number of enrichment

steps and consequently reduce FC loss, thereby making NIPD using FCs more easily achievable.

We also provided proof of concept of the feasibility of using rare FCs in NIPD through micromanipulation of single cells (Article I, Chapter IV). Satisfactory genomic coverage can be obtained from whole genome amplification of a few cells coupled with QF-PCR analysis. Detection and utilization of as few as five FCs in MB can be quite enough to provide > 90% accuracy in detection of fetal sex and major chromosomal aneuploidies and potentially single gene disorders. Clinically, QF-PCR analysis of short tandem repeats can provide a viable alternative to array-comparative genomic hybridization in NIPD using FCs without a significant increase in health expenditure.

The currently available results are sufficiently eloquent to indicate that FCs could be accessible from MB and could be used for analysis of fetal aneuploidies and inherited mendelian genetic disorders. Unrelenting progressive improvement of the sensitivity and specificity of strategies for detection and isolation of rare events combined with the development of fast and reproducible methods of enrichment, will certainly lead to optimization of a universal test of NIPD through the use of FCs. The possibility of diagnosing fetal genetic disorders, with virtually no risk either to the mother or to the pregnancy, would represent a considerable advance in fetal medicine. This approach would make it possible to offer a systematic prenatal diagnostic test for relatively common genetic aneuploidies such as that of Down syndrome (trisomy 21), Edwards syndrome (trisomy 18), Turner syndrome (monosomy X) as well as single gene disorders to all pregnant women without having to select them according to risk criteria of age, values of the serum markers or echographic measures. At the end of our work, let us stress the fact that offering NIPD to all pregnant women will always be justifiable as it could prevent the birth of children with untreatable genetic disorders. However, it will remain for a long time indivisible from the concept of a medical interruption of pregnancy.

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List of articles

1. **Emad A**, Ayub S, Samassekou O, Grégoire M-C, Gadji M, Ntwari A, Lamoureux J, Hemmings F, Krabchi K, Drouin R: Efficiency of manual scanning in recovering rare cellular events identified by fluorescence in situ hybridization (FISH): simulation of the detection of fetal cells in maternal blood. *J Biomed Biotechnol* 2012; 2012:610856. (Emad et al., 2012)

2. **Emad A[1]**, Bouchard E[1], Lamoureux J[1], Dutta A[2], Klingbeil U[2], Drouin R[1]: Validation of automatic scanning of microscope slides in recovering rare cellular events: application for detection of fetal cells in maternal blood. *Prenat Diagn 34(6): 538-546.* (Emad et al., 2014b)

3. **Emad A**, Drouin R: Evaluation of the impact of density gradient centrifugation on fetal cell loss during enrichment from maternal peripheral blood. *Prenat Diagn 34(9): 878-885.* (Emad and Drouin, 2014)

4. **Emad A[a]**, Lamoureux J[a], Ouellet A[b], Drouin R[a,c]: Rapid aneuploidy detection of chromosomes 13, 18, 21, X and Y using QF-PCR with few microdissected fetal cells. (In press in *Fetal Diagnosis and Therapy*). (Emad et al., 2014a)