A BIOLOGICAL MODEL OF FETOMATERNAL HAEMORRHAGE FOR THE DEVELOPMENT OF FIRST-TRIMESTER NON-INVASIVE PRENATAL DIAGNOSIS

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SUMMARY

Background

Non-invasive methods for obtaining intact fetal cells would permit accurate prenatal diagnosis for aneuploidy and single gene disorders without attendant risks associated with invasive procedures. In chromosomally abnormal fetus such as trisomy 18, existence of fetal primitive nucleated erythroblasts during second trimester and at birth has not yet been identified. If proven, it could be of great clinical significance, as the existence of these cells could indicate not only the presence of a chromosomally abnormal fetus but also imply that the ε-globin primitive fetal nucleated erythroblasts are also ideal cell to be targeted in the second trimester for non-invasive prenatal diagnosis.

Various fetal cell enrichment protocols are available; their efficiency has not been tested appropriately in an *in-vitro* model system and it is important to evaluate the intrinsic protocol efficiency *in-vitro* before studying its enrichment efficiency in an *in-vivo* model.

To date, there is no biological model to determine the *in-vivo* efficiency of any new fetal cell enrichment protocol.

Cell-free fetal DNA has been investigated as a marker for feto-maternal haemorrhage at surgical termination of pregnancy and we planned to use this fetal genetic material as corroborating evidence of the quantity of feto-maternal haemorrhage.

Study Objectives

(i) To evaluate the presence of ε-globin-positive primitive fetal nucleated erythroblasts in trisomy 18 syndrome fetus during second trimester and at birth.

Trisomy 18 was selected as a model to prove this hypothesis. The choice of T18 as an aueploid model to base our investigations was based upon the assumption that the haemoglobin switch is similar in all aneuploidies, and that the placental interface is similar in its leakiness for both T18 and T21. We acknowledge that T18 is rarer than T21 and choosing T18 will limit our ability to gauge what happens in a T21 pregnancy which is more clinically relevant, it was due to the availability of T18 cases in our unit at that point in time and lack of any T21 cases, that we chose to undertake T18 to prove our hypothesis.

(ii) To evaluate efficiency of a new fetal cell enrichment protocol in *in-vitro* model system.

(iii) To develop an *in-vivo* model of biological feto-maternal haemorrhage that could be used to evaluate efficiency of new fetal cell enrichment protocol for non-invasive prenatal diagnosis in the first trimester.

Hypotheses

1. Embryonic epsilon-globin positive nucleated red blood cells persists beyond the second trimester using T18 as a model.

2a. Surgical termination of pregnancy can be used to evaluate efficiency of a new fetal cell enrichment protocol in *in-vitro* model system.

2b. Surgical termination of pregnancy can be used as an *in-vivo* model to study enrichment efficiency of a novel non-invasive prenatal diagnosis method in the first trimester.

Methods

To address the first hypothesis pure sample of fetal blood was obtained via cordocentesis from a viable trisomy 18 fetus (n = 1) and karyotypically normal fetuses (n = 4) during the second trimester. Cord blood samples were obtained from three trisomy 18 and twenty karyotypically normal neonates at term vaginal birth delivery. The fetal blood and cord blood samples were processed using Ficoll 1077 and Percoll 1083 density gradient centrifugation, respectively followed by anti-GPA selection on MACS to obtain glycophorin-A-(GPA)-positive fetal erythroblasts. Morphology of primitive fetal nucleated erythroblasts was evaluated by Wright stain and presence of ε-globin within the cytoplasm was determined using immunocytochemical staining.

To address hypothesis 2a, primitive fetal erythroblasts were isolated from products of conception obtained in the first trimester after surgical termination of pregnancy and were spiked into adult peripheral blood and processed through the three-step enrichment method comprising of first step Percoll 1118 density gradient, second-step enrichment method using anti-CD45/anti-GPA on MACS followed by treatment with ammonium chloride/1mM acetazolamide cocktail lysis buffer. FNRBCs recovered were calculated and identified by Wright's staining and ε-globin immunocytochemistry and ICC-cFISH.

To address hypothesis 2b, two maternal blood samples were collected from each patient: 20 ml prior to surgical termination of pregnancy and another 20 ml within 5 minutes after the surgical procedure (n = 10). They were processed immediately using our three-step enrichment protocol. Cells enriched were cytospun for identification. Fetal gender was confirmed by cFISH on FNRBCs and trophoblasts prepared from trophoblastic villi obtained from same patients.

Cytospun slides were Wright's stained for morphological identification of FNRBCs. Slides containing NRBCs as identified by Wright's staining were de-stained and examined for the presence of fetal ε-globin positive primitive erythroblasts by immunocytochemistry. The locations of these cells were recorded. Slides were then destained in xylene, rinsed in distilled water, put through ICC-cFISH, to examine gender of ε-globin cells.

Both total and cffDNA were quantified using real-time quantitative PCR. TaqMan amplification reactions were set up in a reaction volume of 25 µl. Each reaction contained 1x Taqman Universal-Master-Mix, 240 nM of each amplification primer, 100 nM of the corresponding TaqMan probe and 5 µl of the extracted plasma DNA. Thermal cycling for both *SRY* and β-globin was initiated with 2-min incubation at 50˚C, which followed the first denaturation step for 10 min at 95˚C and then 55 cycles of 95˚C for 15s and 60˚C for 1 min.

Results

Compared to normal controls, the proportion of fetal nucleated red blood cells was found to be 16- and 14-fold higher in a second trimester trisomy 18 fetus (n = 1) and in trisomy 18 neonates at birth (n = 3), respectively. Epsilon-globin-positive primitive fetal nucleated erythroblasts were found both during second trimester and at birth, indicating continued gene expression of epsilon-chain in trisomy 18 aneuploidy.

The three-step enrichment protocol to be tested for its efficiency in *in-vitro* model consisted of Percoll 1118 density-gradient-centrifugation (first-step), anti-CD45 or anti-GPA alone or as anti-CD45/anti-GPA combination (second-step) and selective erythrocyte lysis by combination of NH_4Cl/acetazolamide buffer at the ratio of 1:2 (third-

step). Using anti-CD45/anti-GPA combination gave a pure population of red blood cells compared to their use alone, making the former suitable as the second-step enrichment method. Selective lysis during third step with the combination of NH_4Cl/acetazolamide buffer showed 96% of adult erythrocytes to be lysed and 93% of fetal nucleated red blood cells intact. Thus, a three-step protocol was adopted to test its efficiency in *in-vitro* model.

The efficiency of the three-step enrichment system in *in-vitro* model was 37% (95% CI, 28.5%-45.6%; n = 8). Enriched primitive fetal erythroblasts were accurately identifiable by both ε-globin light microscopic immunocytochemistry and ICC-cFISH.

Using surgical termination of pregnancy as an *in-vivo* model, significantly more FNRBCs and ε-globin positive primitive erythroblasts were recovered from post-termination of pregnancy maternal blood samples (3 and 2.8-fold, respectively). A significant positive correlation was also observed between gestational age and increase in FNRBCs (r=0.66; p=0.03) and ε-globin positive cells (r=0.65; p=0.04).

Increase in FNRBCs in maternal blood after surgical termination of pregnancy was associated with a concomitant increase in ε-globin positive fetal primitive erythroblasts (correlation co-efficient, r=0.82; p=0.004).

In pregnancies with male fetuses (n=8), a significant increase in FNRBCs (3 fold) and ε-globin positive primitive fetal erythroblasts (2.8 fold) was seen after the surgical termination of pregnancy.

There was no change in either the total DNA or the cell-free male fetal DNA levels in maternal plasma after, compared with before, surgical termination of pregnancy.

Therefore, surgical post-termination of pregnancy enrichment of ε-globin positive primitive fetal erythroblasts can estimate the efficiency of that particular enrichment system in on-going pregnancies. It shows that an enrichment protocol can expect three-fold fewer fetal cells in an ongoing first trimester pregnancy than the number of cells enriched from first trimester maternal blood obtained after surgical termination of pregnancy.

A mathematical relationship between the distribution of epsilon positive fetal erythroblasts in pre- and post-termination maternal blood was derived as follows: Observed number of pre-termination primitive FNRBCs = co-efficient between pre- and post-termination primitive FNRBCs x observed number of post-termination primitive FNRBCs. The co-efficient of our model is 0.35 (95%CI, 0.26-0.44) and the regression coefficient, R^2 is 0.89.

Our observations suggest that an accurate *in-vivo* enrichment efficiency of any new enrichment protocol could be developed using maternal blood obtained within 5 minutes after first trimester surgical termination of pregnancy.

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LIST OF ABBREVIATIONS

DNA	Deoxy-ribonucleic acid
cFISH	Chromosomal fluorescence *in-situ* hybridisation
AFP	α-feto protein
AMCA	7-amino-4-methylcoumarin-3-acetic acid
β-hCG	β-subunit of human chorionic gonadotropin
BFU-e	Burst forming units-erythroid
BSA	Bovine serum albumin
CA	carbonic anhydrase
CCD	Cooled coupled device
CFU-e	Colony forming units-erythroid
CGH	Comparative Genomic Hybridisation
CRL	Crown-rump length
CVS	Chorionic villus sampling
DAPI	Diamidino-2-phenyl-indole
DMD	Duchenne Muscular Dystrophy
EDTA	Ethylene-diamine tetraacetic acid
FACS	Fluorescence-activated cell sorting
FISH	fluorescence *in-situ* hybridisation
FITC	Fluorescein isothiocyanate
FNRBC	Fetal nucleated red blood cell
g	Centrifugal g force or grams
GPA	Glycphorin A
Hb	Haemoglobin
HbF	Haemoglobin F; Fetal haemoglobin

HBSS	Hank’s balanced salt solution
HLA	Human leukocyte antigen
ICC	Immunocytochemistry
LCR	Locus control region
Mab	Monoclonal antibody
MACS	Magnetic-activated cell sorting
MHC	Major histocompatibility complex
mRNA	Messenger RNA
MSC	Mesenchymal stem cell
NICHD	National Institute of Child and Health Disease
NRBC	Nucleated red blood cell
NT	Nuchal Translucency
OAPR	Odds of being affected given a positive result
OSCAR	One-stop clinic for assessment of fetal risk
PAPP-A	Pregnancy associated plasma protein A
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFNRBC	Primitive fetal nucleated red blood cell
Rh	Rhesus
rpm	Revolutions per minute
RT-PCR	Reverse transcriptase-PCR
SRY	Sex Reversal Y
SSC	Salted sodium citrate
PBST	Phosphate buffered saline;Tween 20
μE3	Unconjugated oestriol
ZFY	Zinc-finger Y

Chapter 1: Introduction

1.1 Overview

Without prenatal diagnosis, 1 in 50 babies are born with serious physical or mental handicap, and as many as 1 in 30 with some form of congenital malformation (Harper, 1998). These may be due to structural or chromosomal abnormalities, or single gene disorders. The diagnosis of aneuploidy, monogenic disorders and fetal rhesus D status requires invasive testing by amniocentesis, chorionic villus biopsy or fetal blood sampling. These tests carry a procedure-related risk of miscarriage of 1-4% (Tabor et al., 1986; Canadian Collaborative CVS-Amniocentesis Clinical Trial Group 1989; Rhoads et al., 1989; MRC Working Party 1991; Buscaglia et al., 1996; Wald et al., 1998).

In contrast to monogenic conditions which are largely confined to certain ethnic groups or clustered within families, over 90% of the structural or chromosomal abnormalities arise in pregnancies with no specific risk factors. Thus, while prenatal diagnosis for single gene disorders is concentrated on at-risk populations, low-risk populations are offered universal screening for structural anomalies and aneuploidy. Whereas second trimester screening for structural malformations by ultrasonography may at the same time be diagnostic, current prenatal screening for chromosomal abnormalities using biochemical and sonographic markers for aneuploidy is more an antenatal risk-estimation exercise (Chitty, 1998).

The diagnosis of aneuploidy and single gene disorders depends upon recovery of fetal cells and fetal DNA (deoxyribonucleic acid), but the hazard of fetal loss associated with

current invasive methods limits the uptake of these procedures by women identified at increased risk by screening tests (Chitty, 1998).

Observations that cell-free fetal DNA and intact fetal cells can enter and circulate within maternal blood have raised the possibility of non-invasive access to fetal genetic material that would allow the prenatal diagnosis of chromosomal and monogenic disorders (Lo et al., 1990; Walknowska et al., 1969).

Use of circulating fetal DNA has progressed from an idea to clinical application of prenatal diagnosis of fetal RhD status by molecular analysis of maternal plasma (Lo et al., 1998a) mainly because of the significant quantity of fetal DNA within maternal plasma and serum (Lo et al., 1998b) compared with the rarity of intact fetal cells within maternal blood (Bianchi et al., 1997). However, the usefulness of fetal DNA in prenatal genetic diagnosis is limited to a few paternally-inherited monogenic conditions whereas recovery of intact fetal cells would allow accurate genetic diagnoses of all aneuploidies and single gene disorders.

Among the fetal cells identified to-date, fetal nucleated red blood cells (FNRBCs) are the favoured target cells at present because they are the predominant nucleated cell type in the first and early second trimester of pregnancy, they are mononuclear and suitable for chromosomal fluorescence *in-situ* hybridisation (cFISH), their limited lifespan makes them unlikely to persist across pregnancies (Pearson, 1967) and unlike trophoblasts, which demonstrate confined placental mosaicism in 1% of cases (Hahnemann and Vejerslev, 1997), these cells reveal the representative fetal genotype. Recent evidence suggests that ε-globin is the ideal fetal cell marker for non-invasive prenatal diagnosis using fetal cells derived from maternal blood (Choolani et al., 2001; Al-Mufti et al.,

2001; Choolani et al., 2003; Mavrou et al., 2003), and Choolani and colleagues (Choolani et al., 2001; Choolani et al., 2003) have addressed the issue of specific identification of fetal origin of first-trimester primitive nucleated erythroblasts using embryonic ε-globin and the simultaneous fluorescence labelling of this marker with cFISH for non-invasive prenatal diagnosis.

Despite this breakthrough (Choolani et al., 2001; Choolani et al., 2003), enriching these rare cells from maternal blood still remain a challenge: there is only 1 fetal nucleated cell per 10^7 maternal nucleated cells (Bianchi et al., 1997) and five hundred-fold less if compared with maternal anucleate red blood cells. As little can be done to alter the frequency of fetal erythroblasts in maternal blood, researchers have focussed on using various enrichment methods that could help them in enriching these cells from maternal blood for non-invasive prenatal diagnosis. Current enrichment protocols incorporate a magnetically-activated cell sorting (MACS) or fluorescence activated cell sorting (FACS) step to deplete or select target cell groups. In either case, density gradient centrifugation is used as the first-step to deplete maternal anucleate red blood cells. However, the efficiency of enrichment protocols used to-date for this rare cell isolation remains to be established. Few methods have been adopted to test the efficiency of enrichment systems for fetal nucleated erythroblast isolation from maternal blood for non-invasive prenatal diagnosis. While some have attempted to determine the efficiency by applying the protocols directly on maternal blood, others used invasive procedures as *in-vivo* model system to determine the efficiency. This may not be an ideal strategy as the numbers of cells obtained are too small to reflect the efficiency of the enrichment system. In this thesis, I explored the possibility of using termination of pregnancy as an *in-vitro* as well as an *in-vivo* model system to study the efficiency of an enrichment system for first-trimester non-invasive prenatal diagnosis. I also explored the prospect of termination of

pregnancy as a model system to study first-trimester non-invasive prenatal diagnosis using cell-free fetal DNA as a biological marker.

1.2 Current methods of prenatal diagnosis

Current methods of prenatal diagnosis of chromosomal abnormalities and single gene disorders involve diagnostic tests. Screening tests are designed to be non-invasive, safe, sensitive and applicable to a low-risk population. Many prenatal screening tests however, have high false-positive rates. At a detection rate of 60%, 70%, 80% and 90%, the false positive rate of double test is 5.5%, 9.2%, 15.9% and 29.9%, respectively. The false positive rate of triple test is 2.7%, 5.5%, 11.1% and 24.1%, respectively. The false positive rate of quadruple test is 1.6%, 3.3%, 7.0% and 16.5%, respectively (Wald and Hackshaw, 2000). In contrast, diagnostic tests are accurate but may be invasive.

Prenatal diagnosis of structural malformations which occur in 2.1% of pregnancies (Bricker et al., 2000) can be diagnosed non-invasively using ultrasonography. Since most structural abnormalities occur in the low-risk population, routine screening scans are offered to all pregnant women in Singapore between 20-22 weeks. The sensitivity of this technique, which ranges from 2.3-100%, depends on the gestational age, scanning policy, skill of the operator, type of machines being used and the type of anomalies included in the evaluation.

Currently, the diagnosis of chromosomal, monogenic disorders and most fetal rhesus D status requires invasive testing by amniocentesis, chorionic villus sampling (CVS) or fetal blood sampling (FBS). These tests carry a small but definite risk of procedure-

related fetal loss. As such, non-invasive screening tests have been devised to identify high-risk population for targeted diagnostic invasive testing.

1.3 Screening for chromosomal disorders

A screening test is not diagnostic. It distinguishes between those who are at high risk of being affected from those not being affected. The more effective the screening test, the better the discrimination. The three screening parameters relevant to the mother are detection rate which is the proportion of affected individuals with a screen positive result, false-positive rate, which is the proportion of unaffected individuals with a screen positive result and OAPR (the odds of being affected given a positive result).

In Down syndrome screening, a woman is judged screen positive if her risk of having an affected pregnancy based on her age and marker levels exceeds a specified cut-off level. Usually the cut-off level is chosen to yield a 5% false-positive rate and the corresponding detection rate is determined.

The expected rate of major chromosomal anomalies in live births of a given population is 0.65% including trisomy 21 with a frequency of 0.12% (Hsu, 1992). For this reason, invasive testing is confined to a selected population at high-risk for chromosomal anomalies in order to reduce the total number of chromosomally normal healthy fetuses lost due to the procedure.

Trisomy 21 is the commonest chromosomal aneuploidy seen to reach viability and carries a significant risk of long-term morbidity and mental handicap. It has an estimated birth prevalence of 1.3 per 1000 live births (Cuckle et al., 1991). Trisomy 13 and 18, on the other hand are usually lethal in-utero or in the first few months of life, and have a birth

prevalence of 0.15 and 0.08 per 1000 live births, respectively (Robert, 2000). Therefore most prenatal screening programmes have been designed to detect Down syndrome.

Maternal Age: Maternal age is being used to estimate the risk of chromosomal abnormalities based on the fact that the risk increases with maternal age and a cut-off level of 35 years (when the risk of Down syndrome is around 1 in 280) in United Kingdom has been placed, and all women at or above 35 years at the time of delivery are offered invasive testing (Surbek and Holzgreve, 2001). Cut-off level of 35 was used initially in UK, as that equates to 5% of all deliveries in UK and it happen to correspond to 1 in 280 risk of down syndrome, a decision made by department of health. Considering the fact that 36,900 deliveries took place in Singapore in 2004 (Ministry of Health, Singapore, 2004), and 20% of all pregnancies were above 35 years of age, and the Down syndrome prevalence being 1.17/1000 live births (Lai et al., 2002), the expected number of cases of Down syndrome occurring in 2004, are 43. As 30% of cases of Down syndrome occur in women above 35 years of age (in U.K) (Chitty, 1998; Wald et al., 1998), 7380 women may have undergone amniocentesis to identify 11 cases of Down syndrome. Therefore, to identify 1 case of Down syndrome 671 women needed to undergo amniocentesis and 7 of them may have had procedure-related miscarriage. Further, 70% of affected neonates are born to mothers under 35 years of age, whom will be classified as low low-risk using maternal age alone.

Strategies which have been developed to estimate the risk for chromosomal anomalies in individual pregnancies includes serum biochemical markers and ultrasound measured nuchal translucency (NT) so as to improve the sensitivity and specificity of detection of Down syndrome. Information from these tests are analysed together with the maternal age to determine the woman's individual risk of having a baby with Down syndrome. It

is known that, about 80% of pregnant women would choose to have a screening test for Down syndrome if available and 90% of those with affected fetuses would terminate the pregnancy (Wald et al., 1998).

Biochemical markers: Biochemical screening in the second trimester include hormonal markers such as alpha-fetoprotein (AFP), unconjugated oestriol (uE_3), free β-subunit of human chorionic gonadotrophin (β-hCG) and dimeric inhibin-A. In 1984, low maternal serum AFP levels were found to be associated with Down syndrome (Merkatz et al., 1984; Cuckle et al., 1984). Later, free β-hCG level was found to be raised (Bogart et al., 1987) and the uE_3 reduced in Down syndrome fetuses (Canick et al., 1988; Wald et al., 1988a). In 1988, the three were combined with maternal age and were coined a name of triple test (Wald et al., 1988b). In 90's, a new second trimester marker dimeric inhibin-A was described (Van Lith et al., 1992; Wald et al., 1996; Wald et al., 1997a), the levels of which was found to be twice as great as those in unaffected pregnancies of the same gestational age (1.91 multiples of the median) (Wald and Hackshaw, 2000). Multiple of the median is a measure of how far an individual test result deviates from the median. MoM is used where the median is highly variable. The test is usually performed between 14-21 weeks as the double test (AFP, β-hCG), triple test (AFP, β-hCG, uE_3) or quadruple test (AFP, β-hCG, uE_3, inhibin-A). At a false positive rate of 5%, the detection rates are 58%, 69% and 75% respectively, for the double, triple and quadruple test and an OAPR of 1:65-50, depending on whether two, three or four biochemical parameters are used (Wald et al., 1998).

Nuchal Translucency screening: Measurement of fetal nuchal translucency (NT) thickness provides an effective screening for Down syndrome in the first-trimester (Szabo and Gellen, 1990). The mechanism of this increased NT (an echolucent space) is

not understood but seems to be related to minor cardiac anomalies in chromosomally abnormal fetuses (Hyett et al., 1999). NT measurements increase with gestational age. Five percent of normal fetuses have NT measurements greater than 2.2mm to 2.8mm at crown-rump lengths (CRLs) of 38 and 84mm, respectively (Pandya et al., 1995). Subsequently, 14 studies were published from different research groups over a period of 4 years. Snijders et al. (1998) looked at a sufficient number of patients to allow assessment of the sensitivity of the method (96,127 patients between 10-14 weeks). They took maternal age and NT into account in the calculation of risks. They demonstrated that at a false-positive rate of 8.3%, NT screening detected 82% of fetuses with Down syndrome. For a false positive rate of 5%, the detection rate was 77%.

Combined test: Wald and Hackshaw (1997b) reported a combination of four parameters for use in the first trimester (10-14 weeks) screening for Down sydnrome: maternal age, first-trimester maternal screening marker Pregnancy Associated Plasma Protein A (PAPP-A) which was found to be 60% lower (0.38 multiples of the median in pregnancies with Down syndrome), free β-hCG which was 80% higher in affected pregnancies (1.83 multiples of the median) and NT. They demonstrated a detection rate of 85% using the combined test at 5% false-positive rate. A recent prospective study (Bindra et al., 2002; Spencer et al., 2003) have demonstrated that screening for trisomy 21 in a one-stop clinics for early assessment of fetal risk (OSCAR) setting by the combination of four parameters at 11-14 weeks gestation is associated with a detection rate of 90% for a false-positive rate of 5%.

Recently, Cicero and colleagues (2001) reported that in about 70% of fetuses with trisomy 21, the nasal bone is absent at the 11^{th} to 14^{th} week ultrasound scan. When this marker was incorporated together with NT, maternal age, free-βhCG and PAPP-A in the

first-trimester screening, a 90% detection rate could be retained with a simultaneous ten-fold reduction in the false-positive rate from 5% to 0.5%. For a 5% false-positive rate, the detection rate could increase to 97% (Cicero et al., 2003).

Integrated test: Since first- and second-trimester serum screening and NT assessment are independent measures of the risk of a Down syndrome pregnancy, adjusting the a priori risk of maternal age, recent efforts have attempted to combine first - and second-trimester serum screening into the integrated test. This involves both the first- and second-trimester tests and holds the information, finally providing the patient with a single screening risk estimate result. The integrated test involves first-trimester PAPP-A and NT and second-trimester free-βhCG, AFP, uE_3 and inhibin-A to derive a single risk figure for the test (Wald et al., 1999). Recently, a prospective Serum, Urine and Ultrasound Screening Study (SURUSS) were carried out on 47,053 singleton pregnancies (including 101 pregnancies with Down syndrome). NT measurements were also taken. Serum and urine samples were collected between 9 and 13 weeks, and again between 14 and 20 weeks of pregnancy. For an 85% Down syndrome detection rate, the false-positive rate for the integrated test (NT and PAPP-A) at 11 completed weeks of pregnancy, and uE_3, AFP, free β-hCG and inhibin-A in the early second trimester, serum integrated test (without NT), combined test (NT with free β-hCG and PAPP-A at 11 weeks), quadruple test (AFP, uE_3, free β-hCG and inhibin-A) and NT alone at 11 weeks were 0.9%, 2.7%, 4.3%, 6.2%, and 15.2% respectively. All tests included maternal age. Using the integrated test at an 85% detection rate, there would be six diagnostic procedure-related unaffected fetal losses following amniocentesis per 100,000 women screened compared with 35 using the combined test or 45 with the quadruple test. It was concluded that integrated test offers the most effective and safe method of screening for women who attend in the first trimester followed by serum-integrated test. While the

quadruple test is most effective for women who first attend in the second trimester, the authors were not in the favour for retaining the double or triple tests, or NT alone (with or without maternal age) in antenatal screening for Down syndrome (Wald et al., 2003).

Recently, a US National Institute of Child Health and Human Development (NICHD) funded large trial named the FASTER (First and Second Trimester Evaluation of Risk for Aneuploidy) trial conducted at 15 centres in United States (Dolan, 2004). The trial involved 33,557 pregnant women at 15 centers who underwent combined first-trimester screening with NT measurement and serum testing for the biochemical markers PAPP-A and free β-hCG between 10^{+3} to 13^{+6} week gestation. The women also underwent second-trimester quadruple screening between 15 and 18 weeks gestation. The trial revealed that integrated screening yields 90% sensitivity in screening for Down syndrome, with a 5.4% false-positive rate.

Integrated screening strategy however has remained controversial. This is because the first-trimester results need to be withheld from women until the second-trimester markers have been measured. Further, clinical staff may find non-disclosure of high-risk first-trimester results unacceptable, believing that the interests of individual patients may be better served by immediate counselling for invasive prenatal diagnosis (Herman et al., 2002). Similarly, the patients may put considerable pressure on the clinicians to reveal intermediate findings since for social and personal reasons they would prefer to terminate an abnormal fetus as early as possible (Copel and Bahado-Singh, 1999). Also there is a possibility of loss of follow up of the patients and finally if they fall in high-risk category at around 16 weeks, they may have to undergo termination of pregnancy using intra-amniotic prostaglandin instillation or prostaglandins or dilatation and evacuation, which is more complicated then termination of pregnancy in the first trimester. These concerns

have provoked others to recommend a policy of routine disclosure (Herman et al., 2002) despite the concomitant considerable increase in false-positive rate for any given detection rate (Wald et al., 2003; Cuckle and Arbuzova, 2004).

Recently, Wright and co-workers (2004) have reported contingent screening for Down syndrome. To present the first and second-trimester Down syndrome screening strategy, the second-trimester marker determination is contingent on the first-trimester results. Unlike the integrated test, which requires all women to have markers in both trimesters, this allows large proportion of the women to complete screening in the first trimester. The authors developed contingent sequential screening by using statistical modelling to define optimal first-trimester upper and lower risk cut-offs which describes three types of results, namely the positive for early diagnosis, negative with screening complete and intermediate needing second-trimester markers. Multivariate Gaussian modelling with Monte Carlo simulation was used to estimate the false-positive rate for a fixed 85% detection rate. Simulation refers to any analytical method meant to imitate a real-life system, especially when other analyses are too mathematically complex or too difficult to reproduce. Without the aid of simulation, a spreadsheet model will only reveal a single outcome, generally the most likely or average scenario. Spreadsheet risk analysis uses both a spreadsheet model and simulation to automatically analyze the effect of varying inputs on outputs of the modeled system. One type of spreadsheet simulation is Monte Carlo simulation, which randomly generates values for uncertain variables over and over to simulate a model. Model parameters were taken from the SURUSS trial. It was concluded that contingent screening can achieve results comparable with the integrated test with second-trimester screening being avoided in up to 80% of the unaffected pregnancies. However, both the strategies need to be evaluated in large-scale prospective studies particularly in relation to psychological impact and practicability.

The optimal screening method for Down syndrome has yet to be decided. Compared with screening by maternal age alone, detection rates have improved significantly to 95% with the introduction of the additional screeing modalities from 30%. Unfortunately, the invasive testing rate remains high at 5%. As 36,900 deliveries took place in Singapore the previous year (2004), 1845 women may have undergone invasive procedure to identify 37 cases of Down syndrome with consequent 18 miscarriages associated with the procedure.

1.4 Screening for Genetic Disorders

Missed or late diagnoses make estimations of the incidence of genetic disease at birth less accurate, but it has been estimated that it may be as high as 1.7% (Polani, 1973). Of the many genetic diseases described to-date, only a few warrant more than a family history as a method of prenatal screening and most of these conditions are autosomal recessive disorders These include cystic fibrosis amongst Caucasians, Tay-Sachs diseases within the Ashkenazi, Sickle cell disease in individuals of African descent and the thalassaemias in Mediterranean, Middle East and Asian people. Within these at-risk populations, the parents-to-be may be screened using biochemical or genetic analysis to determine their carrier status. The tests may investigate mutations on the gene, altered protein production or altered protein function. Invasive prenatal testing is offered to couples at increased risk (>/= 25%) of having an affected fetus.

1.5 Diagnosis of Chromosomal and Genetic Disorders

In contrast to screening test, definitive diagnostic test for these conditions require invasive testing by amniocentesis, chorionic villus sampling (CVS) or fetal blood sampling (FBS). The method used depends upon the gestational age, the condition being

investigated, tests already performed, the expertise available, the risks of the procedure and parental preference.

1.5.1 Invasive procedures for diagnosis of chromosomal and genetic disorders

Amniocentesis

Amniocentesis is usually performed at 15 to 17 weeks of gestation when the uterus is an abdominal organ that can be sampled with minimal risk of injury to the maternal bowel. Before the development of real-time ultrasound, amniocentesis was performed blindly and was responsible for anecdotal cases of exsanguinations, intestinal atresia, gangrene, fistulae, brain damage and blindness in the fetus. Real-time ultrasound enabled greater precision and its use is now a standard care. Prior to amniocentesis, ultrasound examination is performed to evaluate fetal number and viability to confirm gestational age by fetal biometric measurements, to estimate amniotic fluid volume and to establish placental localisation. While avoidance of the placenta during the ultrasound-guided procedure reduces the likelihood of maternal rhesus (Rh) alloimmunisation in Rh-negative women, in 2-3% of second trimester amniocentesis, a fetal-to-maternal haemorrhage of at least 0.1ml still occurs and this haemorrhage leads to Rh-isoimmunisation in 2.1-5.4% of the at-risk pregnancies (Bowman and Pollock, 1985). This complication is preventable by prophylactic administration of anti-D immunoglobin to Rh-negative women before amniocentesis (Bradbenberg et al., 1989). Following the scan, a needle insertion site is selected and a 22-guage spinal needle is introduced into the amniotic cavity through an aseptically prepared area on the maternal abdomen and amniotic fluid is aspirated.

In the mid 70s it was suggested that amniocentesis before 15 weeks is associated with an unacceptable procedural and culture failure rate (Golbus et al., 1979). The advent of high-resolution ultrasound and direct needle guidance made the amniotic sac accessible as early as 7 weeks (Barbara and Wapner, 2002). This, together with the development of improved tissue culture methods, improved laboratory capability requiring less fluid and fewer cells for cytogenetic analysis, led numerous investigators to a reconsideration of amniocentesis earlier in gestation (Hanson and Tennant, 1990; Nevin et al., 1990; Penso et al., 1990; Stripparo et al., 1990; Thayer et al., 1990; Hanson et al., 1992; Jorgensen et al., 1992). However, recent randomised trials have demonstrated a number of concerns related to early amniocentesis performed prior to 14 weeks, the most important being a 10-fold increase in the risk of severe talipes equinovarus (The Canadian Early and Mid-Trimester Amniocentesis Trial Group, 1998). The CEMAT group comparison of early amniocentesis and mid-trimester sampling demonstrated a twofold-increased risk of fluid leakage with the earlier procedure. When this occurred, there was a 15% incidence of talipes equinovarus. Without fluid leakage, the incidence of talipes was still approximately 10-fold higher than had been expected. Also, early amniocentesis demonstrated a 1.7% increased risk of pregnancy loss compared with the mid-trimester amniocentesis.

The primary concern for patients undergoing prenatal testing is the chance that the procedure will lead to the loss of the desired pregnancy. The first major prospective study of genetic amniocentesis, which included 1040 subjects and 992 matched controls, was conducted by the US National Institute of Child Health and Human Development (NICHD) (National Registry for Amniocentesis Study Group, 1976). Of all women who underwent amniocentesis, 3.5% experienced fetal loss between the time of the procedure and delivery compared with 3.2% of controls. The small difference was not statistically

significant and disappeared completely when corrected for maternal age. In Canada, a collaborative group conducted a cohort study but did not include a concurrent control group (Simpson et al., 1976; Medical Research Council, Canada, 1977). The pregnancy loss rate was 3.2%, a frequency similar to that reported in the US collaborative study.

A British collaborative study (United Kingdom Medical Research Council Working Party on Amniocentesis, 1978) found that the rate of fetal loss following amniocentesis was significantly higher than in controls (2.6% versus 1%). In this study, however, a common indication for amniocentesis was elevated maternal serum alpha-fetoprotein (MSAFP) now recognised on its own as a factor associated with increased fetal loss and adverse perinatal outcome. After excluding subjects undergoing amniocentesis for that indication, the loss rates between subject and control groups narrowed to less than 1%, although still a statistically significant difference (NICHD Consensus Conference on Antenatal Diagnosis, 1979).

The relevance of the collaborative studies cited above has been questioned because they were not conducted with high quality ultrasonography as defined by today's standards, nor was concurrent ultrasonography even universally applied. The Danish group, the only study, which is based on large prospective, randomised controlled trial, addressed this criticism in the 1980s and found even in the hands of experienced operators the excess risk of miscarriage due to amniocentesis, was as high as 1% (Tabor et al., 1986). Recently, Seeds (2004) reviewed reports of more than 1000 amniocentesis to establish an estimate of procedure-related pregnancy loss after mid-trimester amniocentesis. They found amniocentesis with concurrent ultrasound guidance in controlled studies was associated with the procedure-related pregnancy loss of 0.6% above the baseline risk of 1.08% due to natural loss without amniocentesis among control patients.

Chorionic Villus Sampling

CVS is usually performed in one of two approaches between 10-13 weeks. In the original, transcervical approach, the procedure is performed with the patient in the lithotomy position. A catheter or biopsy forceps is inserted through the cervix (aseptically prepared) into the placental substance. Transabdominal CVS, as now widely performed, is similar to amniocentesis in that a needle is inserted through the maternal abdomen into the long axis of the placenta. In both procedures, a tissue sample is drawn by negative pressure into a heparinised syringe containing nutrient medium.

Fresh sample of fetal chorionic villi are contaminated by maternal decidua and blood. Thus, in order to prevent culturing maternal decidual cells, the tissues need to be separated by microdissection (Roberts et al., 1988). The incidence of confined placental mosaicism, a discrepancy between the chromosomal complement of the fetus and its placenta that occurs due to post-zygotic mitotic errors during embryonic development in CVS specimens is 1% (Hahnemann and Vejerslev, 1997). In these cases, fetal aneuploidy needs to be confirmed by a second invasive procedure (amniocentesis or fetal blood sampling) before termination of pregnancy can be considered.

It is generally accepted that CVS is a slightly more risky procedure than mid-trimester amniocentesis, with a procedure related miscarriage rate around 2%. Three large prospective collaborative studies compared the risks of these two procedures. In the Canadian (Canadian Collaborative CVS/Amniocentesis Clinical Trial Group, 1989) and US (Rhoads et al., 1989) trials, an excess risk of fetal loss of 0.6% and 0.8% respectively for CVS, was found not to be statistically significant. Both these trials were conducted in only a few centres: the Canadian trial in 11 and the US trial in seven. In contrast, 31

centres participated in the European randomised trial (MRC Working Party, 1991) with an average of 52 cases recruited in each. In this more widespread study by (possibly) less experienced operators, the risk of fetal loss due to CVS was 4.6% higher than amniocentesis (95% CI, 1.6% - 7.5%).

Transcervical CVS is associated with a greater risk of fetal loss than transabdominal CVS. Meta-analysis of randomised trials suggests that transcervical CVS has a 3.7% excess fetal loss rate compared with mid-trimester amniocentesis (Wald et al., 1998). There was also a non-significant trend for the transabdominal approach to be associated with a lower risk of miscarriage compared with transcervical CVS, making its safety comparable to mid-trimester amniocentesis (Smidt-Jensen et al., 1992; Wald et al., 1998). The transabdominal approach is now preferred and there is a case for CVS to be performed in tertiary referral centres where there is considerable expertise. Thus fetal loss from CVS is at least as high as that of amniocentesis, and at least on a global scale, probably higher.

With CVS, as with amniocentesis, severe complications such as septicaemia in the mother or limb reduction defects in the fetus are rare but subchorionic haematomata and vaginal spotting occur in up to 4% of cases after transcervical CVS (Brambati et al., 1987). Since the first report of limb reduction abnormalities, such as the oromandibular-limb hypogenesis syndrome, occurring after CVS (Firth et al., 1991) several large studies have failed to confirm this association (Hsieh et al., 1995; Froster and Jackson, 1996). In the largest of these studies published from the World Health Organisation International Registry and based on data from 63 centres, 77 (0.06%) of the 138,996 children born after CVS, had limb-defects. This suggested there was no increase in risk compared with the general population. The latest WHO report continued to show the same results

(WHO/PAHO consultation on CVS, 1999). Early cases of CVS however were not included in these studies and it has been suggested that procedures performed prior to 10 weeks may carry a risk of severe limb defects as high as 2% (Jenkins and Wapner, 1999).

Fetal Blood Sampling

Fetal whole blood is used for the extraction of fetal DNA while fetal white blood cells (lymphocytes) can be cultured rapidly to obtain metaphase spreads for karyotyping. However, the need to use fetal blood for prenatal diagnosis is declining as (i) abnormalities are increasingly identified by screening and diagnostic methods earlier in pregnancy, and (ii) as molecular testing replaces biochemical and haematological assays, and (iii) as rapid exclusion aneuploidy can now be achieved in more accessible and earlier samples by molecular methods.

Fetal blood sampling procedure is associated with a risk of miscarriage of about 2% (Buscaglia et al., 1996). The risk increases if the procedure is performed before 18 weeeks (Orlandi et al., 1990) or in growth restricted or hydropic fetuses (Maxwell et al., 1991). It is thought that the procedure-related loss rate is about 1% (Weiner and Okamura, 1996; Tongsong et al., 2000) and that the main causes of fetal loss are rupture of membranes (pre-term, pre-labour), chorioamnionitis, puncture of the umbilical artery, bleeding from the puncture site and prolonged bradycardia.

1.5.2 Laboratory analysis of fetal material after invasive testing

Amniocentesis: Amniotic fluid contains cells, enzymes, metabolites and cell-free deoxy-ribonucleic acid (DNA). Amniotic fluid cells can be cultured to contain metaphase spreads for karyotyping or analysed directly by chromosomal fluorescence *in-situ*

hybridisation (cFISH) (Klinger et al., 1992; American College of Medical Genetics, 1999). Enzymes and some metabolites from cultured and uncultured cell and from the supernatant can be used to detect inborn errors of metabolism but has largely been replaced by molecular testing. The DNA extracted from cultured and uncultured cell can be used for analyses by polymerase chain reaction (PCR). Amnio-PCR, the amplification and quantification of specific regions within the DNA of uncultured amniocytes using PCR, is being considered as an alternative to conventional karyotyping because the results are available within two days of the amniocentesis (Levette et al., 2001; Leung et al., 2003). The amniotic fluid also contains fetal DNA suitable for PCR-based diagnosis. Recently, the cell-free fetal DNA extracted from amniotic fluid can be analysed using comparative genomic hybridisation (CGH) micro-array to identify fetal sex and aneuploidy (Larrabee et al., 2004).

CVS: The chorionic villi represent a source of fetal DNA that can be obtained in the first trimester without the need for cell culture. The average sample contains 15-30 mg of villous tissue. Uncultured mononuclear cytotrophoblast cells from such samples, when exposed to colchicine, generally contain a sufficient number of cells in mitotic metaphase to allow rapid karyotyping and banding studies (Simoni et al., 1983). The mesenchymal core of the villi can be cultured and accurately karyotyped in the conventional fashion. Since enzymes are present in sufficient quantity to allow direct measurement or bioassay, biochemical testing for metabolic disease is possible in fresh uncultured villi; again this has largely been replaced by molecular testing.

Fetal Blood Sampling: Fetal whole blood is used for the extraction of fetal DNA while fetal white blood cells can be cultured readily to obtain metaphase spreads for karyotyping. However, the need of FBS for prenatal diagnosis has now been replaced by

methods which can be carried out earlier in pregnancy such as CVS and amniocentesis. Further, rapid prenatal diagnosis using molecular methods such as FISH can now be applied to these more accessible and earlier samples (Lapidot et al., 1996; Pierlugi et al., 1996).

With the availability of new biochemical and ultrasound screening markers for identifying women at high-risk of carrying an aneuploid fetus, detection rates have improved significantly. However, the number of women undergoing unnecessary invasive procedures and the subsequent loss of a healthy fetus remain a cause of concern. On the other hand, definitive diagnosis of fetal chromosomal and monogenic disease requires access to fetal cells and/or fetal DNA which can be currently obtained by invasive methods. These procedures carry the risk of miscarriage and therefore limit the uptake of prenatal diagnosis for these conditions. Hence, there is a need for a risk-free non-invasive method for prenatal diagnosis.

1.6 Developments on non-invasive prenatal diagnosis of chromosomal and single gene disorders

The demonstration that fetal cells (Walknowska et al., 1969) and fetal DNA (Lo et al., 1990) can be obtained from maternal blood, and that chorionic cells that shed into the maternal cervix can also be retrieved (Shettles, 1971), raised the possibility of obtaining fetal genetic material for prenatal diagnosis without the need for invasive testing. The debate as to which fetal material (cells or DNA) and which source (blood or trans-cervical aspirate) to use continues, most investigators in this field currently analyse maternal blood because it contains both fetal DNA and fetal cells and is less likely to be contaminated by foreign cells or DNA (e.g. spermatozoa). There is relatively much more

cell-free fetal DNA in maternal blood than intact fetal cells (Lo et al., 1998b), but the usefulness of cell-free fetal DNA is limited to paternally-inherited fetal conditions for which the mother is not a carrier. Analysis of intact fetal cells would allow a wider range of diagnoses to be made, including the detection of fetal aneuploidy. However, their rarity in maternal blood has hindered the development for non-invasive prenatal diagnosis. It is likely that any clinical test ultimately developed that analyses fetal material within maternal blood will use both cell-free fetal DNA and intact fetal cells to determine the chromosomal and genetic status of the fetus.

1.6.1 Cell-free fetal DNA in maternal plasma

The demonstration of relatively large amounts of circulating cell-free fetal DNA in maternal blood (Lo et al., 1997) revealed yet another source of fetal genetic material that could be used for non-invasive prenatal diagnosis (Bianchi, 1998). Fetal DNA can be extracted from maternal plasma and serum (Lo et al., 1997; Houfflin Debarge et al., 2000; Honda et al., 2001). It is detectable as early as 7 weeks and increases as pregnancy advances (Lo et al., 1997). This sharp increase in fetal DNA in maternal plasma and serum in the late pregnancy stage could be related to gradual breakdown of the maternal fetal interface/placental barrier (Bianchi, 2000a; Bianchi, 2000b). Chan et al. (2003) performed serial analysis of fetal DNA concentrations in late pregnancy and observed a mean increase of 29.3% of fetal DNA each week during the late third-trimester.

The mean half-life of fetal DNA in maternal blood has been estimated by real-time quantitative PCR (Taqman system, Applied Biosystems, Foster City CA) in postnatal samples to be 16.3 min (range 4-30 min) suggesting that the level of fetal DNA in maternal blood reflects a real-time balance between production and clearance (Lo et al.,

1999a) and that the placenta is the likely source of cell-free nucleic acids circulating in the maternal blood. Fetal DNA was reliably detected in up to 80% of cases in as little as 10 μl of maternal plasma (Lo et al., 1997) and comprised 3.4% and 6.2% of total DNA in maternal plasma, in early and late pregnancy respectively (Lo et al., 1998b). The concentration of fetal DNA in the plasma fraction of maternal blood is 21-fold greater than in the cellular fraction (Lo et al., 1998b). Together, these findings suggest that the source of the fetal DNA in maternal blood is more likely to be the continous cellular remodelling at the maternal-placental-fetal interface rather than immune destruction or apoptotic disintegration of the fetal cells that enter and circulate within maternal blood (Sekizawa et al., 2000; Van Wijk et al., 2000; Kolialexi et al., 2001). In their recent finding (Zhong et al., 2002), the group were not able to observe any relationship between fetal nucleated erythroblast numbers and cell-free fetal DNA levels in the first- and second-trimester normal pregnancies by a combination of XY FISH on enriched fetal cells and real-time PCR on the plasma DNA. Similarly, no change in fetal DNA levels in maternal plasma were observed for up to 24 hours in the collection tube following drawing of blood (Angert et al., 2003). These results indicate that cell-free fetal DNA could most likely be coming from the placenta and may not be the result of fetal cell trafficking. Evidence also comes from strong correlation noted between fetal DNA and hCG concentration when measured from the same maternal sample (Sekizawa et al., 2001; Ohashi et al., 2002).

It has been suggested that the amount of fetal DNA in maternal plasma is increased in cases of Down syndrome (Lo et al., 1999b; Zhong et al., 2000). These studies aimed to expand on an earlier observation of a six-fold increase in fetal-cell DNA equivalents in maternal blood when the fetus was trisomic for chromosome 21 (Bianchi et al., 1997). In two of these studies (Bianchi et al., 1997; Lo et al., 1999b) the results were confounded

by the fact that in most pregnancies complicated with Down syndrome, maternal blood was obtained after amniocentesis. In all three studies, although the amount of total fetal DNA between aneuploid and euploid fetuses was significantly different, the number of valid comparisons was small and there was considerable overlap. Recently, a 1.7 (Lee et al., 2002) and a 1.8 fold (Wataganara et al., 2003) increase in fetal DNA in maternal serum was observed in trisomic pregnancies as compared to controls. In contrast, no difference was observed in the fetal:maternal DNA ratio in maternal plasma of patients with affected trisomy 21 fetuses compared to normal controls (Hromadnikova et al. (2002a). Similarly, no difference was found in the fetal DNA concentrations in pregnancies carrying euploid (n=55) and aneuploid (n=9) fetuses (Ohashi et al., 2002). This could be because they found a large variation in the normal range (18-fold difference between the maximum and minimum values). There is also a daily variation in the amount of free fetal DNA present in the plasma of the same mother (Hahn et al., 2001). Recently, a scientific group (Farina et al., 2003) evaluated the use of circulating serum fetal DNA as a second-trimester maternal serum marker of Down syndrome. They found the median fetal DNA concentration to be 1.7-fold greater in Down syndrome cases than in controls. When incorporated with the quadruple marker screen, non-invasive aneuploidy detection improved to 86% at a fixed 5% false-positive rate.

Before circulating fetal DNA can be evaluated as a method of first-trimester screening for fetal trisomies, consistent and reproducible results are first required for increased concentration of fetal DNA in aneuploid pregnancies detected from maternal plasma and serum. The above studies are limited by fetal gender, and widespread implementation of fetal DNA quantitation awaits development of accurate gender-independent markers.

The relatively high fetal DNA in maternal plasma and serum has permitted the non-invasive prenatal diagnosis of fetal rhesus (Rh) D status (Lo et al., 1998b). It has now shown to be highly accurate by several groups and has been introduced as a routine service by the British National Blood Service since 2001 (Finning et al., 2004). This is the first routine use of non-invasive DNA-based prenatal diagnosis. Other genetic applications of fetal DNA in maternal plasma include the detection of myotonic dystrophy (Amicucci et al., 2000) achondroplasia (Saito et al., 2000), cystic fibrosis (Gonzalez-Gonzalez et al., 2002), Huntington disease (Gonzalez-Gonzalez et al., 2003), congenital adrenal hyperplasia (Rijnders et al., 2001; Chiu et al., 2002) and β-thalassaemia (Chiu et al., 2004; Li et al., 2005). The main limitation in all these examples is that the fetal genes or mutations detected are paternally-inherited and genetically different from corresponding sequences in the mother. 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 to non-invasive prenatal diagnosis of the common aneuploidies or monogenic disorders.

1.6.2 Transcervical sampling of fetal cells

Shedding of the trophoblastic cells from the placenta into the cervical canal and its subsequent retrieval for non-invasive prenatal sex determination was observed more than three decades ago (Shettles et al., 1971). This pioneering observation was confirmed by a scientific group four years later (Rhine et al., 1975) who correctly predicted the fetal sex in 31 out of 36 cases examined.

The advent of PCR and cFISH revolutionised molecular diagnostics a decade ago. Utilising this technology, the first prenatal diagnosis of a fetus with trisomy 18 by the use of FISH probes on 26% of the trophoblast cells were reported (Adinolfi et al., 1993). The cells were obtained by transcervical flushing. Since then, transcervical fetal cells have been actively investigated as a source of genetic material for non-invasive prenatal diagnosis (Adinolfi, 1995; Adinolfi and Sherlock, 1997, Adinolfi and Sherlock, 2001). Mucus sampling is considered as the best option because unlike transcervical cell sampling technique that involves the flushing of the endocervical or the uterine cavity, it seems to be deprived of any invasiveness. Use of cytobrush for mucus collection makes this sampling method similar to a Pap smear, which is not considered harmful in pregnancy (Rodeck et al. 1995; Fejgin et al. 2001). However, transcervical sampling has been found to be more effective in fetal cell recovery (Chang et al., 1997; Cioni et al., 2002) than the less invasive cervical mucus aspiration (Daryani et al., 2000) but has also been implicated in fetal limb reduction defects (Chou et al., 1997). Recently, Ian Findlay and his group were able to demonstrate the efficiency of serially enriching fetal cells from pap smears and identifying such cells as fetal using single cell DNA fingerprinting (International Genetics Congress, Melbourne 2003). Although efforts to use transcervically derived fetal cells for non-invasive prenatal diagnosis continue (Cirigliano et al., 1999; Ergln et al., 2001), poor recovery of fetal cells (Overton et al., 1996; Cioni et al., 2003), contamination by foreign genetic material (Daryani et al., 1997) and considerable variation in the composition and quality of recovered material (Miller et al., 1999) limit its applicability for non-invasive prenatal diagnosis.

1.6.3 Fetal cells in maternal blood

In 1893, Schmorl first documented the presence of fetal-derived trophoblast sprouts in the lungs of women who died from complications of eclampsia. Although, similar observations were reported by Douglas and co-workers (1959) and Attwood and Park (1960), the definitive proof that fetal cells circulate in maternal blood came in 1969 when lymphocytes carrying an X and a Y chromosome were detected in the peripheral blood of pregnant women (Walknowska, 1960). Convincing evidence of the existence of fetal cells in maternal blood came in 1990 with the advent of sophisticated molecular genetic techniques such as cFISH and PCR (Simpson and Elias, 1993). Currently, the research in this area not only focuses on the understanding of their biological role and effect in the mother but also how to isolate and use these for non-invasive prenatal diagnosis.

1.6.3.1 Candidate target cells for non-invasive prenatal diagnosis

Trophoblasts: Trophoblast cells are formed very early in gestation, have a short replication time and circulate in the maternal blood as a consequence of their invasive and proliferative nature. These cells have a unique morphology, which permits definitive microscopic identification (Pertl and Bianchi, 1999) and secondly, they do not have to cross the placenta in order to reach the maternal blood. In fact, specific placental cells actively breach the maternal vessels and enter into maternal circulation extensively during the first trimester (Oudejans et al., 2003). In contrast to cells of the erythroid and lymphoid lineage which originate from pregnancies and can persists in maternal circulation several years after delivery (Bianchi et al., 1993), trophoblast cells are not programmed for long-term circulation in the blood and are unlikely to persist after delivery including subsequent pregnancies (Van Wijk et al., 1996).

The use of trophoblast cells for non-invasive prenatal diagnosis has met with several difficulties. Trophoblast deportation into maternal circulation does not appear to be a phenomenon common to all pregnancies (Sargent et al., 1994). When it does occur, the cells are cleared rapidly by the pulmonary circulation (Attwood and Park, 1960; Benirschke, 1994). Their extraembryonic origin as part of the placenta implies that trophoblast cells are likely to exhibit confined placental mosaicism in 1% of cases sampled (Hahnemann and Vejerslev, 1997) and any prenatal diagnostic screening test relying exclusively on these cells may not reflect the true fetal karyotype. Similarly, syncytiotrophoblast cells which are multi-nucleate, do not give accurate results when chromosomes are analysed by FISH.

Perhaps the greatest obstacle in using trophoblast cells for non-invasive prenatal diagnosis has been the development of specific monoclonal antibodies against trophoblast cell surface antigens. In the early 80s, Trophoblast Like Cells were identified in the peripheral maternal blood through immunocytology in the antecubital veins of six of ten women between 8 weeks gestation and full term (Goodfellow & Taylor, 1982). Two years later, identification of syncytiotrophoblast cells from maternal blood was reported using H315 antigen through flow cytometry (Covone et al., 1984). Later, the same group (Covone et al., 1988) and others (Bertero et al., 1988; Pool et al., 1989) were not able to detect any nucleated elements that were clearly reminiscent of the free inter-villous trophoblast cells. They observed H315 antigen can be absorbed onto maternal lymphocytes and most of the Trophoblast Like Cells were leukocytes of maternal origin, which had absorbed trophoblast membrane antigens. A similar finding was observed (Adinolfi et al., 1988) when most H315+ cellular elements did not react with Y-chromosome specific probes regardless of whether the fetus was male. They affirmed that maternal blood cells adsorbed H315 as they circulated through placenta.

In an attempt to find monoclonal antibodies (MAbs) that are truly specific for trophoblast antigens, 6800 antibodies were generated and screened (Mueller et al., 1990). They found five MAbs FDO161G, FDO66Q, FDO338P, FDO78P and FDO93P appeared to react specifically with a surface membrane protein antigen residing on both villous syncytiotrophoblast and non-villous cytotrophoblast cells, but not with any other cells or serum components in maternal blood. Two of the former, defined a single trophoblast membrane surface protein. The labelled cells were gathered using magnetic beads and proved their fetal origin by PCR amplification of a Y-chromosome-specific DNA sequence. Of 13 women carrying male fetuses, 12 had accurate prediction of fetal sex by this method. Later, the same group demonstrated not only the morphology of the syncytiotrophoblast cells enriched from maternal blood but also identified paternally-inherited mutations for β-thalassaemia (Hawes et al., 1994a; Hawes et al., 1994b). However, because of the lack of general availability of these MAbs, other research groups had never reproduced this work.

The problems resulting from the adsorption of trophoblast antigens onto the cell surface of maternal leukocytes were overcome with the newer marker such as HASH2 (Van Wijk et al., 1998), HLA-G233 (Van Wijk et al., 1996; Loke et al., 1997; Van Wijk et al., 2001), and MAb 340 (Durrant et al., 1996; Lim et al., 1999; Lim et al., 2001). The proof of principle has however been demonstrated solely on the male fetuses using XY cFISH (Lim et al., 1999, Lim et al., 2001; Van Wijk et al., 2001) or by amplification of the Y-chromosome-specific DNA sequence after initial density gradient centrifugation and purification with or without magnetic-activated cell sorting (MACS) (Durrant et al., 1996; Van Wijk et al., 1996; Van Wijk et al., 2001). It is yet to be seen how the trophoblast cells enriched from a female fetus would be differentiated from maternal nucleated cells using cFISH as an identification marker if a gender-independent non-

invasive prenatal diagnosis has to be achieved. Not all groups have been successful in detecting circulating trophoblast from maternal blood. Using newer markers such as HASH-2, HLA-G, human placental lactogen and other known trophoblast antigens, one scientific group was unable to isolate and identify any trophoblast cells within maternal blood (Schueler et al., 2001). Recent demonstration of the novel method of enrichment of trophoblast cells by the isolation by size of epithelial tumor cells method complemented by micro-dissection (Vona et al., 2002) is yet to be verified by independent investigators. On the other hand, trophoblast-derived cells enriched from maternal blood using anti-HLA-G antibody were able to proliferate under *in-vitro* culture condition (Guetta et al., 2005). However, a male fetal cell was observed in a woman carrying a female pregnancy.

Therefore, it is essential that the fetal nucleated cell type selected as the target for non-invasive prenatal diagnosis is a terminally differentiated species with a limited lifespan and no capacity to proliferate. Such a cell type would reflect the genetic status of only the current pregnancy.

Leukocytes: The landmark study that conclusively described that fetal cells do circulate in maternal blood was published by Walknowska et al. (1969). The group demonstrated the presence of a Y chromosome in mitogen-stimulated lymphocytes obtained from pregnant women who were carrying male fetuses. The presence of Human Leukocyte Antigen (HLA) on lymphocytes provided another means of successfully isolating fetal lymphocytes from maternal blood (Herzenberg et al., 1979; Iverson et al., 1981). In contrast other group could locate only maternal cells after metaphase had been induced (Tharapel et al., 1993). Most investigators focussed on fetal lymphocytes but others (Zillacus et al., 1975; Wessman et al., 1992) raised the possibility of using fetal

granulocytes as targets for non-invasive prenatal diagnosis, and suggested that fetal granulocytes transfer into the maternal compartment from as early as seven weeks and on a regular basis. They found fetal granulocytes comprised on average 0.13%, and occassionally as much as 0.26%, of all mononuclear cells in maternal blood. These results suggest a surprisingly high frequency of these cells, especially since they are uncommon in pure fetal blood before 20 weeks, but alternatively they might be artefactual, reflecting non-specific hybridisation of the Y-chromosome probe. Available data therefore do not support the use of granulocytes as suitable targets for non-invasive prenatal diagnosis.

One of the earlier attractions of fetal leukocytes was their ability to proliferate *in-vitro.* It is interesting that this propensity for fetal white blood cells to proliferate is now regarded as a disadvantage (since it is thought that they can proliferate *in-vivo* in maternal organs) and has limited the development of this cell type for use in non-invasive prenatal diagnosis. Numerous groups have described the persistence of fetal leukocytes in the maternal circulation after delivery. While some found the persistence of fetal lymphocytes until one year after delivery (Schroder et al., 1974), others observed it in the samples from women 5-7 years (Ciaranfi et al., 1977) and 27 years post-partum (Bianchi et al., 1996). Thus, there is concern that enriched leukocytes may be the remnants of previous pregnancies and does not represent the true fetal genetic status in the current pregnancy.

Studies of fetal white cells in the maternal circulation for non-invasive prenatal diagnosis are limited by the abundance of contaminating maternal white cells, which excludes the application of white-cell-specific-markers but requires the application of individual specific antibodies for isolation such as HLA antibodies. This may be impractical for

routine prenatal diagnosis because relying on HLA antigens requires not only known paternity, but also an informative polymorphic couple (Pertl and Bianchi, 1999).

Thus, the cell type chosen for non-invasive prenatal diagnosis should be short lived within the mother, have no or limited capacity to proliferate, and have unique cell surface markers to facilitate enrichment in all pregnancies.

Haemopoietic progenitors and stem cells: One way to overcome the relative scarcity of fetal cells enriched from maternal circulation is by *in-vitro* cultivation. This would allow selective amplification of the target cells and would provide a larger number of cells for prenatal genetic diagnosis. Two cell types that fall under this category are the haemopoietic progenitors and stem cells.

Erythroid progenitor cells of male fetal origin derived from fetal liver were co-cultured with a 100-fold excess of peripheral blood mononuclear cells from a non-pregnant woman in an erythropoietin-enriched medium (Lo et al., 1994). A 26-fold increase in the number of male fetal cells was observed. This was followed by culture of maternal peripheral blood samples containing male fetuses in the second trimester, which showed preferential expansion of fetal cells over maternal cells. Subsequently, a successful enrichment and culture of early committed fetal erythroid progenitor cells CFU-E (colony forming unit-erythroid) and M-BFU-E (mature burst forming unit-erythroid) with 18% purity was observed (Valerio et al., 1996). This was followed by successful culture of fetal hematopoietic cells in maternal blood of women carrying aneuploid fetuses (Valerio et al., 1997a; Valerio et al., 2000). On the other hand, Chen et al. (1998) were not able to reproduce the results. Two methods of fetal cell selection were tested on 27 samples from pregnant women between 9-17 weeks gestation and five non-pregnant controls.

One method was based on using different concentrations of erythropoietin ligand in culture and the other was based on the conditions similar to Valerio et al. (1996). The erythroid colonies obtained under either culture conditions were from maternal progenitors, and not of fetal origin.

A two-phase liquid culture system for fetal erythroid progenitor cells were developed (Han et al., 1999). The liquid culture consisted of erythropoietin-independent and erythropoietin dependent phases followed by HbF staining. One blood sample from a woman who was pregnant with a male fetus at 10 weeks gestation showed the presence of fetal cells by PCR, but no XY cells were observed by metaphase analysis. A study of liquid-phase culture of fetal erythroid progenitor cells by the same group (Han et al., 2001) led to similar results. On the other hand, single clones of fetal erythroid progenitor cells from maternal blood were isolated and cultured (Tutschek et al., 2000). PCR (locus: amelogenin Amg XY) showed the presence of male fetal cells. Some of the colonies were mixed (fetal/maternal cells) and some contained pure fetal cells. The presence of mixed clonal colonies and the use of PCR rather than FISH were subsequently questioned (Campagnoli et al., 2001a). Others could not reproduce Tutschek's work (2000) and culture of progenitor cells from all 16 male pregnancies did not show Y-bearing clones and all colonies were maternal in origin (Zimmermann et al., 2002). They observed that Tutschek results could well be due to errors in their complex PCR assay, indicating that alleles might have been incorrectly amplified and presumed to be fetal.

Optimisation of fetal cell selection methods and culture conditions were made using model mixture experiments whereby adult non-pregnant blood was spiked with a known number of fetal cells (Bohmer et al., 1999). They reported the standardisation of serum content conditions for culture of FNRBCs in a co-culture model. When applied to

maternal blood samples, fetal cells were not detected by FISH, PCR and sorting for HbF in 24 samples including known male pregnancies and trisomies (Bohmer et al., 2002).

Fetal cells that express the CD34 antigen remain a target as they proliferate more rapidly in culture than similar cells of adult origin (Eridani et al., 1998). FACS-sorted CD34+ haemopoietic progenitor cells were expanded from maternal blood samples (Little et al., 1997). They obtained a 2-5 fold expansion of CD34+ fetal cells after 5 days of culture. Recently, first trimester CD34+ fetal haematopoietic progenitors were selectively expanded *in-vitro* in model cultures (Campagnoli et al., 2002). On the contrary, no XY cells were identified in culture of CD34+ enriched and non-enriched progenitor cells from 17 first-trimester maternal blood samples, of which 10 were confirmed male pregnancies (Manotaya et al., 2002). Others identified fetal CD34+ cells in all samples from women carrying a male fetus by the presence of Y-chromosome-sequences detected by FISH and nested-PCR (Guetta et al., 2003). Culturing of CD34+ cells resulted in a significant increase in fetal cell numbers and the CD34+ cells were more primitive in the maternal circulation than cells that had already committed to the erythroid differentiation pathway. However, they also observed male CD34+ fetal cells from previous pregnancies in both non-pregnant women and those pregnant with a female fetus and the number of these cells persisting from previous pregnancies increased after culture over maternal CD34+ cells.

A spiking model in which 3, 10, 36 or 71 male cord blood CD34+ cells were diluted in 400,000 female CD34+ peripheral blood cells were used and showed a 1500-fold expansion of fetal cells in culture (Jansen et al., 2000). When the same culture conditions and cytokine combination were applied to 100 maternal samples with gestational age ranging between 7-16 weeks, 65 of which were from women carrying male fetuses, there

was no preferential growth of fetal haemopoietic progenitor cells. In a similar study, a spiking model was used of fetal erythroid cells, which were cultured and subjected to high-performance liquid chromatography (HPLC) and FISH analysis. Although the results were promising in model mixture experiment, application of the same method to 26 pregnant samples of maternal blood including 13 confirmed male fetuses did not show any Y-positive cells (Huber et al., 2000).

The culture of fetal haematopoietic cells from maternal blood samples has not been achieved in a reproducible manner. This could be due to the fact that the occurrence of expandable fetal haematopoietic progenitor cells in maternal blood is an extremely rare event. Another reason could be that they require some as yet unidentified culture conditions. Further, the finding that fetal haematopoietic progenitor cells can persist in the maternal circulation after delivery constitutes a potentially serious caveate for targeting such cells for non-invasive prenatal diagnosis. This observation also represents a long-term low-grade chimeric state in women.

Mesenchymal stem cells: Fetal mesenchymal stem cells (MSCs) have been identified in the fetal blood, liver and bone marrow as early as the seventh week of gestation Campagnoli et al., 2001b). Their mesenchymal phenotype is demonstrated by the expression of specific markers such as SH2 – CD105 and SH3 and 4 – CD73 and expression of vimentin, fibronectin and vascular cell adhesion molecule (VCAM-1). The multi-lineage potential of MSCs can be demonstrated under appropriate culture conditions by their differentiation into osteocytes, adipocytes, and chondrocytes. The group (Campagnoli et al., 2001b) subsequently developed optimal protocols for fetal MSC enrichment in model mixture experiment. When applied to the peripheral blood of pregnant women obtained after termination of pregnancy, they could detect MSC in only

one of 20 samples (O'Donoghue et al., 2003). This therefore, precludes their clinical use for non-invasive prenatal diagnosis. Further, their unique property to differentiate and proliferate makes us believe that like other stem cells, there is a possibility that they too may sequester in the organs of the mothers and persist in the circulation from previous pregnancies and continue to circulate in maternal blood in the current pregnancy.

Endothelial cells: Recently, few groups have looked into the possibility of culturing endothelial cell progenitors presumably of fetal origin from maternal blood. Endothelial cell progenitors from maternal blood were tried to be cultured, but no XY cells were detected in women carrying male fetuses based on FISH analysis and the endothelial cell progenitors appeared to be of maternal origin (Rafii 2000). Others hypothesised endothelial precursor cells might enter the maternal circulation (Gussin et al., 2002). FISH analysis revealed nuclei with XX signals where the women were carrying male fetus indicating that the primitive endothelial cells were maternal in origin. In their subsequent report (Gussin et al., 2004), the authors enriched cells from maternal blood on the basis of their expression of CD133 and CD105, and placed both the positive fraction and the depleted fraction in culture in conditions favouring endothelial for 7 and 30 days, respectively. No PCR products consistent with the presence of male cells were detected in women carrying male fetuses. This indicates that CD133+ and CD105+ cells appear to be of maternal rather than fetal in origin. Recently, they tried to determine optimal concentrations of colony stimulating factor-1 (CSF-1), placental growth factor (PlGF) and transforming growth factor-beta 1 (TGF-beta1) for preferential expansion of fetal endothelial cells in model culture. These conditions when applied to maternal blood samples, did not demonstrate any fetal cells based on PCR for SRY in women carrying male fetuses (Gussin et al., 2005). Therefore, fetal endothelial cell progenitors have not

been successfully enriched to-date from maternal blood for non-invasive prenatal diagnosis.

Erythroblasts: First trimester fetal blood contains two types of nucleated erythocytes: primitive fetal nucleated erythroblasts and definitive NRBCs (Palis and Segel, 1998; Keller et al., 1999). The primitive cells, derived from the yolk sac (Palis and Yoder, 2001) are large and have a high cytoplasmic:nuclear ratio. In contrast, definitive erythroblasts, produced in the fetal liver and bone marrow, are smaller with a lower cytoplasmic:nuclear ratio and extrude their nuclei on maturation (Keller et al., 1999).

The search for a fetal cell target that has a limited life span (Pearson, 1967) and can be distinguished morphologically from its counterparts in maternal blood (Bischoff et al., 2002) led to the selection of the fetal nucleated erythrocyte. Other advantages of this cell type that have made it the target cell of choice for most investigators include the following: (i) they are consistently present in maternal blood during pregnancy (Parano et al., 2001); (ii) they represent 10% of all the red blood corpuscles in a fetus of 11 weeks and 0.5% in a fetus of 19 weeks (Torricelli and Pescucci, 2001); (iii) are full complements of nuclear gene cells detectable early during pregnancy (Liou et al., 1993); (iv) are abundant in the first and second trimester fetal blood, (Thomas and Yoffey, 1962) as the erythrocyte line develops earlier in gestation than the white cell line (Pertl & Bianchi, 1999); (v) are mononucleated (Kelemen et al., 1979) and (vi) carry developmentally-specific markers such as fetal and embryonic haemoglobins. In contrast to the leukocytes, they express several unique antigens such as CD71 receptor (Bianchi et al., 1990; Miltenyi et al., 1990; Ganshirt-Ahlert et al., 1992), erythropoietin receptor (Valerio et al., 1997b), thrombospondin receptor (CD36), glycophorin-A (Bianchi et al., 1993), I/i blood group antigen, HAE9 (Savion et al., 1997) and FB3-2, 2-6B/6, H3-3

(Zheng et al., 1997) that allow their enrichment from maternal circulation, not just those in HLA-informative couple.

An increase in NRBCs within maternal blood in the presence of Rh-incompatibility was observed after termination of pregnancy and amniocentesis (Clayton et al., 1966). These cells were presumed to be fetal in origin based upon staining for fetal haemoglobin (Kleihauer et al., 1957) and that the frequency of NRBCs in the fetus in early gestation had been noted to be relatively high (Thomas and Yoffey, 1962). Independent confirmation that NRBCs within maternal blood were of fetal origin was obtained much later. In 1990, NRBCs were flow-sorted for transferrin receptor (CD71) expression and PCR was performed for a Y sequence from the peripheral blood of pregnant women prior to amniocentesis. After enrichment, Y sequences were found in six of eight pregnancies in which women were carrying male fetuses (Bianchi et al., 1990). A year later, the cells were sorted not only for CD71 but also for cell size, cell granularity, and glycophorin-A (GPA) positivity. Using nested-PCR for a Y-specific sequence, 12 of 12 male fetuses and five of six female fetuses were correctly identified in flow-sorted samples (Wachtel et al., 1991).

The first prenatally detected fetal aneuploidy of trisomy 18 by enrichment of fetal erythroblasts from maternal blood was demonstrated at 10^{+8} weeks of gestation before CVS sampling procedure (Price et al., 1991). They analysed inter-phase cells by FISH with chromosome-specific-probes. This was followed by proof of principle demonstration of aneuploidy by several other groups (Bianchi et al., 1992; Simpson and Elias, 1993; Ganshirt-Ahlert et al., 1993; Simpson and Elias, 1994). The general availability of MAbs against red cell antigens, the relative ease of enrichment protocols and the demonstration of successful cytogenetic diagnosis in fetal nucleated red blood

cells (FNRBCs) isolated from maternal blood make erythroblasts arguably the target cell type of choice for non-invasive prenatal diagnosis.

To-date, fetal material that has been targeted for non-invasive prenatal diagnosis includes fetal DNA or fetal cells. The usefulness of the former is limited to paternally-inherited fetal conditions for which the mother is not a carrier. On the other hand, the latter could allow a wider range of diagnoses to be made, including the detection of fetal aneuploidy. The source through which fetal cells can be obtained is transcervical or from maternal blood. The risk of miscarriage associated with the transcervical route and contamination by foreign genetic material makes maternal blood the ideal source of enriching the cells. Of all the fetal cells enriched so far from maternal blood, fetal nucleated erythroblasts are the most suitable target for non-invasive prenatal diagnosis. Although, fetal anucelated red blood cells also cross over into maternal circulation but they do not contain any nucleus and therefore cannot be used for non-invasive prenatal diagnosis.

1.6.4 Non-invasive prenatal diagnosis using fetal nucleated erythroblasts in maternal blood

The accuracy of prenatal diagnosis using fetal nucleated erythroblasts enriched from maternal blood depends upon the specificity of their identification. The presence of a cell surface antigen that is highly specific for fetal erythroblasts would be ideal since it could be used both to isolate these cells from maternal blood and to identify their fetal origin. Such a fetal cell marker is not yet available (Huie et al., 2001). It was thought that NRBCs are rare in maternal blood (Pembrey et al., 1973). Recent enrichment systems have demonstrated that a much larger population of NRBCs that originate in the maternal bone marrow circulate in pregnancy (Slunga-Tallberg et al., 1995). This implied that the

earlier assumption that morphology could be used to identify fetal NRBCs was incorrect. It also meant that a marker for fetal, as opposed to maternal, NRBCs needed to be identified (Zheng et al., 1999). Fetal haemoglobin has been used to differentiate maternal from fetal NRBCs, but 20% of all HbF-positive NRBCs in maternal blood are maternal in origin (de Graaf et al., 1999) and are found in all pregnancies (Lim et al., 2001). Thus, if fetal nucleated erythroblasts are to be used as target cells for accurate non-invasive prenatal diagnosis, a more specific identification system needs to be developed.

1.6.4.1 Identification of fetal origin of erythroblasts enriched from maternal blood

The three NRBCs identifier studied so far are the fetal and embryonic haemoglobins γ-, ζ- and ε-globins. In 1993, Zheng and co-workers confirmed the origin of FNRBCs by labelling intracytoplasmic, developmental-specific fetal haemoglobin. Since then, most groups identify fetal erythroblasts by using MAb labeling of γ-globin (DeMaria et al., 1996; Mavrou et al., 1997; Oosterwijk et al 1998a; de Graaf et al., 1999; Samura et al., 2000; Lim et al., 2001), or confirming the presence of HbF by the Kleihauer test (Rodriguez et al., 1999; Wang et al., 2000; Rodriguez et al., 2001; Parano et al., 2001; Martel-Petit et al., 2001). Even if a suitable enrichment technique was established which enriches NRBCs, using γ-globin as a fetal cell identification marker will not promote a gender independent non-invasive prenatal diagnosis. This is because production of γ-globin (HbF) is increased during acute erythroid expansion such as in pregnancy (Pembrey et al., 1973; Popat et al., 1977), during recovery from iron deficiency anaemia (Dover et al., 1979) and in patients with β-thalassaemia (Olivieri, 1999, Weatherall, 2000).

Anti-γ globin antibody (DeMaria et al. 1996) and Kleihauer test (Martel Petit et al. 2001) directed against fetal haemoglobin, is highly fetal specific. These findings were based on enrichment of male cord blood NRBCs from artificial mixtures in non-pregnant adult female blood. This is true in a model mixture experiment where HbF positive NRBCs are unlikely to be found in non-pregnant adult blood. In contrast, nucleated erythroblasts were enriched from maternal blood obtained within 30 minutes after transcervical CVS and fetal gender was predicted within γ-globin positive cells by XY cFISH (Oosterwijk et al., 1998a). Of the 10 cases in which the fetuses were confirmed to be XY by karyotyping, in one the γ-globin positive cells were XX. Similarly, using HbF as a fetal cell identification marker, 22 percent of all HbF positive NRBCs enriched from maternal blood of male pregnancies were found to be of XX karyotype on cFISH, indicating that these cells were maternal in origin (de Graff et al., 1999). FNRBCs were enriched from CVS supernatant and the gender was correctly identifed by cFISH. HbF positive NRBCs with two XX signals however, were also detected in the CVS supernatant (Mavrou et al., 1997). In 21 percent of pregnant women carrying male pregnancies, all of the HbF positive NRBCs on Kleihauer test had two X chromosome signals present on cFISH analysis suggesting maternal origin of NRBCs (Rodriguez et al., 1999). All γ-positive cells enriched in 46 percent of pregnant women between 6-23 weeks contained two X chromosome signals consistent with females (Samura et al., 2000). In one case report, it was observed that 54 percent of γ-positive cells enriched from a pregnant woman carrying a male fetus were maternal in origin with 2 XX signals (Zheng et al., 1995). In another study, 433 nucleated γ-globin positive candidate fetal cells in 13 maternal blood samples obtained after termination of pregnancy were recovered. Of these, 42 XX cells (9.7%) were found in 11 of the 13 samples (84.6%) (Wang et al., 2000). HbF positive XX NRBCs were found in the maternal blood of 100% of the 23 subjects carrying male

fetuses (Lim et al., 2001). In the only publication that demonstrated 100% specificity while staining for HbF (Parano et al., 2001), a judegment call was required by the observer/operator as to the pinkness of candidate cells (intensity of HbF staining in fetal vs. maternal NRBCs) and only the brightest staining cells were transferred by micromanipulation onto fresh glass slides for cFISH. A system, which is operator dependent as this, is prone to error if applied in routine clinical practice.

Based on the leaky expression of γ-globin in adults, the use of the embryonic ζ-globin was instead suggested (Cheung et al., 1996). They established the principles by the non-invasive prenatal diagnosis of one case each of sickle cell anaemia and β-thalassaemia in micromanipulated, ζ-globin positive NRBCs enriched from maternal blood between 10-12 weeks. While ζ-globin is definitely less likely to be present in adult blood cells, its expression is not completely switched off after the embryonic period. It was shown that ζ-gene transcription continues in the definitive erythropoiesis by detecting ζ-mRNA in fetal liver (Yagi et al., 1986). Synthesis of ζ-chains could be detected in fetal definitive erythrocytes between 15-22 weeks gestation and in definitive erythrocytes of normal newborn cord blood (Kutlar et al., 1987; Chui et al., 1989). ζ-globin was present in 53% of definitive erythrocytes between 15-22 weeks and in 34% at term (Luo et al.,1999). ζ-globin transcript was also demonstrated within the peripheral blood of healthy individuals (Albitar et al., 1989). Others found ζ-globin chains in adults with the α-thalassaemia trait (Chung et al., 1984; Chui et al., 1986; Tang et al., 1992). The consistency of this finding suggested that ζ-globin could be used as a marker applicable to the general population for the α-thalassemia-1 haplotype with a cis-deletion of both α-globin genes on chromosome 16 (Chui et al., 1986). These findings suggest that the expression of ζ-globin is not

strictly restricted to the fetus and may not be useful as a fetal cell specific identification marker.

After the initial idea of using embryonic instead of fetal globins (Cheung et al., 1996), the presence of ε-globin positive male fetal erythroblasts was demonstrated in two post-CVS maternal blood samples (Mesker et al., 1998). This publication emphasised the possibility of first trimester non-invasive diagnoses of fetal aneuploidy and single gene disorders using ε-globin as a fetal cell specific marker. Later, the specificity of ε- and ζ-globin was compared in CVS supernatant and it was found that embryonic ε-globin was more reliable and specific for the detection of embryonic NRBCs than anti-ζ (Mavrou et al., 1999). Subsequently, an indirect evidence was provided that ε-globin expression is more tightly regulated than ζ-globin (Luo et al., 1999). They found an 18-fold greater expression of ζ- compared with ε-globin when fetal erythroblasts were cultured *in-vitro*. Using a sensitive reverse transcriptase-PCR (RT-PCR) method, ζ-globin transcripts was observed in the CD71 positive mononuclear cell fraction of the peripheral blood of 3 out of 20 non-pregnant women while ε-globin transcripts were found in none (Hogh et al., 2001). A similar result was obtained (Boye et al., 2001) whereby differential display RT-PCR method was used to compare transcription differences between the mononuclear cell fraction of adult female blood and trophoblast tissue, which included fetal nucleated erythroblasts but not in adult blood cells. No differential expression of ζ-globin was noted by this method, confirming the presence of ζ-globin mRNA within adult blood cells. In their study on CVS washings (Voullaire et al., 2001) ε-globin nucleated red blood cells was found to be increased at 10 weeks and a rapid decline was observed by 13 weeks of gestation. On the other hand, study on pure fetal blood samples in the first trimester demonstrated that 50% of the fetal primitive nucleated erythroblast still

contained ε-globin as late as 12 weeks gestation and reached negligible levels by 14th week of gestation (Choolani et al., 2001). The group also investigated the presence of ε-globin positive fetal nucleated erythroblasts in the bone marrow aspirate from healthy adult donor and found no ε-globin positive erythroblasts. In their study on chromosomally normal fetal blood samples (Al-Mufti et al., 2000) 97% of cells were reported to contain ε-globin chain at 10 weeks and 45% of cells still exhibited ε-globin chain at 12 weeks. It was suggested that rare adult NRBCs might also contain ε-globin (Lau et al., 2001), but their findings could have been confounded by autoflourescence artefact (Choolani et al., 2002). Recently, it was reported that primitive fetal nucleated erythroblasts is a suitable FNRBCs to be targeted in the first trimester of pregnancy as the ε-globin intracytoplasmic protein is present in 100% of primitive fetal erythroblasts and rarely in definitive NRBCs (Choolani et al., 2003).

These data suggest no definitive evidence that ε-globin is produced in adult blood cells or in definitive erythroblast indicating ε-globin to be more tightly regulated than γ-globin. It also indicates that ε-globin is a highly specific marker for fetal nucleated erythroblast identification and could be used for first-trimester non-invasive prenatal diagnosis.

1.6.4.2 Diagnosis of chromosomal and monogenic disorders using fetal nucleated erythroblasts enriched from maternal blood

The three most important molecular techniques that have allowed genetic analysis of enriched fetal cells are PCR (Saiki et al., 1985), RT-PCR (Rappolee et al., 1988) and cFISH (Pinkel et al., 1988). The ability of PCR to amplify minute quantities of DNA (even single copy) over a billion-fold has been exploited by several investigators to

demonstrate the possibility of prenatal diagnosis of monogenic disorders using fetal cells enriched from maternal blood (Camschella et al., 1990; Suzumori et al., 1992; Lo et al., 1993; Takabayashi et al., 1995; Sekizawa et al., 1996; Cheung et al., 1996; Watanabe et al., 1998; Samura et al., 2001). In cells expressing a particular gene, there are many more copies of the RNA (ribonucleic acid) message compared with only one or two alleles within the genome. Thus, RT-PCR for fetal mRNA (messenger RNA) is more sensitive than PCR amplification of genomic DNA (Al-Mufti et al., 1998). cFISH allows the detection of anueploidy and chromosomal rearrangements in inter-phase nuclei. It has been used to detect most of the major fetal aneuploidies within fetal cells isolated from maternal blood (Price et al., 1991; Elias et al., 1992; Bianchi et al., 1992; Ganshirt-Ahlert et al., 1993; Simpson and Elias, 1993; Simpson and Elias, 1994; Elias et al., 1996; Pezello et al., 1997; Oosterwijk et al., 1998c; Al-Mufti et al., 1999; Rodriguez de Alba et al., 1999; Hromadnikova et al., 2002a; Mavrou et al., 2003).

PCR: Camschella et al. (1990) first demonstrated fetal inheritance in fetal cells, by showing Hb Leporn-Boston by PCR amplification of putative sequences in the buffy coat of maternal peripheral blood. The Lepore-Boston gene is a hybrid δ-β gene arising from a 7 kb deletion between codon 87 of exon 2 of the δ-gene and the 5′ end of intron 2 of the β-gene (Flavell et al., 1978). The gene confers a thalassaemia phenotype. The investigators designed primers that flanked the δ-β junction to amplify a 356 bp product if the mutant gene were present. They diagnosed the inheritance of the mutant gene in two fetuses and confirmed this by CVS. This was the first time a clinical condition in the fetus was demonstrated by PCR amplification of maternal blood.

Subsequently, the feasibility of fetal sex determination by Y-sequence amplification (Suzumori et al., 1992) and fetal RhD genotyping by nested amplification of a 262 bp

segment of the RhD gene in rhesus-negative mothers (Lo et al., 1993) respectively, was demonstrated. It was initially thought that determination of the fetal RhD status would be one of the more important applications of fetal cells enriched from maternal blood (Geifman-Holtzman et al., 1996; Geifman-Holtzman et al., 1998; Geifman-Holtzman et al., 2000). However, it is now known that this can be successfully and more easily performed by analysing cell-free DNA in maternal plasma (Lo et al., 1998a).

Whether cells or cell-free DNA are used, the detection of fetal RhD status by analysis of maternal blood has confirmed the importance of PCR. It allowed the first clinical application of non-invasive prenatal diagnosis. Non-invasive confirmation that the fetus is RhD negative would reassure parents that the fetus is not at risk and would avoid any unnecessary use of RhD immune globulin and avoid the risks of invasive diagnosis in alloimmunised women whose partner is heterozygous for RhD.

RT-PCR: It was hypothesised that since there are many more copies of mRNA of an expressed gene than there are the genomic sequences, RT-PCR amplification of message should be more sensitive than PCR amplification of genomic DNA (Al-Mufti et al., 1998). They observed a doubling in sensitivity for the non-invasive prenatal diagnosis of fetal RhD status using RT-PCR. In contrast, PCR detection of fetal DNA in maternal plasma was found to be more sensitive than RT-PCR detection of fetal RNA (Poon et al., 2000). They suggested that this was because fetal RNA is more susceptible to enzymatic degradation in maternal blood than fetal DNA. Thus the value of RT-PCR in non-invasive prenatal diagnosis remains controversial.

cFISH: The place of cFISH in non-invasive prenatal diagnosis has been firmly established. The use of this technique was successfully demonstrated in the prenatal

diagnosis of autosomal trisomies 18 and 21 (Price et al., 1991). These observations were extended by diagnosing fetal trisomy 21 in the maternal blood of a 42-year old woman taken before CVS (Elias et al., 1992). Subsequently, trisomy 21 fetus was identified from maternal blood before termination of pregnancy (Bianchi et al., 1992). Following this report, five cases of trisomy 18 and ten cases of trisomy 21 were confirmed in NRBCs enriched from maternal blood (Ganshirt-Ahlert et al., 1993). Sex chromosome mosaicism was detected by cFISH in fetal cells enriched from maternal blood (Bischoff et al., 1995). Further, it was shown that, sex chromosome aneuploidy could also be detected reliably (Cacheux et al., 1992; Al-Mufti et al., 1999). The latter group was also successful in identifying aneuploidy cases between 10-14 weeks of gestation, including 36 cases of trisomy 21, 24 cases of triosmy 18 and 10 cases of trisomy 13. Similarly, one (Pezzello et al., 1997) and three cases (Al-Mufti et al., 1999) of fetal triploidy was diagnosed by cFISH on enriched fetal cells and confirmed by CVS. On the other hand, 4 cases of trisomies on FNRBCs enriched from maternal blood (Rodriguez et al., 1999) and 18 cases of trisomy 21 between 12-25 weeks of gestation was demonstrated (Hromadnikova et al., 2002a). Also, 4 cases of trisomy 21 after amniocentesis and before termination of pregnancy in the second trimester was successfully demonstrated (Mavrou et al., 2003). Some investigators have attempted to increase the number of chromosomes that could be analysed by inter-phase FISH by performing simultaneous multicolour cFISH (Bischoff et al., 1998) or by sequential hybridisation of chromosome pairs (Zhen et al., 1998).

Simultaneous immunophenotyping and cFISH: Several groups (Zheng et al., 1993; Oosterwijk et al., 1998b) suggested the simultaneous visualisation of fetal cell identifier and cFISH, after enrichment of NRBCs using either cell sorting with MACS (Zheng et al., 1993) or triple density gradient (Oosterwijk et al., 1998b). The authors showed that

the combination of immunophenotyping with mouse monoclonal anti-HbF antibody using Vector red (Zheng et al., 1993) or blue substrate, (Oosterwijk et al., 1998b) and fluorophores for FISH analysis allowed the identification of FNRBCs within the excess of maternal cells. Using this combined immunoenzymatic labeling of fetal antigens and cFISH have however, proved problematic as it is necessary to register the location of γ-globin-positive cells with a graticule and relocate each cell for analysis after cFISH (Zheng et al., 1993), cumbersome switching between transmission and fluorescence modes on a fluorescence microscope (Pazouki et al., 1996), and performing a simultaneous *in-situ* detection technique that allows co-visualisation of the fetal cell identifier and any genetic signals (Price et al., 1992; Kibbelaar et al., 1992; Oosterwijk et al., 1998b). Furthermore, if the globin were labeled using a light microscopy dye and the hybridisation probes with fluorophores, exposure of the latter to the bright light of a transmission microscope bulb would bleach the cFISH signals quickly, which could lead to inaccurate diagnosis. Since most genomic probes are fluorescently labeled, some investigators used fluorescent dyes to label the fetal cell marker and cFISH signals but found that when anti-γ-globin antibody was conjugated to fluorescein isothiocyanate (FITC), the intense green cytoplasmic staining precluded the use of FITC-labelled cFISH probes because the two labels could not be satisfactorily distinguished from each other and also limited the detection sensitivity by FACS due to haem auto-fluorescence (DeMaria et al., 1996). The above studies were also limited by the fact that some of the HbF positive cells enriched from maternal blood showed two copies of X chromosome, which suggested that the cells were maternal in origin. Therefore, even if the above shortcomings are overcome, correct identification of fetal cells is still the linchpin of a non-invasive prenatal diagnostic technique. The identification system should be fetally

specific, gender-independent and reliant on developmental differences between mother and fetus.

Based on the above information of ε-globin being a fetal cell identifier, successful enrichment of ε-globin positive primitive fetal nucleated erythroblasts was demonstrated from maternal blood using immunoenzymatic staining followed by FISH for gender identification and chromosomal aneuploidy (Mavrou et al., 2003). The hybridisation efficiency using X- and Y-chromosome specific probes was only 64%, while the efficiency with chromosome-21-specific probe was 54.7%. In 2001, Choolani and colleagues demonstrated that labelling the ε-globin intracellular fetal cell antigen with the blue fluorescent dye AMCA (7-amino-4-methylcoumarin-3-acetic acid) not only circumvents the problems of haem auto-fluorescence, which thus far had been the limiting factor preventing simultaneous visualisation of the fetal cell identifier and molecular genetic signals (DeMaria et al., 1996; Oosterwijk et al., 1998b) but also enhances cFISH efficiency and limits further fetal cell loss. Previous attempts at reducing or correcting auto-fluorescence, which involved mathematical manipulations of pixel intensity (Szollosi et al., 1995) or concomitant use of dyes absorbing certain emitted wavelengths (Mosiman et al., 1997) met with little success. The group (Choolani et al., 2001) has been successful in developing a novel technique which consists of immunolabelling of the highly specific ε-globin positive first-trimester primitive fetal nucleated erythroblasts followed by cFISH.

This recent breakthrough (Choolani et al., 2001; Choolani et al., 2003) has addressed the issue of specific identification of fetal primitive nucleated erythroblasts of first trimester origin using embryonic ε-globin and the simultaneous fluorescence labelling of this marker with cFISH for non-invasive prenatal diagnosis. However, the first phase that is,

enrichment of target cells from the mother's blood remains a challenge. Due to the scarcity of fetal nucleated erythroblasts in the maternal blood (1 in 10^7 to 1 in 10^9 maternal nucleated cells) (Smits et al., 2000), several strategies to exploit the differences in physical, chemical and biological properties of individual cells have been used to enrich fetal erythroblasts from maternal blood.

1.6.4.3 Various approaches for the enrichment of fetal nucleated erythroblasts from maternal blood

Charge flow separation: The charge flow separation instrument has been utilised by some investigators to enrich FNRBCs (Wachtel et al., 1996; Wachtel et al., 1998; Shulman et al., 1998). In their technique, the investigators employed a horizontal cross-flow fluid gradient opposing an electric field in a separation chamber. Cells flow vertically, perpendicular to the gradient and the electric field. According to their characteristic surface charge densities, the different cell types are separated from each other and are focused in different compartments, where they are directed into waiting collection tubes. Though the initial results reported by the authors were encouraging (Wachtel et al., 1996), when carried out on a large sample size of 225 patients between 10-18 weeks of gestation, no NRBCs could be recovered from one-third of patients and from the remaining cases the authors inferred that 30% of NRBCs in maternal blood were fetal in origin (Wachtel et al., 1998). On the contrary, it is likely that most of the cells recovered by this technique were not fetal, as only 17 samples were evaluated by FISH with chromosome-specific probes. This view was affirmed in their recent review article (Wachtel et al., 2001) where the authors indicated from their unpublished data that their earlier figure was an overestimate for the number of fetal cells that can be recovered by charge flow separation.

Density Gradient Centrifugation: The trend to use density gradient centrifugation as the first-step to eliminate/reduce the overwhelming abundance of maternal red blood cells followed a publication in 1990 (Bhat et al., 1990). They demonstrated using cord blood samples that if Histopaque-1077 were used alone, 82.6% of NRBCs would pellet, indicating that 1077 density gradient though eliminates most of the erythrocytes and polynuclear leukocytes, also depletes NRBCs as well. They showed that if a discontinous double density gradient of Histopaque 1077 and 1119 were prepared, less than 0.5% of NRBCs would collect at the top of the interface and 78.6% would settle at 1119. They found that Histopaque-1077 when used alone or in combination with 1119 could also deplete FNRBCs. Subsequently, the authors developed discontinuous triple density gradient protocol (Bhat et al., 1993) by layering Histopaque 1119 at the bottom, Histopaque 1107 in the middle, and Histopaque 1077 on the top in a model mixture experiment where the adult blood was spiked with cord blood. The enriched cells were sorted by flow-cytometry using anti-CD71 antibody followed by anti-CD13 anti-CD45 antibody. They reported a 25-fold enrichment of NRBCs with triple density gradient as compared to single density gradient. In fact, this result represents the efficacy of the entire protocol rather than of the first step. It would have been more appropriate to identify the number of NRBCs enriched at the first step so as to know the exact efficacy of the first-step enrichment method. Since then, the choice of density gradients used in the fetal cell enrichment protocols has been guided by model mixtures using cord blood NRBCs.

Following the above findings (Bhat et al., 1993) numerous researchers have been using triple density ficoll gradient as part of their enrichment protocol to isolate fetal erythroblasts from maternal blood (Ganshirt-Ahlert et al., 1993; Ganshirt-Ahlert et al., 1998; Pezello et al., 1997; Oosterwijk et al., 1998a; 1998c**;** Kuo et al., 1998; Al-Mufti et

al., 1999; Al-Mufti et al., 2003). Recently, the efficacy of triple density gradient was compared with that of single density gradient Ficoll 1077 in the same group of patients and it was observed that triple density gradient had better efficacy then single density gradient (Al-Mufti et al., 2004). The study is limited by the samples being processed across the entire protocol starting from the first-step enrichment method to second-step enrichment rather than determining the efficiency of the gradient centrifugation at the first step alone. Other groups used either single density gradient Ficoll 1077 (Price et al., 1991; Bianchi et al., 1992; Cheung et al., 1996; Hromadnikova et al., 2002a; Hromadnikova et al., 2002b) or Ficoll 1119 (Martel-Petit et al., 2001) or Ficoll 1090 (Kitagawa et al., 2002) or Ficoll 1083 (Mavrou et al., 2003) for FNRBCs enrichment from maternal blood for gender and aneuploidy analysis. Several others preferred to use double density gradient comprising of Ficoll 1077 and 1109 (Rodriguez de Alba et al., 2001) or 1077 and 1083 (Parano et al., 2001) for enrichment of fetal nucleated erythroblasts from maternal blood.

The efficiency of single density gradient Ficoll 1077, 1098, 1110 and 1119 were studied by spiking cord blood with non-pregnant female adult peripheral blood in the model mixture experiment (Troeger et al., 1999). It was found that the yield of erythroblast was higher with Ficoll 1119 and was preferred to be used as the first-step enrichment method. Subsequently, the efficacy of single Histopaque-1090 was compared with 1119 for maximum recovery of FNRBCs directly on maternal blood instead of testing the protocol in the model mixture experiment (Samura et al., 2000). They reported the median number of FNRBCs separated by the 1119 density gradient was significantly higher than that by 1090. The result of the study was however confounded by the fact that all the samples were collected immediately after termination of pregnancy and by inclusion of aneuploidy pregnancies. Such a finding do not reflect the efficiency of the enrichment

protocol used because firstly, invasive procedures itself cause fetal blood to pass into the maternal circulation (Brambati et al., 1986). Secondly, in aneuploidy fetuses, the placental feto-maternal barrier is disturbed due to impaired development and function of placental villi leading to an increased feto-maternal transfusion (Genest et al., 1995). An altered haematopoiesis is also indicated in the early trisomic embryos (Thilaganathan et al., 1995) leading to an increased NRBCs content in fetomaternal transfusion. Therefore, it is not clear whether the significant recovery of FNRBCs was due to the Histopaque gradient 1119 or due to the fetomaternal haemorrhage secondary to the procedure or whether it was the biology of the aneuploidies which worked in favour of the test. Also, the post-procedure results are of limited clinical significance if the enrichment technique needs to be brought into actual clinical practice.

Other groups looked into the possibility of using Percoll as a suitable gradient for fetal nucleated erythroblast enrichment from maternal blood. The efficacy of single Percoll density gradient 1090 and 1083 was assessed for fetal cell enrichment on maternal blood (Sekizawa et al., 1999). It was observed that 1.4 times more MNCs were being separated at the first-step 1090 density. The results were confounded by all the samples being obtained after termination of pregnancy. Unlike this study, other group (Smits et al., 2000) assessed the efficacy of different Percoll gradients as the first-step for enrichment of FNRBCs in model mixture experiment whereby cord blood was mixed with adult peripheral blood. They observed an increase in the recovery of fetal nucleated erythroblast from 3.23% to 68.56% with an increase in single density Percoll gradient from 1075 to 1098 respectively. Use of double Percoll gradient (Percoll 1075 and Percoll 1085) did not lead to any significant erythroblast enrichment. Despite this finding, others considered double density gradient as successful for FNRBCs enrichment from maternal blood (Takbayashi et al., 1995; Sekizawa et al., 1996, Watanabe et al., 1998).

Recently, Choolani and his group (2003) exclusively studied the buoyant densities of first-trimester primitive fetal nucleated erythroblasts enriched from fetal blood samples. They observed even with the most commonly used Ficoll such as 1077 and 1119 being used on patient samples for fetal cell enrichment, 91.4% and 68.3% of NRBCs respectively, settle down in the erythrocyte pellet at the first-step density gradient centrifugation process. They also observed the median recovery of fetal nucleated erythroblast from mixtures in maternal blood was superior with Percoll 1118 with 64.1% recovery as compared to 35.3% using Ficoll 1119. They therefore, recommended the use of Percoll 1118 as the first-step method for primitive fetal nucleated erythroblasts enrichment from maternal blood.

Cell Sorting by targeting surface antigen: FACS and MACS are the two most commonly employed systems in the field of non-invasive prenatal diagnosis. Both FACS and MACS exploit antigenic differences between cells and are capable of providing either positive selection (selection for those cells desired for analysis) or negative selection (selection against cells not desired for analysis). FACS first used over two decades ago for this purpose (Herzenberg et al., 1979), is able to enrich cells with high purity so that slides with sorted cells can be readily scanned manually. It also allows multi-parameter sorting: simultaneous analysis of several criteria on a single cell and can be adapted for use with intracytoplasmic antigens. The disadvantages of the technology are its cost, expensive maintenance, requirement for specially trained laboratory personnel, limitation in the number of cells that can be put through the system in a reasonable amount of time and fading of the fluorochrome if exposed to cFISH after sorting and cell loss.

MACS has gained popularity because of its speed of operation and ease, and is faster and less expensive bench-top technique better suited to process larger cell numbers. It can

also be performed in most laboratories without trained staff and high maintenance costs. The antibodies conjugated to magnetic beads can be directed against the cells sought for enrichment. These beads attaching the desired cell type are retained under a magnetic field when cells in solution pass through (positive selection). Cells that lack the antigen in question fail to attach to beads, are not retained in the magnetic field and pass through unimpeded to be discarded. After releasing the magnetic field, cells attached to the beads can then be collected for subsequent analysis. In addition the converse can be performed: desired cells are allowed to pass through and non-desired cells are retained under the magnetic field. Although both negative and positive selections can be performed on the same population of cells in the same experiment, enrichments must be performed in series because cell selection can be based on only one antigen at a time. When studying the absolute numbers of fetal cells recovered, it has been shown that MACS is at least as effective as and more specific than FACS (Wang et al., 2000). Recently, National Institute of Child Health and human Development Fetal Cell Isolation Study (NIFTY) I data suggests that feal cell recovery and detection were better using MACS than with FACS (Bianchi et al., 2002).

No surface antigens specific for fetal erythroblasts have been identified to-date (Huie et al., 2001). FNRBCs were first enriched from peripheral blood of pregnant women using anti-CD71 antibody against transferrin receptor (Bianchi et al., 1990). The transferrin receptor (CD71) is present on all cells that incorporate iron including monocytes, activated lymphocytes and trophoblast. It is also known to be expressed on erythroid cells from the burst forming unit–erythoid stage up to and including the reticulocyte stage (Loken et al., 1987a). This signifies that CD71 antigen is not specific to FNRBCs but is also found on maternal NRBCs as well (Bianchi et al., 1994; Slunga-Tallberg et al., 1996; Zheng et al., 1997). Poor specificity of CD71 antibody was reported (Ganshirt-Ahlert et

al. 1992), indicating that alone it is insufficient for the isolation of FNRBCs. Similarly, about 1 in 1000 nucleated cells was identified to be fetal after density gradient centrifugation and MACS with anti-CD71, indicative of a considerable contamination by maternal CD71+ cells (Troeger et al., 1999). Despite these limitations, numerous groups to-date have still been using CD71 to enrich FNRBCs from maternal blood, compromising the purity of the enriched fraction (Price et al., 1991; Bianchi et al., 1992; Ganshirt-Ahlert et al., 1993; Ganshirt-Ahlert et al., 1998; Elias et al., 1992; Chueng et al., 1996; Al-Mufti et al., 1999; Al-Mufti et al., 2003; Rodriguez de Alba et al., 2001; Mavrou et al., 2003).

A study was conducted to demonstrate whether using anti-CD36, anti-CD71 or GPA in combination or alone would be effective in enriching FNRBCs from maternal blood in the first and second trimester (8-19 weeks) (Bianchi et al., 1993). CD36 was selected because it recognises a 90 kD glycoprotein corresponding to the thrombospondin receptor (Edelman et al., 1986) which is expressed during the colony-forming unit erythroid (CFU-E) stage of erythroid differentiation. GPA was selected as it is the major sialoglycoprotein of the erythroid membrane (Loken et al., 1987b) and is expressed on all erythrocytes. Blood samples from 47 women were flow sorted using FACS for three different antibodies to fetal erythrocyte antigens. They observed that using anti-CD71, gender prediction was 57% correct while it was 88% correct with anti-CD36. Anti-GPA when used alone or in combination with anti-CD71 or anti-CD36 improved gender prediction to 100%. This indicates anti-GPA alone or in combination with anti-CD36 or anti-CD71 improves the yield rather than using anti-CD36 or anti-CD71 alone. Several groups tried to verify the results of Bianchi and co-workers (1993) by using combination antibodies for enrichment of fetal cells from maternal blood. A combination of anti-CD36/anti-GPA and anti-CD71/anti-GPA was used to flow-sort FNRBCs from maternal

blood across all the three trimesters (Sohda et al., 1997). More than 90% of NRBCs by were found by either combination. MACS with the combination of anti-CD36/anti-GPA and anti-CD71/anti-GPA were used to enrich fetal nucleated erythroblasts from maternal blood in the second trimester (15-19 weeks) (Campagnoli et al., 1997). They observed no difference in either method in terms of percentage of fetal cells recovered. The efficacy of anti-GPA, anti-CD71 and anti-CD36 were assessed in the model mixture experiment using MACS (Troeger et al., 1999). This was followed by testing the efficiency of these antibodies on maternal clinical samples. They observed the recovery of NRBCs was significantly higher with anti-GPA than with anti-CD71 and using anti-CD36 was not as successful as anti-GPA.

Although fetal nucleated erythroblasts have been enriched from maternal blood using anti-CD36 (Bianchi et al., 1993; Bianchi et al., 1994; Campagnoli et al., 1997; Sohda et al., 1997; Troeger et al., 1999), it is likely that the NRBCs exhibiting this surface antigen were definitive erythroblasts in origin. This is because, although the targeted samples were between 8-19 weeks gestation (Bianchi et al., 1993), the authors have not mentioned how many of them belonged to the first trimester and it is possible that the CD36 positive cells may have been enriched from the patient samples obtained in the second trimester. In another study (Sohda et al., 1997) patients were enrolled across all the three trimesters while Campagnoli et al. (1997) recruited the patients only from the second trimester. In contrast, Troeger et al. (1999) have not documented the gestational age of the patients. Thus it remains plausible that the difference in sensitivity they observed was a result of different cell populations being selected.

As fetal erythroblasts are negative for CD45 antigen, Zheng and co-workers (1993) investigated the utilisation of using anti-CD45 so as to deplete maternal leukocytes in the

MACS after first-step enrichment process. CD45 is present on the cell surface of all mature human leukocytes and is not expressed on immature and mature erythrocytes. A method of enriching NRBCs by negative MACS using anti-CD45 alone was deployed by only a few research groups (Zheng et al., 1995; Slunga-Tallberg et al., 1995; Samura et al., 2000). On the other hand, Hromadnikova et al. (2002a) and Mavrou et al. (2003) used a combination of depleting antibodies CD45 and CD14, as CD14 recognises antigens on monocytes (Bianchi et al., 1998) and anti-CD45, respectively. This was followed by positive selection of FNRBCs by using non-specific CD71 antibody.

The cell surface antigen properties of exclusively first-trimester primitive fetal nucleated erythroblast in pure fetal blood were recently studied (Choolani et al., 2003). They observed the antigenic profile of these cells were similar to adult erythrocytes, being 100% positive for GPA and 100% CD36 and CD45 negative. In contrast to adult NRBCs 96% of which express CD71 (Loken et al., 1987a), they reported only 68% of primitive fetal erythroblasts were weakly positive for CD71 as compared with 100% of definitive erythroblasts. On MACS sorting for pure fetal blood, the mean recovery of primitive fetal erythroblasts was greatest using anti-GPA and poorest with anti-CD71, the most frequently used antibody for enriching fetal erythroblasts from maternal blood. This was subsequently confirmed in the model mixture experiment where the yield and the purity were higher with anti-GPA then with anti-CD71. This finding was in line with other study (Troeger et al., 1999) that anti-GPA was indeed superior and indicate that its utilisation would eventually enrich both primitive and definitive fetal erythroblasts. The authors (Choolani et al., 2003) also observed anti-GPA when used with anti-CD45 depletion gave a yield similar to that of using anti-CD45 alone (76%) albeit with 24% loss of target cells and 100% purity. The group suggested the use of anti-CD45/anti-GPA as a second-step enrichment method.

Enzymes: Though**,** several enzymes such as 2,3-biphosphoglycerate (Von Koskull and Gahmberg, 1995) and thymidine kinase (Hengstschlager and Bernaschek, 1997) have been used to differentiate fetal from maternal red cells after enrichment, the most extensively studied has been carbonic anhydrase enzyme (Saunders et al., 1997). The selective lyses of adult anucleated red blood cells are based on enzyme difference between maternal and fetal red blood cells. Maternal cells contain carbonic anhydrase I and II whereas fetal cells only contain carbonic anhydrase II. By adding substrate (ammonium chloride and potassium bicarbonate), ammonium bicarbonate is formed within the maternal cells, which will lyse due to the uptake of water (de Graaf et al., 1999) and are therefore more susceptible to ammonium chloride lysis than fetal erythroblasts (Alter et al., 1979) as carbonic anhydrase activity is at least five-fold greater and acetazolamide permeability about ten-fold less in adult compared with fetal red blood cells (Boyer et al., 1976).

Selective lysis of maternal red blood cells was first used for the diagnosis of haemoglobinopathies where globin chains extracted from a highly purified population of fetal red blood cells were analysed by electrophoresis, a technique in which significant fetal cell loss could be tolerated. Ammonium chloride lysis of erythrocytes is known to effect functional studies in white cells (Webster and Pockley, 1993; Ridings et al., 1996). It was believed that the effect on erythroblast remains unchanged and they are intact during this process. Subsequently, several research groups tried to study the affect of ammonium chloride on FNRBCs and adult anucleated red blood cells and to find out whether it could be introduced as the first-step enrichment process. An enrichment protocol was developed from the model mixture experiment where first-trimester fetal erythroblasts isolated from CVS washings were spiked with adult peripheral blood Voullaire et al., 2001). Ammonium chloride for selective lysis of adult erythrocytes was

used as the first-step enrichment process. This was followed by double-density Percoll and MACS MS column using anti-CD71 antibody. They were able to recover 70% of FNRBCs in their model system. When applied to the actual clinical samples in the first trimester they found no FNRBCs in all the 15 patient samples taken before CVS inclusive of two aneuploid cases (Voullaire et al., 2003). Similarly, differential lysis was carried out by another group (Huber et al., 2000). However, none of the primitive erythroid cells were found to be fetal. Despite methods that were able to detect presumably 1 to 2 fetal cells from among 750,000 maternal cells and surveying 100 cases for a minimum of 750,000 cells each, no actual fetal cells were found (Huber et al., 2000). On the other hand three-step enrichment procedure comprising bulk separation method, lysis buffer technique as a second-step enrichment method followed by triple percoll gradient was used directly on patient samples (de Graaf et al., 1999). They were not able to enrich FNRBCs from 30% of cases. Selective lysis of adult erythrocytes using ammonium chloride in their model mixture experiment was attempted as the first-step enrichment method (Choolani et al., 2003). They observed a lysis of 88% adult erythrocytes with no lysis of first-trimester FNRBCs when cell mixtures were exposed to 10:1 v/v ammonium chloride/1mM acetazolamide for 30 minutes. Despite this promising result, a significant clumping between unlysed erythrocytes and enriched primitive NRBCs rendered a poor morphology of the target cells and made it impossible to be recognised during fetal cell identification assay technique. Their findings indicate that ammonium chloride lysis technique may not be an ideal method to be used as a first-step enrichment method as FNRBCs in some way are altered by the lysis procedure and there might be an alteration in the erythroblast membrane properties. It may however be useful if incorporated after the initial density gradient and MACS step, when maximum number of adult erythrocytes is already depleted.

Till to-date, no fetal cell enrichment protocol has demonstrated superiority over the other. This is because, the choice of protocols have been guided by (i) testing the efficiency directly on patient blood samples in the first and second trimester where the initial number of target cells remain unknown (ii) using invasive procedures as *in-vivo* model system where, the numbers obtained before and after the procedure is too small to reflect the efficiency of a system and (iii) model mixtures using cord blood NRBCs (Bhat et al., 1990; Troeger et al., 1999; Smits et al., 2000) rather than the cells actually to be targeted for non-invasive prenatal diagnosis: first-trimester erythroblasts.

1.6.4.4. Testing enrichment protocols in maternal blood

In any fetal cell enrichment protocol, two efficiencies need to be defined: *in-vitro* enrichment efficiency and *in-vivo* enrichment efficiency. The *in-vitro* enrichment efficiency can be readily obtained from experiments on model mixture of the target cells in maternal blood. The *in-vivo* enrichment efficiency is harder to define since these would vary with different protocols, and the exact number of fetal cells circulating in maternal blood can never be known with certainity. Using various combinations of the above-mentioned enrichment methodologies, numerous groups applied their protocols on maternal blood samples to enrich fetal nucleated erythroblasts (Table 1), without testing its efficiency in *in-vitro* model system. In their *in-vitro* model mixture experiment using CVS (Voulliare et al., 2001) they observed the efficiency of their protocol to be approximately 70%. However, in an *in-vivo* model, no FNRBCs could be enriched in the pre-CVS maternal blood samples (Voulliare et al., 2003). Others used pre- and post procedure maternal blood samples to test the efficiency of enrichment protocols (Table 2). CVS was the most commonly used procedure. This could help them identify the number of fold increase in the FNRBCs in the maternal blood and therefore, the number

of target cells to be expected to circulate in an ongoing pregnancy in the first and second trimester. Triple density gradient (Ficoll 1077, 1110, 1119) and MACS of anti-CD71 MAb was used (Ganshirt-Ahlert et al., 1993) to determine the total number of enriched erythroblasts before and after invasive procedures. They observed no significant differences. The authors did not document the kind of invasive procedures used. In their subsequent study (Ganshirt-Ahlert et al., 1998) they again reported no difference and the invasive procedures included in this study were CVS, amniocentesis and fetal blood sampling. These studies were limited by the fact that the pre and the post procedure blood samples were obtained from two different groups of women. The range of gestational age of the pregnancies examined had not been documented as there is a biological variation in the number of FNRBCs present in maternal blood at different gestations (Bianchi et al., 1991; Ganshirt-Ahlert et al., 1994; Sohda et al., 1997; Lim et al., 2001). Further, the distribution of normal and abnormal karyotype in the pregnancies examined before and after the invasive procedures was not given and it is known that in trisomic pregnancies the number of fetal cells in maternal blood is increased (Genest et al., 1995).

Other group (Jansen et al., 1997) isolated fetal cells by Ficoll 1077 single density gradient centrifugation and maternal white cells depletion with anti-CD14/anti-CD45 followed by anti-CD71 MACS enrichment. They then applied FISH for the Y chromosome from blood samples obtained immediately before and after CVS in women carrying karyotypically normal male fetuses at 11-14 weeks gestation. In only 8 cases, CVS was associated with an increase in the number of fetal cells (1-175). Before CVS, the cell numbers ranged from 0-2. In 9 cases no fetal cells were isolated before and after CVS. This inconsistency in FNRBC recovery before and after CVS could most likely be due to

the use of inefficient enrichment protocol as 91.4% of FNRBCs are lost in the pellet with the use of Ficoll 1077 single gradient centrifugation (Choolani et al., 2003).

Quantifification of NRBCs were carried out in blood samples taken both before and after CVS from the same group of 20 patients carrying male euploid pregnancy between 10^{+3} and 13^{+4} weeks of gestation (Oosterwijk et al., 1998a). The authors used triple density gradient as the first-step enrichment protocol. They found a 12-fold increase in the number of NRBCs with a mean number of 1.8 NRBCs in the Pre-CVS and 21.7 NRBCs in the post-CVS group. However, the study had numerous limitations. Firstly, the data in the pre-CVS group was skewed towards zero with 70% of cases demonstrating no cells. Under the non-gaussian distribution, median is a better statistical marker for reporting (Pre-CVS: median = 0; range = 0-8; Post CVS: median = 10; range = 4-100). Secondly, the number of NRBCs was detectable on the basis of HbF staining, which is a non-specific fetal cell marker, as 20% of all HbF positive NRBCs in maternal blood are of maternal origin in the first trimester (de Graaf et al., 1999). No subsequent analysis with FISH was carried out to prove the fetal origin of NRBCs and hence the numbers, before and after CVS. In their subsequent case report, (Oosterwijk et al., 1998c) the authors reported 52 HbF positive NRBCs before and 80 after the CVS procedure with a 1.5-fold increase in the number post-CVS. When cFISH was carried out to determine the fetal origin, only 58% of pre-CVS and 42% of post-CVS cells showed one X and one Y signal, suggesting that a fraction of HbF positive NRBCs could be of maternal origin.

Table 1.1: Enrichment of fetal nucleated erythroblasts from maternal blood

Authors	Year	GA	Density	Deplete	Select	Detection
Price et al	1991	1st & 2nd trimester	Ficoll 1077		CD71/GPA	cFISH /PCR
Bianchi et al cFISH	1992	19	Ficoll 1077		CD71	
Elias S et al	1992	10^{+}			CD71/GPA	cFISH
Chueng et al	1996	10-12	Ficoll 1077		CD71	Immunfluorescence staining for zeta/gamma-globin/PCR
Pezello et al	1997	15^{+6}	Triple density Ficoll gradient		CD71	cFISH
Kuo et al	1998	5-32	Triple density Ficoll gradient			KB
Al-Mufti et al	1999	12	Triple density Ficoll gradient		CD71	KB/cFISH
Rodriguez et al	2001	10-20	Double density Ficoll 1077 and 1109		CD71	KB/cFISH
Parano et al	2001	16-18	Double density Ficoll 1077 and 1083			KB/cFISH
Martel et al	2001	14-29	Ficoll 1119		CD71/GPA	KB/cFISH
Hromadnikova et al	2002	19-21	Ficoll 1077	CD14/45	CD71	cFISH
Kitigawa et al	2002	11-16	Ficoll 1090			Lectin/cFISH
Mavrou et al	2003	11-14	Ficoll 1083	CD45	CD71	IHC for epsilon-globin/cFISH
Choolani et al	2003	$8\text{-}10^{+6}$	Percoll 1118	CD45	GPA	Simultaneous immunofluorescence staining for epsilon-globin and cFISH

GA: Gestational age; Density: Type and density of solution used in density gradient centrifugation; CD: Types of cell development antibodies used; GPA: Glycophorin A; cFISH: Chromosomal fluorescence *in-situ* hybridisation; PCR: Polymerase chain reaction; KB: Kleihauer-Betke staining for fetal haemoglobin; IHC:Immunohistochemistry

Table 1.2: Testing efficiency of enrichment protocols by using invasive procedures as *in-vivo* model system

Authors	Year	GA	Density	Deplete	Select	Detection
Ganshirt et al	1993	Not known (before & after invasive procedure)	Triple density Ficoll gradient		CD71	cFISH
Ganshirt et al	1998	Not known (before & after Amnio, CVS, FBS)	Triple density Ficoll gradient		CD71	H & E staining
Jansen et al	1997	11-14 (before & after CVS)	Ficoll 1077	CD14/45	CD71	cFISH
Oosterwijk et al	1998	10^{+3}-13^{+4} (before & after CVS)	Triple density Ficoll gradient			IHC for HbF
Oosterwijk et al	1998	11 (before & after CVS)	Triple density Ficoll gradient			IHC for HbF/cFISH
Raghad et al	2003	11-14 (before & after CVS)	Triple density Ficoll gradient		CD71	KB/ Immunoflouresecence staining for epsilon- gobin/gamma-globin/cFISH
Christensen et al	2003	9-14 (before & after CVS)	BS /Lysis/Triple density Percoll gradient	CD45	CD71	Immunoflouresecence staining for epsilon-globin/gamma-globin/cFISH

GA: Gestational age; Density: Type and density of solution used in density gradient centrifugation; CD: Types of cell development antibodies used; GPA: Glycophorin A; cFISH: Chromosomal fluorescence *in-situ* hybridisation; H & E: Haematoxylin and Eosin staining; KB: Kleihauer-Betke staining for fetal haemoglobin; IHC: Immunohistochemistry; BS: Bulk separation by centrifugation using a specialised tube

The study was confounded by the inclusion of trisomic pregnancy, which could have also influenced the number of FNRBCs in maternal circulation before the procedure.

An increase in fetomaternal cellular trafficking of up to three-fold following CVS was reported (Al Mufti et al., 2003). They followed the enrichment protocol as used by the above mentioned group (Ganshirt-Ahlert et al., 1993). The authors included all karyotypically abnormal trisomy 18 cases in their study, which could have confounded the number of NRBCs enriched before the invasive procedure. Further, the authors used Kleihauer-Betke method, immunocytochemistry staining using monoclonal fluorescein isothiocynate (FITC) conjugate fluorescent antibody against ε- and γ-gamma globin chains and Y-signal cFISH to calculate the enriched fetal erythroblast before and after the procedure. Kleihauer-Betke staining against γ-globin chain is not specific for accurate fetal erythroblast identification and so as the immunohistochemistry, as both could stain HbF positive maternal NRBCs (section 1.6.4.1) and therefore can confound the number of FNRBCs enriched from maternal circulation before and after the CVS.

Christensen et al. (2003) studied the efficiency of their enrichment protocol using CVS between 9-14 weeks on women carrying male fetus. However, the study design was not appropriate. Firstly, the samples were acquired from two different groups of women. Secondly, same enrichment protocols were not applied to all the patients. Thirdly, the volume of blood samples analysed varied from patient to patient. Fourthly, in post-CVS samples, antibody staining against γ and ε chains was used as compared to pre-CVS samples where antibody to ε-chain was used. Only 72% of all NRBCs stained against γ-chain were identified to be of fetal origin on cFISH. Subsequently, in pre-CVS samples, only one cell was identified from among 18 cases.

The above groups tried to assess the efficiency of enrichment protocols in the first and second trimester using the invasive procedure as an *in-vivo* model system. This may not be an ideal strategy as the numbers of cells obtained are too small to reflect the efficiency of the enrichment system. The studies also differed from each other in some way, making direct comparisons unreliable. The results were further confounded by:

1. Using enrichment protocols, the efficiency of which was tested by other group (Bhat et al., 1990) in model mixture experiments. The cells of interest mostly came from cord blood samples. Cord blood FNRBCs and definitive erythroblasts have identical morphology and similar biological and physical properties. However, they differ significantly from primitive fetal nucleated erythroblasts (Choolani et al., 2003) which are now considered the target for first trimester non-invasive prenatal diagnosis (section 1.6.4.2).
2. Non-uniformity of protocol for FNRBCs enrichment used among different groups
3. Inclusion of aneuploid pregnancies in study design
4. Usage of non-specific fetal cell markers during the enrichment and identification process
5. Application of enrichment protocols across varying gestational ages
6. Pre and post procedure samples obtained from two different groups
7. Targeting only male pregnancies.

1.7 Experimental aims and hypotheses

Development of non-invasive prenatal diagnosis has potential to reduce, or even eliminate, the risk of miscarriage from invasive diagnostic testing by CVS, amniocentesis and FBS. Fetal DNA can be retrieved from maternal blood in the plasma or cellular

fraction. Using DNA in the plasma fraction, only a few paternally inherited single gene differences can be diagnosed whereas the isolation of fetal nucleated erythroblasts permits the diagnosis of any fetal aneuploidy or monogenic disorder. If such a technology is available in the first trimester, women having invasive diagnostic testing would increase the uptake of test (Zamerowski et al., 2001).

Non-invasive prenatal diagnosis using a cell-based strategy can be divided into 3 phases: (i) enrichment of fetal cells from maternal blood (ii) identification of enriched cells as fetal and (iii) making the genetic diagnosis. The latter two phases have been addressed by demonstrating ε-globin, a developmentally-specific embryonic globin, as a fetal nucleated erythroblast specific marker in normal and aneuploidy pregnancy in the first-trimester for non-invasive prenatal diagnosis (section 1.6.4.2). However, two problems need to be addressed:

(a) First, it remains to be identified whether ε-globin positive primitive fetal nucleated erythroblasts persist in the blood of chromosomally abnormal neonates at birth. Since, 45-50% of ε-globin positive erythroblasts are still found to be present in euploid fetal circulation as late as 12 weeks gestation (Al-Mufti et al., 2000; Choolani et al., 2001), it is likely to be enriched from pregnant women's blood sample carrying euploid (Al-Mufti et al., 2001; Choolani et al., 2003) and aneuploidy fetuses in the first trimester such as trisomy 13 (Choolani et al., 2003) and trisomy 18 (Al-Mufti et al., 2003), respectively. Recently, it was observed that these cells persist in blood of chromosomally abnormal fetuses (Trisomy 13, 18, 21 and triploidy) up to 28 weeks gestation (Al-Mufti et al., 2000). Enrichment of ε-globin positive primitive fetal nucleated erythroblasts from women in the second trimester known to be carrying trisomy 21 fetus was demonstrated after the amniocentesis and before the termination of pregnancy (Mavrou et al., 2003).

These observations indicate that embryonic ε-globin gene is not switched off in chromosomally abnormal fetuses and such target cells could persist into the second trimester. It was hypothesised that such cells could persist up to term in aneuploidy fetuses. Trisomy 18 was selected as a model to prove this hypothesis. The choice of T18 as an aueploid model to base our investigations was based upon the assumption that the haemoglobin switch is similar in all aneuploidies, and that the placental interface is similar in its leakiness for both T18 and T21. We acknowledge that T18 is rarer than T21 and choosing T18 will limit our ability to gauge what happens in a T21 pregnancy which is more clinically relevant, it was due to the availability of T18 cases in our unit at that point in time and lack of any T21 cases, that we chose to undertake T18 to prove our hypothesis. If proven, it could be of great clinical significance, as the existence of these cells could indicate not only the presence of a chromosomally abnormal fetus but also imply that the ε-globin primitive fetal nucleated erythroblasts are also ideal cell to be targeted in the second trimester for non-invasive prenatal diagnosis. This thesis looks into, whether the ε-globin positive primitive fetal nucleated erythroblasts persists beyond the second trimester in the trisomy 18 fetus and in trisomy 18 neonates at birth.

(b) Secondly, the first phase, i.e. enrichment of FNRBCs from maternal blood still remains a challenge, as to-date, efficiency of enrichment protocols which have been used for isolating fetal nucleated erythroblasts from maternal blood remains unknown. This is because efficiency of FNRBCs enrichment methods have been tested using cord blood samples, aneupolid fetuses, post-procedure maternal blood samples and pre-procedure maternal blood samples. Most of the protocols were developed on cord blood NRBCs (Ganshirt et al., 1992; 1998; Bhat et al., 1993; Troeger et al., 1999; Smits et al., 2000) in model mixture experiments. Subsequently, they were applied directly on maternal blood samples in on-going prengnacies so as to test its *in-vivo* efficiency without actually

testing its efficiency in an appropriate *in-vitro* model system. This method of testing the efficiency of enrichment protocols is not appropriate because (1) properties of cord blood FNRBCs are similar to that of maternal NRBCs and both contain γ-globin which is a non-specific fetal cell marker and (2) upto 50% of FNRBCs enriched by the protocols are maternal in origin (Troeger et al., 1999; Lim et al., 2001) and (3) the number of target cells obtained by applying enrichment protocols directly on maternal blood is too small to reflect the efficiency of an enrichment system for non-invasive prenatal diagnosis.

Few groups tested the efficiency of the protocols directly on maternal blood samples complicated by aneuploidy (Price et al., 1991; Elias 1992; Bianchi et al., 1992; Pezello et al., 1997; Oosterwijk et al., 1998; Al-Mufti et al., 1999; Parano et al., 2001; Hromadnikova et al., 2002). This is also not appropriate, as the placental feto-maternal barrier is disturbed due to impaired development and function of placental villi leading to an increased fetomaternal transfusion (Genest et al., 1995).

Others used post-procedure maternal blood samples to study the efficiency of their fetal cell enrichment methods (Samura et al., 2000; Wang et al., 2000; Cha et al., 2003; Purwosunu et al., 2006). It is possible to determine the *in-vivo* enrichment efficiency of novel fetal cell enrichment protocols following an invasive procedure since greater number of target cells circulate within maternal maternal blood. It is impossible however to extrapolate how this enrichment protocol would behave in ongoing pregnancies in the absence of any invasive procedure. It is logical to test new fetal cell enrichment protocols after invasive procedures, but no mechanism exist to-date that could predict the behaviour of such a protocol in ongoing pregnancies. Furthermore, if the invasive procedure were a surgical termination of pregnancy performed for non-medical indications in the first trimester, the development of a predictive model would indicate

how this new enrichment protocol would work in healthy, ongoing pregnancies in the first trimester of pregnancy.

First trimester PFNRBCs contain embryonic epsilon-globin, an ideal fetal cell identifier (Choolani et al., 2001; Choolani et al., 2003), and are the promising candidate target cells for non-invasive prenatal diagnosis (Choolani et al., 2001; 2003; Voulliare et al., 2001; Christensen et al., 2003; Mavrou et al., 2003). However, protocols to enrich these cells from maternal blood have been developed at CVS (Al-Mufti et al., 2003; Christensen et al., 2003; Mavrou et al., 2003; Voullaire et al., 2003) and at surgical termination of pregnancy (Choolani et al., 2003). In these studies feal cells were enriched from maternal blood post-procedure, but there was no way to reliably pedict the performance of the enrichment protocol in pre-procedure samples. First trimester surgical termination of pregnancy for non-medical indications is preferable to CVS as the index invasive procedure as the results are less likely to be biased by an overrepresentation of high-risk pregnancies.

Therefore, currently there is no accurate assessment of the efficiency of any enrichment protocol and no standard method is available to evaluate the efficency of new fetal cell enrichment system. There is a lack of any such validated *in-vivo* biological model that can be used to assess the efficiency of a new fetal cell enrichment system. This thesis aims to develop an *in-vivo* model of biological feto-maternal haemorrhage that could be used to evaluate new fetal cell enrichment protocol in standardized fashion in the first trimester. Termination of pregnancy was chosen as a biological model to study first trimester non-invasive prenatal diagnosis because fetal blood sampling and amniocentesis is performed in the second trimester and by this time PFRNBCs reach negligible levels in the fetal circulation. Therefore, it does not give the target cells i.e. primitive fetal

nucleated erythroblasts to study the efficiency of enrichment system in the first trimester. CVS is also not an appropriate model to use because it is an invasive testing method with fetal loss associated with it and the number of FNRBCs extracted for *in-vitro* model is too small to test the efficiency of any enrichment system. Furthermore, CVS is performed only in those pregnancies which are at high-risk of carrying aneuploidy fetus and therefore the results are likely to be biased by an overrepresentation of high-risk pregnancies. On the other hand, surgical termination of pregnancy does not carry any risk to the fetus and the PFNRBCs can be enriched in sufficient numbers from termination of pregnancy tissue for *in-vitro* model mixture experiments to study the efficiency of a novel enrichment system for non-invasive prenatal diagnosis in the first trimester. Though the use of fetal tissue to extract FNRBCs can be further improved using pure fetal blood via cardiocentesis, it depends on the availability of such samples and would be near impossible to gather in Singapore.

Recently, a novel two-step protocol, Percoll 1118 density gradient followed by anti-CD45/anti-GPA on MACS based on first-trimester primitive fetal nucleated erythroblasts was designed (Choolani et al., 2003). The properties of these cells differ significantly from second-trimester definitive nucleated erythroblasts as it contains embryonic ε-globin as a fetal cell specific marker. However, the efficiency of this protocol is not yet been established. This thesis investigates the efficiency of a novel fetal cell enrichment protocol.

Cell free fetal DNA has been investigated as a marker for feto-maternal haemorrhage at surgical termination of pregnancy (Bianchi et al., 2001; Watagnara et al., 2004; 2005) and we plan to use this fetal genetic material as corroborating evidence of the quantity of feto-maternal haemorrhage.

The study was planned in 4 stages: (a) to demonstrate if ε-globin positive primitive fetal nucleated erythroblasts circulate in trisomy 18 fetus beyond the first-trimester and in cord blood of trisomy 18 neonates at birth (b) to evaluate efficiency of a new fetal cell enrichment protocol in *in-vitro* model system (c) to develop an *in-vivo* model of biological feto-maternal haemorrhage that could be used to evaluate efficiency of new fetal cell enrichment protocol for non-invasive preantal diagnosis in the first trimester and (d) to demonstrate cell-free fetal DNA as a marker of feto-maternal haemorrhage using surgical termination of pregnancy in the first trimester.

1.7.1 Hypotheses

1. Embryonic epsilon-globin positive nucleated red blood cells persists beyond the second trimester using T18 as a model.

2a. Surgical termination of pregnancy can be used to evaluate efficiency of a new fetal cell enrichment protocol in *in-vitro* model system.

2b. Surgical termination of pregnancy can be used as an *in-vivo* model to study enrichment efficiency of a novel non-invasive prenatal diagnosis method in the first trimester.

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Human tissue and blood samples

2.1.1.1 Ethical approval for use of human tissue and blood samples

Blood and fetal tissue collection for this research was approved by the Institutional Research Board of National University Hospital, National University of Singapore in compliance with guidelines regarding the use of fetal tissue for research purposes. All research participants gave written informed consent for the collection of their blood samples and fetal trophoblasts tissue and fetal blood samples.

2.1.1.2 First trimester trophoblasts, second trimester fetal blood samples and cord blood

First trimester fetal trophoblasts tissue was collected immediately after termination of pregnancy from within the products of conception. Fetal blood sample was collected by ultrasound-guided trans-abdominal cardiocentesis immediately prior to clinically indicated termination of pregnancy due to chromosomal abnormality in the second trimester. Fetal blood samples were also collected by trans-abdominal cordocentesis from pregnant women undergoing prenatal diagnosis in the second trimester. Cord blood was obtained from chromosomally normal and abnormal newborns at birth.

2.1.1.3 Peripheral blood from pregnant women

Peripheral blood samples (23 ml) were collected from women undergoing termination of pregnancy in the first trimester for non-medical indications. Samples were collected either, before and after termination of pregnancy or immediately after termination of pregnancy.

2.1.1.4 Peripheral blood from healthy male volunteers

Peripheral blood samples (3 ml) were collected from healthy male volunteers.

2.1.2 Antibodies, reagents, media, solutions and kits

All antibodies, reagents, media and kits used in the experiments are listed below together with their source.

2.1.2.1 Antibodies

Antibodies directed against the surface antigens GPA and CD45 conjugated to magnetic beads (Miltenyi Biotech, Germany, GMBH); unconjugated anti ε-globin antibody (Europa Bioproducts, Cambridge, UK); biotinylated goat anti-mouse secondary antibody (Vector Laboratories, Burlingame, CA).

2.1.2.2 Reagents and supplements

Methanol, ethanol, acetone, tri-sodium citrate, sodium chloride (NaCl) and glacial acetic acid (AnalaR, BDH Merck, Poole, UK); Nonidet p40 (NP40) and dextran sulphate (BDH Merck, Poole, UK); Wright stain (Weskor, Utah, USA); ammonium chloride (NH_4Cl)

(StemCell Technologies, London, UK); Hank's Balanced salt solution (HBSS) without calcium and magnesium, phosphate-buffered saline (PBS) and ethylenediaminetetraacetic acid (EDTA) GIBCO/BRL, Invitrogen Life Technologies, Paisley, UK); trypsin (Difco, BD diagnostic systems, Oxford, UK); Tween-20 (polyoxyethylene sorbitan monolaurate; DAKO Corporation, Carpinteria, CA); formamide (Promega, Madison, WI); bovine serum albumin (BSA), normal goat serum, acetazolamide and Ficoll (Histopaque) 1077 (Sigma Chemical Co., Poole, UK); Hepes 1M, Percoll 1.030 g/ml (Amersham Pharmacia Biotech, Buckinghamshire, UK); streptavidin conjugated with AMCA (7-amino-4-methylcoumarin-3-acetic acid), antifade mounting medium with DAPI (diamidino-2-phenyl-indole) and without DAPI (Vectashield) (Vector Laboratories, Burlingame, CA); DPX mountant (RA Lamb, East Sussex, UK); 10% fetal calf serum (ICN Pharmaceuticals, Hampshire, UK).

2.1.2.3 Solutions

Carnoy's fixative (3:1 methanol:glacial acetic acid) was prepared fresh for each experiment. Dilutions of salted sodium citrate (SSC) were prepared from a stock of 20X SSC (3M NaCl, 0.3 M tri-sodium citrate); cFISH denaturation and hybridisation buffer (50% formamide and 10% dextran sulphate in 2X SSC, pH 7.0); MACS cell sorting buffer (0.5% BSA/PBS (W/V) supplemented with 2mM EDTA); 1.5M NaCl.

2.1.2.4 Kits

Alkaline phosphatase Vector Blue substrate labelling kit (Vector Laboratories, Burlingame, CA); chromosome-specific centromeric repeat probes DXZ1, labelled with

SpectrumOrange™, and DYZ1, labelled with SpectrumGreen™ (Vysis XY FISH probes, Vysis, Downer's Grove, IL).

2.1.3 Hardware

All specialised hardware used in the experiments is listed below together with their source.

2.1.3.1 Pipettes, centrifuge tubes, slide storage box and filters

Positive-pressure pipettes (Gilson, Villiers-le-Bel, France); Pipette-Aid motorised (Wolf laboratories, York, UK); 1.5 ml conical-bottom polypropylene tubes (Eppendorf, Cambridge, UK); Falcon 70 μm filters, 15 and 50 ml conical-bottom polypropylene tubes (BD Biosciences, Oxford, UK); slides storage box (BDH Merck, Poole, UK).

2.1.3.2 Immunomagnetic cell sorting equipment

VarioMACS, MACS multistand, miniMACS and midiMACS separation units, MS and LD columns (Miltenyi Biotech, Germany, GMBH).

2.1.3.3 Centrifuges for polypropylene tubes

Bench top centrifuge for 15- and 50 ml polypropylene tubes (Beckman GS-6R); bench top microcentrifuge for 0.5- and 1.5 ml polypropylene tubes (Eppendorf, Cambridge, UK).

2.1.3.4 Cytocentrifuge and water bath

Cytocentrifuge (Cytospin 3, Shandon, Manchester, UK); standard water bath (Grant Instruments, Herts, UK).

2.1.3.5 Chromosomal fluorescence *in-situ* hybridisation block

DNA Engine™ thermal cycling system with tower block adaptor for slides (MJ Research, Waltham, MA).

2.1.3.6 Blood collection tubes, slides, coverslips, haemocytometer, coplin jars, immersion oil and Parafilm™

Vacutainer tubes and needles (BD Vacutainer systems, Oxford, UK); frosted end microscope slides, coverslips, Neubauer haemocytometer and coplin jars (BDH Merck, Poole, UK); low fluorescence immersion oil (Zeiss, Welwyn Garden City Herts, UK); Parafilm™ (American National Can Company, Chicago, IL).

2.1.3.7 Microscopes and filters

Inverted light microscope (Olympus, Hamburg, Germany); Fluorescence microscope (Olympus BX51, Hamburg, Germany); Photometrics cooled charge coupled device camera (CCD) (Roper Scientific, Ottobrunn, Germany); Filter sets: triple bandpass filter cube (DAPI/Green/Red); Orange, Aqua and DAPI single bandpass filter cubes (Vysis, Downer's Grove, IL).

2.1.3.8 Computers and software

Dell computer connected to fluorescence microscope (Olympus BX51); SPSS Software (SPSS Inc., Chicago, IL).

2.2 Methods

2.2.1 Nucleated cell count

Up to 10 µl of a nucleated cell sample was diluted 1 in 2 with 3% glacial acetic acid and 10 µl were loaded onto the Neubauer haemocytometer. Nucleated cells were counted under the inverted microscope. At least 200 cells or four separate areas were examined and the average number of cells, N, per 1 mm^2, (0.1 µl volume) determined and the concentration of nucleated cells in the original sample calculated (N x Dilution x 10^4 per ml).

2.2.2 Cytospin preparation

Up to 3 x 10^4 nucleated cells were re-suspended in 300 µl of 0.5% BSA/PBS (w/v), loaded into a cytospin chamber and centrifuged on to a slide at 500 rpm for 5 min inside the cytocentrifuge. Slides were air dried and processed immediately or stored in foil and Parafilm at -20^{o}C.

2.2.3 Preparation of fetal trophoblast cells and primitive fetal erythroblasts from products of conception

Trophoblast digestion buffer was prepared as follows: 0.182 g trypsin (Difco) and 3.75 ml 1M Hepes (Amersham Pharmacia Biotech) were mixed into 146.3 ml HBSS (Invitrogen Life Technologies) and warmed to 37^{o}C to dissolve trypsin. Products of conception were obtained in the first trimester of pregnancy. The trophoblast tissues were carefully dissected off adjacent deciduas and washed twice in PBS to remove

maternal blood contamination. The cleaned specimen was incubated in 50 ml of trophoblast digestion buffer at 37^{o}C for 30 minutes in a water bath. The cell suspension was transferred into a fresh 50 ml polypropylene tube and the enzyme activity stopped with 5 ml fetal calf serum. The cell suspension was then strained through a 70 μm filter to obtain single cell suspension and centrifuged at 3000 rpm (2060 g) (Beckman GS-6R) with brakes off at room temperature for 10 minutes. The cell pellet was re-suspended in 5 ml of PBS and layered over Percoll 1083 and centrifuged at 3000 rpm (2060 g) (Beckman GS-6R) with brakes off for 20 minutes. A gradient interface layer was obtained and the red cells were pelleted at the bottom. The cells in the interface layer containing the trophoblast cells were collected separately and suspended in 0.5% BSA/PBS. Up to 3x10^{4} cells were re-suspended into 300 μl 0.5% BSA/PBS (w/v) and loaded into cytospin chamber and centrifuged onto a slide at 500 rpm for 5 minutes inside the cytocentrifuge. Slides were air dried and processed for standard XY cFISH (section 2.2.12). Red cells pelleted at the bottom contained primitive fetal nucleated erythroblasts and were suspended in 0.5% BSA/PBS and washed once at 3000 rpm (2060 g) (Beckman GS-6R) with brakes off for 10 minutes to be used for model mixture experiments. The remaining cells were immediately cytocentrifuged on to glass slides (section 2.2.2) to serve as internal positive and negative controls for immunocytochemical staining (section 2.2.10), immunofluoresence staining using AMCA and XY cFISH (section 2.2.13), and combined immunoenzymatic, immunofluoresence and cFISH (2.2.14).

2.2.4 Preparation of adult mononuclear cells using Ficoll 1077 for external negative controls

Blood was obtained from adult male volunteers after informed consent and the samples were diluted 1:1 in PBS and carefully layered over an equal volume of Ficoll 1077 in 15

ml propylene tube. Samples were centrifuged at 1800 rpm (700 g) (Beckman GS-6R) for 30 minutes keeping the brakes off at room temperature. Compact nucleated cell layers at the gradient interface were collected using a 22-gauge needle attached to a 3 ml syringe. Cells collected were transferred separately into clean 15 ml polypropylene tubes, washed twice with 5-10 ml of 0.5% BSA/PBS at 3000 rpm (2060 g) (Beckman GS-6R) for 10 minutes with brakes off at room temperature. The supernatant was carefully transferred and the cell pellet re-suspended in an appropriate volume of 0.5% BSA/PBS for counting of nucleated cells. Up to 10 µl of a nucleated cell sample was diluted 1 in 2 with 3% glacial acetic acid and 10 µl was loaded onto Neubauer haemocytometer. Nucleated cells were counted under the inverted microscope (section 2.2.1). Cells were then cytocentrifuged onto glass slides (section 2.2.2) to serve as external negative controls during immunocytochemical staining (section 2.2.10) and fluorescence AMCA and cFISH (section 2.2.13), and combined immunoenzymatic, immunofluoresence and cFISH (2.2.14).

2.2.5 Wright's stain

Cells cytospun on to glass slides were Wright stained by manual method. The slides were dipped in additive methanol for 90 seconds followed by 1 min each in eosin stain and polychrome methylene blue, respectively. The slides were finally rinsed in distilled water for 30 seconds. Processed slides were air-dried and mounted with coverslips over DPX mountant.

2.2.6 Preparation of Percoll gradients

Percoll is available commercially as a density gradient of 1.130 g/ml. Percoll 1083 and 1118 were prepared from this stock by dilution with 1.5M NaCl (ρ = 1.058 g/ml) (Percoll Methodology and Applications 2nd Edition, Amersham Pharmacia Biotech, Buckinghamshire, UK). Percoll has a very low osmolality (<25 mOs/kg H_2O) and can therefore form density gradients with iso-osmotic solutions (1.5M NaCl) without significantly affecting the physiological conditions. This is important for obtaining preparations of cells with intact morphology.

Percoll gradients of the following density were prepared: 1083 and 1118. Following formula was used to prepare Percoll 1083 g/ml and Percoll 1118 g/ml by mixing combination of 1.5M NaCl, Percoll (1.130 g/ml) and distilled water.

To prepare 50 ml of Percoll at a specified density, the following were mixed together:

5ml 1.5M NaCl solution

Percoll (1.130 g/ml), the volume of which was calculated as follows:

$$V_0 = V \frac{\rho - 0.1\rho' - 0.9}{\rho_0 - 1}, \text{ where}$$

V0 = volume of Percoll (1.130 g/ml) in ml

V = volume of the final working solution in ml (50 ml)

ρ = desired density of the final working solution in g/ml

ρ_0 = density of Percoll stock in g/ml (1.130 g/ml)

ρ' = density of 1.5M NaCl (1.058 g/ml) in g/ml

Distilled water to make up to 50 ml.

2.2.7 Layering the cells

Adult blood samples and mixtures of fetal primitive nucleated erythroblasts in adult blood samples

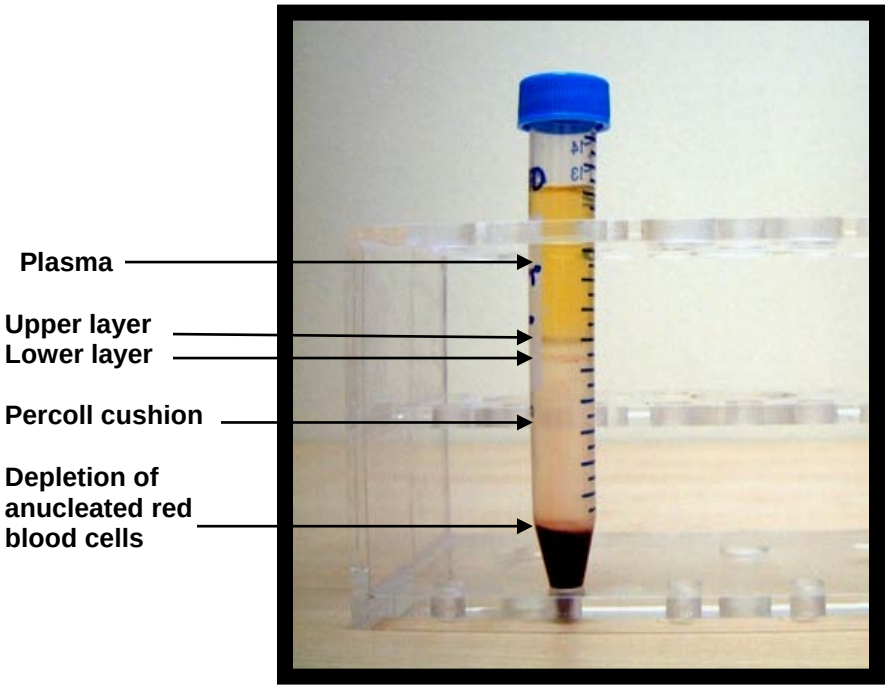
Adult blood samples were diluted 1:1 in PBS and carefully layered over an equal volume of Percoll 1118 in 15 ml propylene tube. All the tubes were coated with 5% BSA so as to avoid the target cells getting adhered to the wall of the tubes. Samples were centrifuged at 2750 rpm (1745 g) (Beckman GS-6R) keeping the brakes off for 30 minutes at 18^{o}C. Two layers were obtained at the gradient interface: an upper and a lower cell layer (Figure 2.1). The layers were collected using a 22-gauge needle attached to a 3 ml syringe. Cells were transferred into clean 15 ml polypropylene tubes, washed twice with 0.5% BSA/PBS at 3000 rpm (2060 g) (Beckman GS-6R) keeping the brakes off for 10 minutes at room temperature. The supernatant was carefully transferred and the cell pellet re-suspended in an appropriate volume of 0.5% BSA/PBS for subsequent processing. This density gradient is applied in this thesis as the first-step enrichment strategy for isolation of primitive fetal nucleated erythroblasts in model mixture experiments and from clinical samples.

Fetal blood samples and cord blood samples

Fetal blood samples and cord blood samples were diluted 1:1 in PBS and carefully layered over an equal volume of Ficoll 1077 g/ml and Percoll 1083 g/ml, respectively, in 15 ml propylene tubes. Samples were centrifuged at 1800 rpm (700 g) and 3000 rpm (2060 g) (Beckman GS-6R) for 30 minutes at room temperature keeping the brakes off,

respectively. Nucleated cell layers at the gradient interface were collected using a 22-gauge needle attached to a 3 ml syringe. Cells were transferred into clean 15 ml polypropylene tubes, washed twice with 5-10 ml of 0.5% BSA/PBS at 3000 rpm (2060 g) for 10 minutes. The supernatant was transferred and the cell pellet re-suspended in an appropriate volume of 0.5% BSA/PBS for subsequent processing (Chapter 3).

Figure 2.1 Two cell layers formed after density gradient centrifugation with Percoll 1118



2.2.8 Centrifugation

The behaviour of Ficoll and Percoll gradients is influenced by the model of the centrifuge, the angle of the rotor used, the rotation speed and the size of the centrifuge tubes. Thus, centrifuge tubes were standardised and the same bench top centrifuges were used in all experiments. Two sizes of conical-bottom polypropylene tubes were used: 1.5 ml (Eppendorf) and 15 ml (Falcon). The centrifugation speeds for two centrifuges

(Eppendorf microcentrifuge and Beckman GS-6R centrifuge) were optimised before specific experiments began. The optimal speed for the microcentrifuge was 3000-5000 rpm (654-1817 g) while for the Beckman GS-6R centrifuge was 1800 rpm (700 g) for Ficoll 1077 g/ml, 2750 rpm for Percoll 1118 (1745 g) and 3000 rpm for Percoll 1083 (2060 g). Lower or higher speeds produced smears of cells rather than compact layers at the gradient interface whereas very high speeds altered cell morphology. The following formula was used to convert rotation speeds into g value:

$$g = 1118 \times 10^{-8} \times R \times N^2, \text{ where}$$

g = relative centrifugal field (multiple of acceleration due to gravity g = 9.80 m/s^2)

R = radius of rotation in cm

N = speed of rotation in revolutions per min (rpm)

2.2.9 Selective lysis of mature anucleate red blood cells

Lysis with ammonium chloride

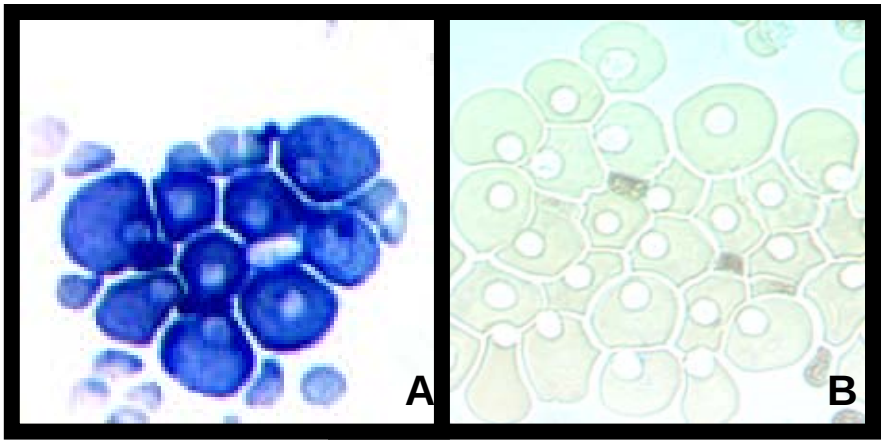
Ice-cold ammonium chloride was mixed with pure populations, or mixtures, of fetal erythroblasts and adult mature anucleated erythrocytes at 1:2 ratios. These were incubated in ice for a specified duration. The durations selected depended upon the aim of the particular experiment. If the activity of carbonic anhydrase within red cells needed to be blocked, this was done using acetazolamide diluted in PBS. Proportions of cells lysed were determined by analysing a small aliquot (10 μl) of the sample in a haemocytometer.

2.2.10 Immunocytochemistry

Expression of intracellular ε-globin (Europa Bioproducts) on first trimester primitive fetal nucleated erythroblasts was determined using alkaline phosphatase immunocytochemistry. Cytospun slides containing FNRBCs isolated from first trimester products of conception were fixed in 1:1 (v/v) methanol:acetone for 5 minutes at room temperature and rinsed in PBS for 2 minutes and air-dried. After rehydration in PBST, the slides were incubated with goat serum (Sigma) diluted 1:10 with PBS for 2 hours. The slides were washed twice with PBST, incubated with anti-ε antibody diluted 1:100 for 60 minutes and washed again twice. Subsequent incubations were with a secondary antibody, biotinylated goat anti-mouse (Vector Laboratories) and with streptavidin conjugated with alkaline phosphatase (Vector Laboratories), both diluted 1:100 and incubated for 30 minutes each. The colour reaction was developed with freshly prepared Vector Blue substrate (Vector Laboratories) for 10 minutes without light. All incubations were in a humidifying chamber at room temperature. All washes were in PBST for 5 minutes each and slides were washed between incubations. The slides were finally rinsed in tap water and dehydrated in 100% ethanol for 30 seconds each, air dried and mounted in Vectashield (Vector Laboratories). Slides were analysed by light microscopy, which demonstrated ε-globin positive primitive fetal nucleated erythroblasts (Figure 2.2A). Subsequently, some of the slides were stained for nuclear fast red stain. After rinsing the slides under tap water for 30 seconds, nuclear fast red stain was applied for 20 minutes followed by rinsing under tap water for 30 seconds and dehydration with 70%, 90% and 100 ethanol for 2 minutes each. Slides were then air-dried and mounted. Slides were examined under light microscopy (Figure 2.3).

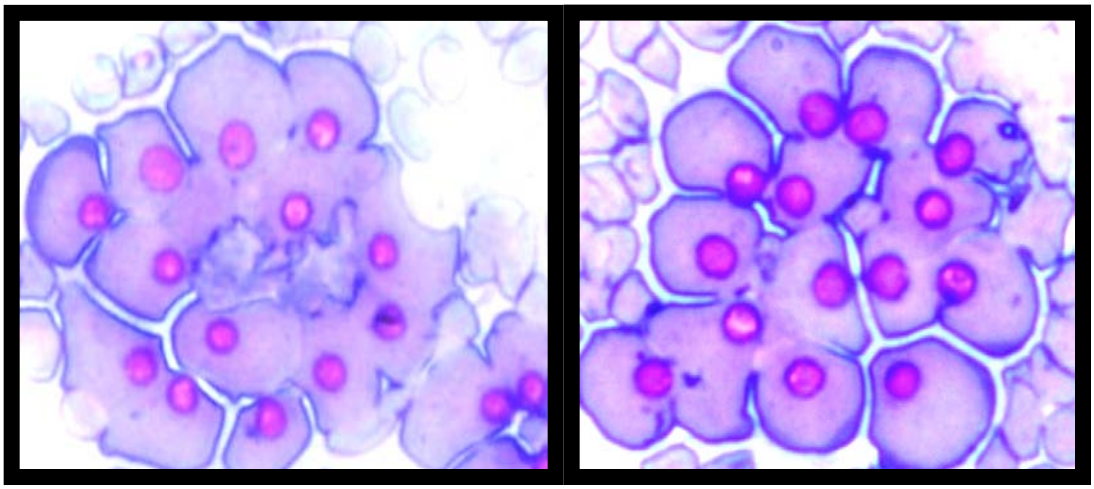
Figure 2.2 Immunocytochemistry on fetal primitive nucleated erythroblasts enriched from products of conception

Internal positive control **Internal negative control**



A: ε-globin positive primitive fetal nucleated erythroblasts (40 x magnifications)
B: Primitive fetal nucleated erythroblasts without primary antibody (40 x magnifications)

Figure 2.3 Nuclear fast red stain on ε-globin positive primitive fetal nucleated erythroblasts enriched from products of conception



(60 x magnifications)

2.2.11 Magnetically-activated cell sorting (MACS)

Positive and negative selection using the MACS immunomagnetic separation system was performed with only modifications to the manufacturer's instructions (Miltenyi Biotech).

Cells to be enriched were suspended in sorting buffer (80 μl per 10^7 total cells) and incubated with the appropriate immunomagnetic bead-conjugated anti-human murine monoclonal antibody (20 μl per 10^7 total cells, Miltenyi Biotech) at 4^{o}C for 30 min and the tubes were tapped at every 5 minutes interval so as to allow maximum binding of the cells to the antibody-coated beads. After washing with buffer, the pellet was re-suspended in sorting buffer and passed through the MS or LD columns attached to the MACS multi-stand via the miniMACS or midiMACS separation units, respectively. Unlabelled cells that passed through the columns were collected as the negative fraction. Cells of the positive fraction, retained within the columns, were collected by positive-pressure elution. After washing, cells were re-suspended in the buffer appropriate for the next stage of investigation such as 0.5% BSA/PBS for cytocentrifugation.

2.2.12 Standard XY cFISH

Cytospun slides, prepared as described in section 2.2.2, were fixed using 5 drops (10 μl each) of Carnoy's fixative and air-dried. Slides were then dehydrated through 70%, 90% and 100% ethanol for 2 minutes and air-dried. Five micro litres of Vysis XY FISH probe diluted 2:3 in the hybridisation buffer were added to each cytospin under a coverglass sealed with Parafilm. Target DNA was denatured on an in situ hybridisation block at 71^{o}C for 7 minutes followed by 4 hours hybridisation at 37^{o}C. Post hybridisation washes included once in 0.4XSSC at 72^{o}C for 2 minutes and once in 2XSSC for 2 minutes at room temperature. Slides were dehydrated through an ethanol series and protected by coverslips mounted over fluorescence antifade medium containing DAPI (Vector Laboratories) and sealed with clear nail varnish. Throughout the procedure, the slides were shielded from direct light. Slides were analysed by epifluorescence microscopy using single band pass filters for Aqua, Orange and DAPI and triple band pass filter set.

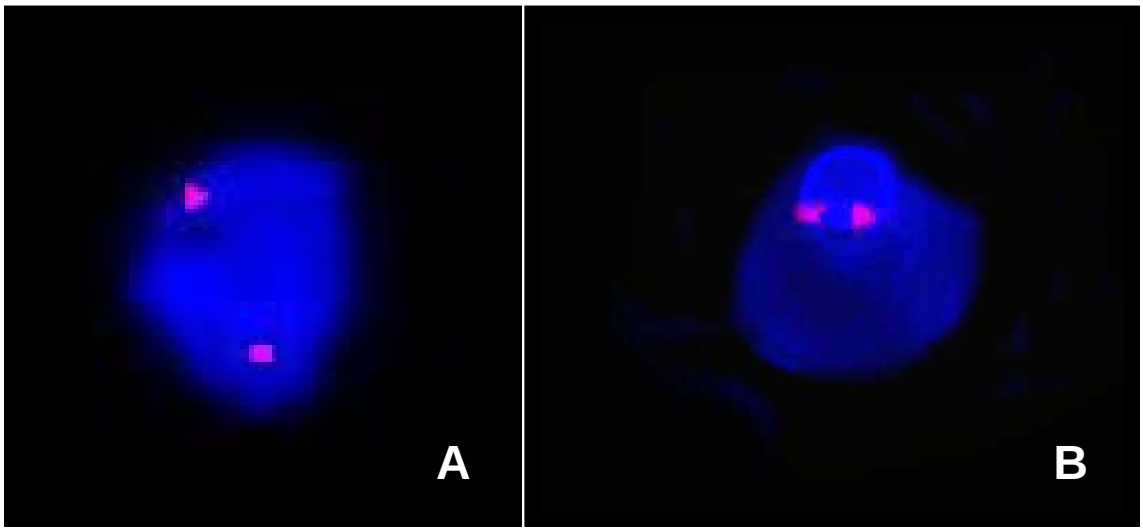
Images were captured using a cooled CCD camera (Roper Scientific). Nuclei with two red or orange signals were classified as female (XX) (Figure 2.4A). Those with one red or orange signal and one green signal were categorized as male (XY).

2.2.13 Simultaneous immunofluorescence cytochemistry and cFISH

Cytospun slides prepared (section 2.2.2) were fixed in 1:1 (v/v) methanol:acetone for 8 minutes at room temperature permeabilised with 0.25% glacial acetic acid in methanol (v/v) and rinsed in PBST. The slides were incubated with goat serum (Sigma) diluted 1:1 with PBS for 2 hours. The slides were washed twice with PBST, incubated with anti-ε monoclonal antibody diluted 1:100 for 60 minutes and washed again twice. Subsequent incubations were with a secondary antibody, biotinylated goat anti-mouse (Vector Laboratories) and with streptavidin conjugated with AMCA (Vector Laboratories) both diluted 1:100 and incubated for 30 minutes each. All incubations were in a humidifying chamber at room temperature. All washes were in PBST for 5 minutes each and slides were washed between incubations. Slides were dehydrated through 70%, 90% and 100% ethanol air dried and prepared for cFISH to the sex chromosomes. Five micro litres of the probe diluted 2:3 in hybridisation buffer were added to the each cytospin under a coverglass sealed with Parafilm. Target DNA was denatured on an in situ hybridisation block at 71^{o}C for 7 minutes followed by 4 hours hybridisation at 37^{o}C. Post hybridisation washes included once in 0.4 X SSC at 72^{o}C for 2 minutes and once in 2 X SSC at room temperature for 2 minutes. Slides were dehydrated through an ethanol series and protected by coverslip mounted over fluorescence anti-fade mounting medium without DAPI and sealed with clear nail varnish. Slides were analysed by epifluorescence microscopy using single band pass filters for Aqua, Orange and DAPI and triple band pass filter set. Images were captured using a cooled CCD camera (Roper

Scientific), which demonstrated simultaneous fetal cell specific ε-globin positive primitive fetal erythroblasts stained with AMCA and the diagnosis of female gender by cFISH (Figure 2.4B).

Figure 2.4 Standard cFISH (A) and simultaneous AMCA and cFISH on fetal primitive erythroblasts (B): XX fetus



A: DAPI staining of the nucleus of primitive fetal erythroblast (40 x magnifications)
B: Accumulation of AMCA around the nucleus and staining of intracytoplasmic ε-globin positive primitive fetal nucleated erythroblast, depicting the morphology of the entire cell (40 x magnifications)

2.2.14 Combined immunocytochemistry-Vector Blue Substrate, Immunofluorescence AMCA and cFISH

Cytospun slides demonstrating ε-globin positive primitive fetal erythroblasts on immuncytochemical staining (section 2.2.10) were processed further for fluorescence staining with AMCA and cFISH analysis for fetal cell identification and gender determination. Slides were destained in Xylene followed by rinse in distilled water for 2 minutes and air-dried. Slides were then washed in PBST twice for 5 minutes and air-

dried. Subsequent incubation was carried out with streptavidin conjugated with AMCA (Vector Laboratories) diluted 1:100 and incubated for 30 minutes. Incubation was performed in a humidifying chamber at room temperature followed by washes in PBST for 5 minutes each. Cells were permeabilised with 0.25% glacial acetic acid in methanol (v/v) and 5 drops (10 μl each) was placed on each slide. Slides were then dehydrated through 70%, 90% and 100% ethanol, air dried and prepared for cFISH to the sex chromosomes as described (section 2.2.12).

2.2.15 Quantitative analysis of cell-free fetal DNA

DNA extraction from plasma samples and Real-Time Quantitative PCR

Whole blood samples were centrifuged at 2800 rpm for 15 minutes and plasma was carefully removed and placed into clean 1.5 ml eppendorf tubes and re-centrifuged at 13000 rpm for 15 minutes. The supernatant was collected in a fresh 1.5 ml eppendorf tube. 800 μl of plasma sample was used for DNA extraction and the remaining was stored at -80^{o}C. DNA was extracted from plasma samples by use of a High Pure PCR Template Preparation Kit (Roche, GmH) using the protocol for peripheral blood. The concentration of fetal DNA in maternal plasma was determined by real-time PCR amplification using a Perkin-Elmer Applied Biosystems 7700 Sequence Detector (Applied Biosystems, Foster City CA), which is essentially a combined thermal cycler/fluorescence detector with the ability to monitor, optically, the progress of individual PCR reactions. The amplification and product-reporting system used was based on the 5´ nuclease assay (the TaqMan assay, Perkin-Elmer). In this system, in addition to the two amplification primers, as in conventional PCR a dual labelled fluorogenic hybridisation probe was also included. One fluorescent dye (6

carboxyfluorescein [FAM] served as a reporter, and its emission spectrum was quenched by a second fluorescent dye (6 carboxy-tetramethylrodamine [TAMRA]). During the extension phase of PCR, the 5′ to 3′ exonuclease activity of the Taq DNA polymerase cleaves the reporter from the probe, thus releasing it from the quencher, resulting in an increase in fluorescent emission at 518 nm. The fluorescent emission of the 96 amplification wells were continuously measured during the DNA amplification process and recorded by computer using Applied Biosystems Sequence Detection System software, version 1.7.

The *SRY* Taqman system consisted of the amplification primers SRY-109F, 5′-TGG CGA TTA AGT CAA ATT CGC-3′; SRY-245R, 5′-TGG CGA TTA AGT CAA ATT CGC-3′; SRY-245R, 5′-CCC CCTAGTACC CTG ACA ATG TAT T-3′; and a dual labelled fluoresecent TaqMan probe SRY-142T, 5′-(FAM) AGC AGT AGA GCA GTC AGG GAG GCA GA (TAMRA)-3′. The *β-globin* TaqMan system consisted of the amplification primers β-globin-354F, 5′-GTG CAC CTG ACT CCT GAG GAG A-3′; β-globin-455R, 5′-CCT TGA TAC CAA CCT GCC CAG-3′; and a dual-labelled fluorescent TaqMan probe β-globin-402T, 5′-(FAM)AAG GTG AAC GTG GAT GAA GTT GGT GG(TAMRA)-3′ The TaqMan probes contained a 3′-blocking phosphate group, to prevent probe extension during PCR. Primer/probe combinations were obtained from Lo et al. (1998). Sequence data were obtained from the GeneBank Sequence Database (accession numbers L08063 [SRY gene] and U01317 [β-globin gene]).

TaqMan amplification reactions were set up in a reaction volume of 25 µl by use of components (except TaqMan probes and amplification primers) supplied in a TaqMan Universal Master Mix (Perkin-Elmer). TaqMan probes and PCR primers were custom-

synthesized by PE Applied Biosystems. Each reaction contained 1 x Taqman Universal Master Mix A; 240 nM of each amplification primer; 100 nM of the corresponding TaqMan probe; 4mM. 5 µl of the extracted plasma DNA was used for amplification. DNA amplifications were carried out in 96-well reaction plates that were frosted, by the manufacturer, to prevent light reflection and were closed by use of caps designed to prevent light scattering (Perkin Elmer). Each sample was analysed in triplicate with the mean value used for further calculations. A standard curve of titrated, commercial male DNA was run simultaneously in parallel with each analysis. For all samples, the β-globin gene was amplified to estimate the total amount of DNA in the sample. Identical thermal profiles were used for both the *SRY* and the *β-globin* TaqMan systems. Thermal cycling was initiated with a 2-minute incubation at 50^{o}C, to allow the uracil N-glycosylase to act, followed by a first denaturation step of 10 minutes at 95^{o}C and then 55 cycles of 95^{o}C for 15 seconds and 60^{o}C for 1 minute. The conversion factor of 6.6 pg of DNA per cell was used, for expression of results as copy numbers.

2.2.16 Enrichment of epsilon-globin positive primitive fetal nucleated erythroblasts from trisomy 18 syndrome fetus beyond first-trimester and from neonates at term

A pure sample of fetal blood was obtained from a viable trisomy 18 fetus at 20 weeks gestation prior to termination of pregnancy. Only one case was obtained during the course of research period. Fetal blood samples were also obtained by ultrasound guided cordocentesis (n = 4) in patients undergoing prenatal diagnosis in the second trimester (gestational age range = 21^{+2}–23^{+1} weeks) the results of which were karyotypically normal (controls). Cord blood samples were obtained from three trisomy 18 and twenty

karyotypically normal neonates (controls) at term vaginal birth delivery. Though the diagnoses of T18 were made in the second trimester through amniocentesis by sending the amniotic fluid for cFISH, because of religious reason, the couples declined for termination of pregnancy and continued till term. Gestational age was calculated from the date of the last menstrual period and confirmed by ultrasound examination in the first trimester. The fetal blood and cord blood samples were processed using Ficoll 1077 and Percoll 1083 density gradient centrifugation respectively (section 2.2.7) and MACS (2.2.11) to obtain glycophorin-A-(GPA)-positive fetal erythroblasts. Morphology of these cells was evaluated by Wright stain (section 2.2.5) and presence of ε-globin within the cytoplasm was determined using immunocytochemical staining (section 2.2.10). FNRBCs isolated from first trimester surgical termination of pregnancies served as internal positive controls whereas cells incubated with the secondary antibody only were used as internal negative controls (section 2.2.3). Mononuclear cells enriched from healthy male volunteer using Ficoll 1077 were used as external negative controls (section 2.2.4). Chromosome aneuploidy was confirmed with fluorescence *in-situ* hybridisation (cFISH) (section 2.2.12).

2.2.17 Effect of ammonium chloride lysis buffer on first-trimester primitive fetal nucleated erythroblasts and on adult anucleate erythrocytes

Pure population of primitive fetal nucleated erythroblasts were extracted from products of conception in the first trimester (Figure 4.1). Fetal nucleated erythroblasts and adult anucleate erythrocytes were then treated with ammonium chloride at the ratio of one volume of cell suspension to 2 volumes of lysis buffer so as to identify the time period at

which the adult erythrocytes start to lyse and the target cells remain intact. All the incubations were carried out on ice. In between observations, reaction mixtures were left on ice.

10 ul of PBS containing 6 x 10^2 first trimester fetal erythroblasts were mixed with ice cold ammonium chloride lysis buffer. The effect of the lysis buffer on FRNBCs was observed across 5 to 40 minutes time interval. The experiment was repeated four times and average percentages of cells remaining intact were calculated.

In the second experiment, pure population of 1.5 x 10^6 adult erythrocytes diluted in 10 ul of PBS were treated with ammonium chloride lysis buffer across 5-40 minutes time interval. Percentage of lysed cells was calculated over an average of four repeat experiments. The test for 95% CI was calculated by repeated measures ANOVA for binary outcome.

2.2.18 Effect of ammonium chloride alone and ammonium chloride/1mM acetazolamide on a model mixture of primitive fetal nucleated erythroblasts and adult anucleated red blood cells

Products of conception were obtained in the first trimester of pregnancy. In the first experiment, 10^4 FNRBCs were spiked with 5 x 10^6 adult erythrocytes (1:500) and exposed to ammonium chloride alone for 5 minutes. The experiments were repeated four times and average percentage of lysed cells was calculated for both cell types.

In the second experiment, the mixture of first trimester fetal erythroblasts and adult anucleated erythrocytes were exposed to ammonium chloride containing 1mM acetazolamide. Average percentage of lysed cells was obtained for four similar experiments. Paired t-test was applied for data analysis.

2.2.19 Optimisation of second-step enrichment method on maternal blood samples

Six maternal blood samples (20 ml each) obtained in the first trimester of pregnancy were processed through Percoll 1118 density gradient as the first-step enrichment protocol (section 2.2.7). At the second-step, three samples were treated with anti-GPA and three with anti-CD45 on MACS, respectively (section 2.2.11). Four maternal samples (20ml each) were processed through Percoll 1118 density gradient centrifugation followed by anti-CD45 and then anti-GPA on MACS and subsequent selective erythrocytes lysis using ammonium chloride/acetazolamide buffer. Slides obtained from all the cases were Wright stained (section 2.2.5) to assess for purity. Fischer exact test was applied for data analysis.

2.2.20 Examination of the efficiency of a novel three-step enrichment protocol for first-trimester non-invasive prenatal diagnosis in *in-vitro* model system

Primitive fetal erythroblasts were isolated from products of conception obtained in the first trimester after termination of pregnancy (section 2.2.3). 10^4 fetal nucleated

erythroblasts obtained from female fetus were spiked in 2 ml of adult male volunteer blood and processed through the first step Percoll 1118 density gradient method (section 2.2.7). The upper and lower layers obtained were processed separately. The supernatant obtained from the two washings were centrifuged at 3000 rpm (2060 g) (Beckman GS-6R) for 10 minutes and any cell pellet obtained was added to the cell suspension of their respective layers. Figure 4.8 shows presence of fetal nucleated red blood cells in the supernatant after the initial density gradient centrifugation step. The cell pellets from the upper and lower layer were processed separately for second-step enrichment method using anti-CD45/anti-GPA on MACS. The positive fraction comprising of pure population of red blood cells after anti-GPA sorting from the upper and lower layer were re-suspended separately in 100 µl of 0.5% BSA/PBS and treated with ammonium chloride/1mM acetazolamide cocktail lysis buffer at the ratio of 1:2 on ice for 5 minutes. The recovery of FNRBCs from upper and lower layer was determined separately by analysing the samples in a haemocytometer. The experiments were repeated eight times so as to test the reproducibility of the results. Distribution of FNRBCs between the upper and lower was analysed using wilcoxon signed-rank test due to the non-normal distribution of the data.

Chapter 3: Presence of ε-globin primitive fetal nucleated erythroblasts beyond the first-trimester and at birth in trisomy 18 syndrome neonates: Implication for non-invasive prenatal diagnosis

3.1 Introduction

The greatest likelihood of enriching embryonic ε-globin primitive fetal nucleated erythroblasts from maternal blood for non-invasive prenatal diagnosis is in the first trimester. This is because ε-globin NRBCs are found to be increased at around 9-10 weeks in euploid fetal blood and 45-50% is still circulating up to 12 weeks gestation (Al-Mufti et al., 2000; Choolani et al., 2001). However, their numbers become negligible by 14 weeks of gestation (Choolani et al., 2001). Following these reports, the same groups have demonstrated the enrichment of these cells from first trimester maternal blood carrying not only euploid fetuses (Al-Mufti et al., 2001; Choolani et al., 2003) but also aneuploidy fetuses such as trisomy 13 (Choolani et al., 2003) and trisomy 18, respectively (Al-Mufti et al., 2003). Al-Mufti et al. (2000) observed the persistence of these cells in blood of chromosomally abnormal fetuses (T18, T21, T13, triploidy) up to 28 weeks gestation. Mavrou et al. (2003) were able to enrich these cells from women carrying trisomy 21 fetuses in the second trimester, after the invasive procedure and before the termination of pregnancy. These findings indicate that embryonic ε-globin positive primitive fetal erythroblasts could persist into the second trimester in chromsomally abnormal fetuses and the ε-globin gene is not switched off. It was hypothesised that such cells could persist not only in the second trimester of

chromsomally abnormal fetus, but also at term. Trisomy 18 was selected as a model to prove this hypothesis. The choice of T18 as an aueploid model to base our investigations was based upon the assumption that the haemoglobin switch is similar in all aneuploidies, and that the placental interface is similar in its leakiness for both T18 and T21. We acknowledge that T18 is rarer than T21 and choosing T18 will limit our ability to gauge what happens in a T21 pregnancy which is more clinically relevant, it was due to the availability of cases of T18 in our unit at that point in time and lack of any T21 cases, that we chose to undertake T18 to prove our hypothesis. With the availability of newer screening protocols (section 1.3), a proportion of pregnant women at risk for aneuploidy pregnancy may test screen positive in the second trimester and enrichment of such cells from this cohort of women could indicate not only the presence of a chromosomally abnormal fetus but also imply that the ε-globin primitive fetal nucleated erythroblasts are ideal cells to be targeted in the second trimester for non-invasive prenatal diagnosis.

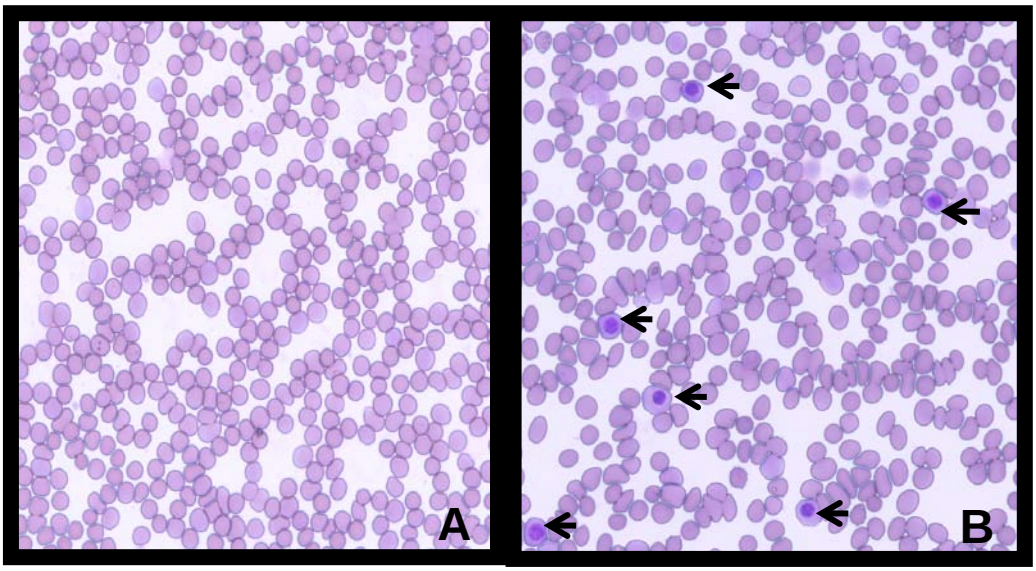
3.2 Enrichment of epsilon-globin positive primitive fetal nucleated erythroblasts from trisomy 18 syndrome fetus beyond first-trimester and from neonates at term (section 2.2.16)

Aim: To demonstrate whether the ε-globin gene expression persists beyond the first trimester in trisomy 18 syndrome fetus and in trisomy 18 syndrome neonates at birth.

Results: The proportion of FNRBCs identified in trisomy 18 syndrome fetus at 20 weeks gestation was 16-fold higher (0.55%; n = 1) as compared to karyotypically normal fetuses (0.034%; n = 4). Figure 3.1 and 3.2 shows numerous FNRBCs on peripheral smear and on MACS sorting after positive selection with GPA in trisomy 18 fetus (Arrows).

Figure 3.1 Peripheral smear from fetal blood of karyotypically normal fetuses (n = 4; GA = 21-23 weeks) and trisomy 18 fetus (n = 1; GA = 20 weeks)

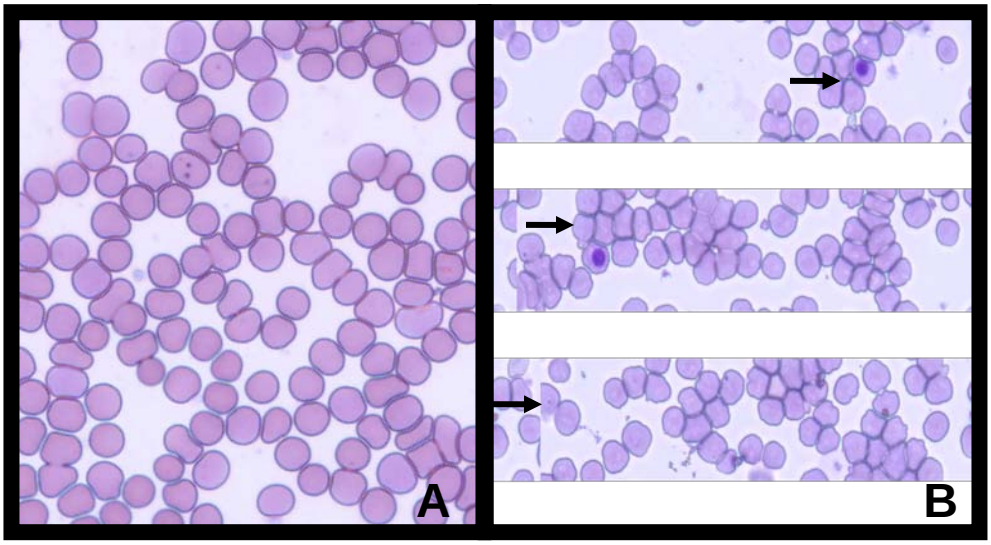
Karyotpically normal fetuses **Trisomy 18 fetus**



A: Absence of fetal nucleated red blood cells on Wright stain in normal fetuses (10 x magnifications)
B: Fetal nucleated red blood cells on Wright stain in trisomy 18 syndrome fetus (10 x magnifications)

Figure 3.2 Red blood cells sorted on MACS using positive selection with Glycophorin A after first step density gradient centrifugation by Ficoll 1077 on fetal blood of normal fetuses (n = 4; GA = 21-23 weeks) and trisomy 18 fetus (n = 1; GA = 20 weeks)

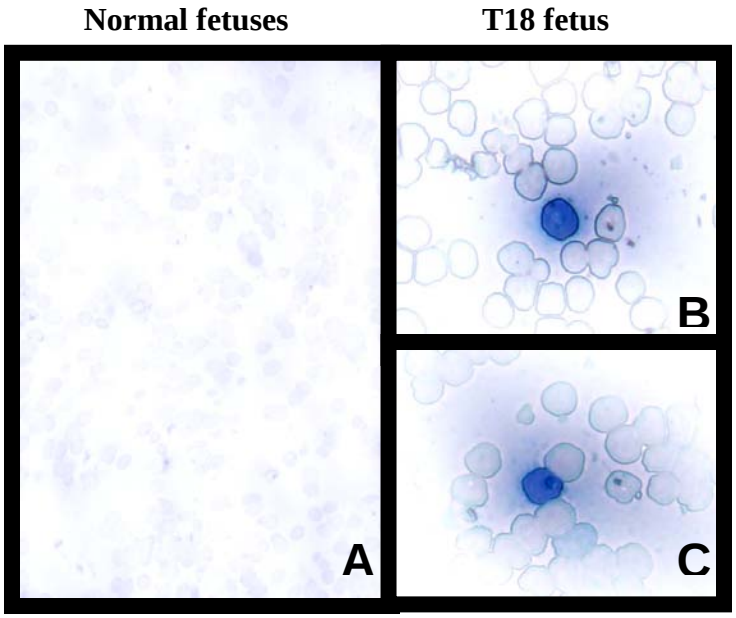
Karyotpically Normal fetuses **Trisomy 18 fetus**



A: Absence of fetal nucleated red blood cells on Wright stain in normal fetuses (20 x magnifications)
B: Fetal nucleated red blood cells on Wright stain in trisomy 18 syndrome fetus (20 x magnifications)

Embryonic ε-globin primitive fetal nucleated erythroblast cells were observed in trisomy 18 syndrome fetus (n = 1). No ε-globin positive cells were noted in the blood samples of karyotypically normal fetuses (n = 4) (Figure 3.3).

Figure 3.3 ε-globin staining (vector blue) of primitive fetal nucleated erythroblasts by alkaline phosphatase immunocytochemistry in a T18 fetus after first step density gradient centrifugation using Ficoll 1077 followed by positive selection with Glycophorin A on MACS (GA = 20 weeks)



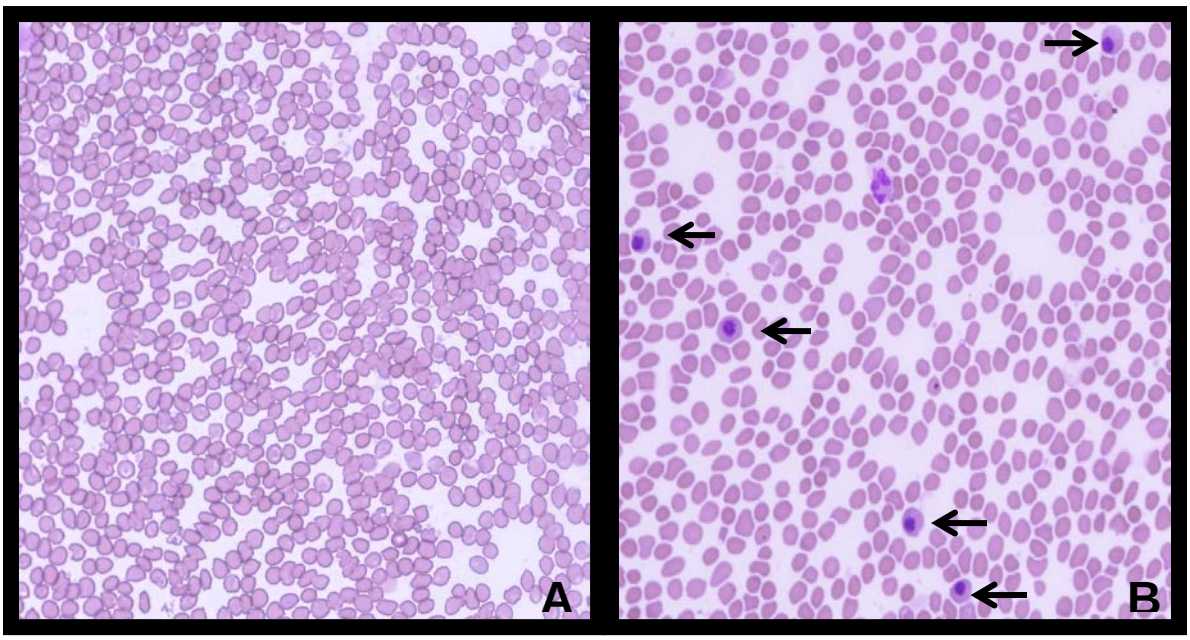
A: All anucleate red blood cells are epsilon-globin negative. No staining with vector blue (20 x magnifications)
B,C: Epsilon-globin (vector blue) staining of fetal nucleated erythroblasts (20 x magnifications)

The proportion of FNRBCs identified in cord blood of affected T18 newborns (n = 3) were found to be 14-fold higher and significantly increased (Mann Whitney U test; median % = 3; Range = 2 - 3.46) as compared with karyotypically normal neonates (n = 20) (median % = 0.22; Range = 0.073 – 0.50; p = 0.001). Quantification of FNRBCs was done over 3 x 10^4 cells. Figure 3.4, 3.5 and 3.6 shows numerous FNRBCs on peripheral smear, density gradient centrifugation and on MACS sorting after GPA in cord blood of trisomy 18 and karyotypically normal newborns (Arrows). Embryonic ε-globin positive primitive nucleated erythroblasts were observed in all the three trisomy 18 neonates (Median = 9; Range = 6-23). No such cells were noted in the cord blood of twenty karyotypically normal newborns at birth (Figure 3.7). FNRBCs isolated from first

trimester surgical termination of pregnancies served as internal positive controls whereas cells incubated with the secondary antibody only were used as internal negative controls. Mononuclear cells enriched from healthy male volunteer using Ficoll 1077 were used as external negative controls and no adult white blood cells were found to be ε-globin positive. Figure 3.8 demonstrates nucleated erythroblast with three signals for chromosome 18 on cFISH.

Figure 3.4 Peripheral smear from cord blood of karyotypically normal (n = 20) and trisomy 18 newborns (n = 3) at birth

Normal newborns **Trisomy 18 newborns**

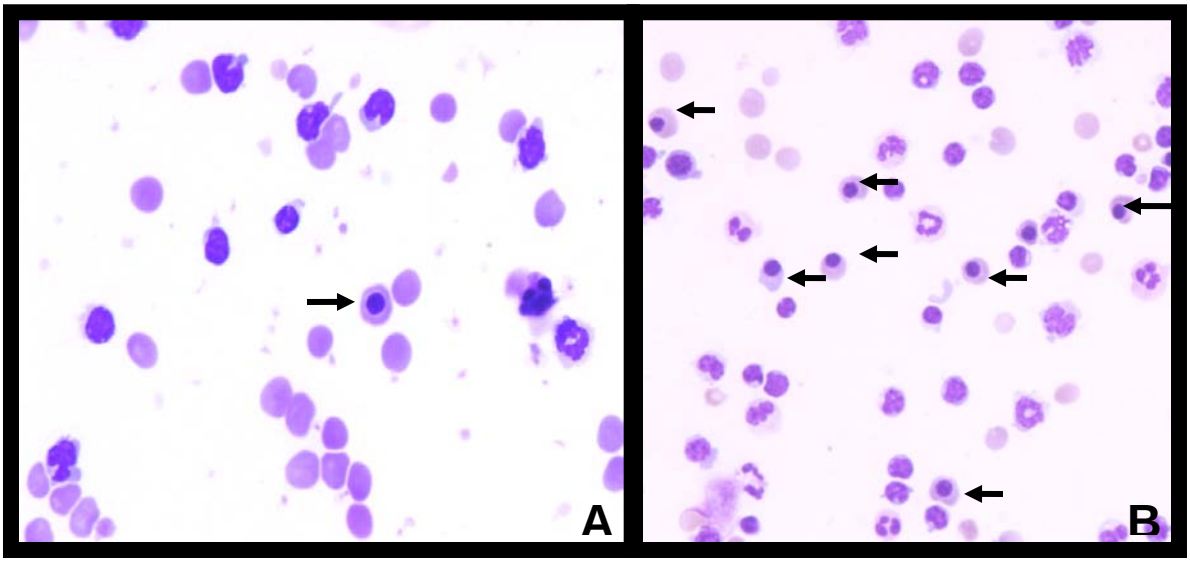


A: Absence of fetal nucleated red blood cells on Wright stain in normal newborns at birth (10 x magnifications)

B: Fetal nucleated red blood cells on Wright stain in trisomy 18 syndrome newborns at birth (10 x magnifications)

Figure 3.5 Distribution of fetal nucleated red blood cells after first step density gradient centrifugation using Ficoll 1077 on cord blood of karyotypically normal (n = 20) and trisomy 18 newborns (n = 3) at birth

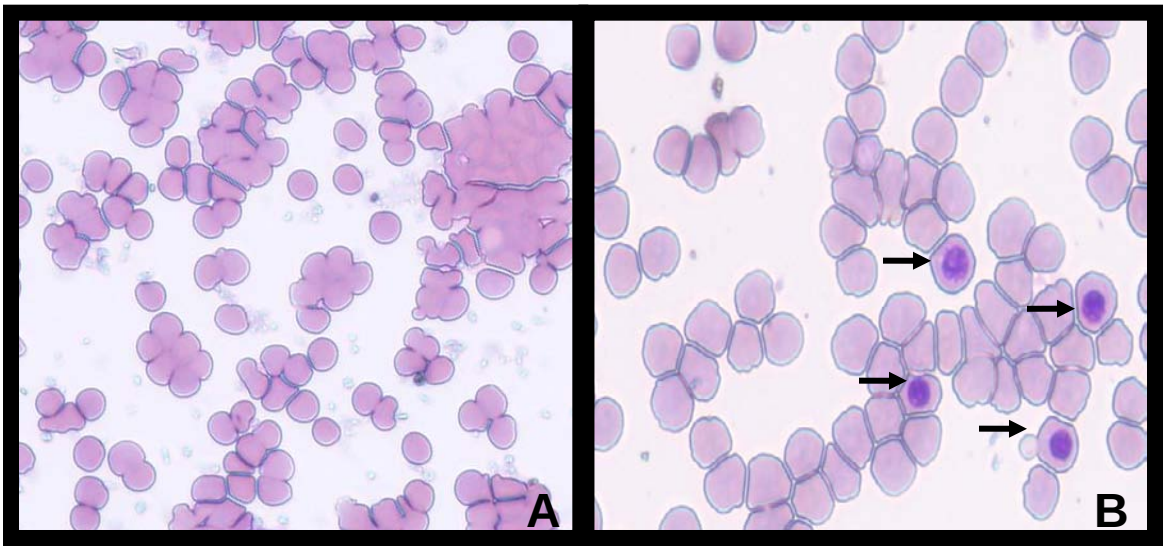
Normal Newborns **Trisomy 18 Newborns**



A: Fetal nucleated red blood cell on Wright stain in normal newborns at birth (20 x magnifications)
B: Fetal nucleated red blood cells on Wright stain in trisomy 18 syndrome newborns at birth (20 x magnifications)

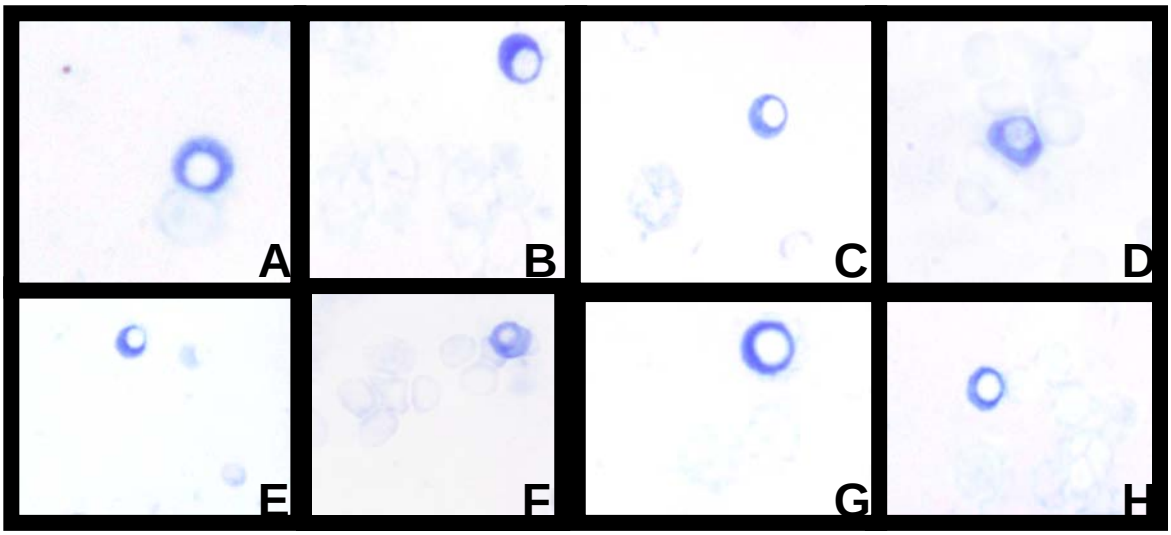
Figure 3.6 Red blood cells sorted by MACS using positive selection with Glycophorin A after first step density gradient centrifugation with Ficoll 1077 on cord blood of karyotypically normal (n = 20) and trisomy 18 newborns (n = 3) at birth

Normal newborns **Trisomy 18 newborns**



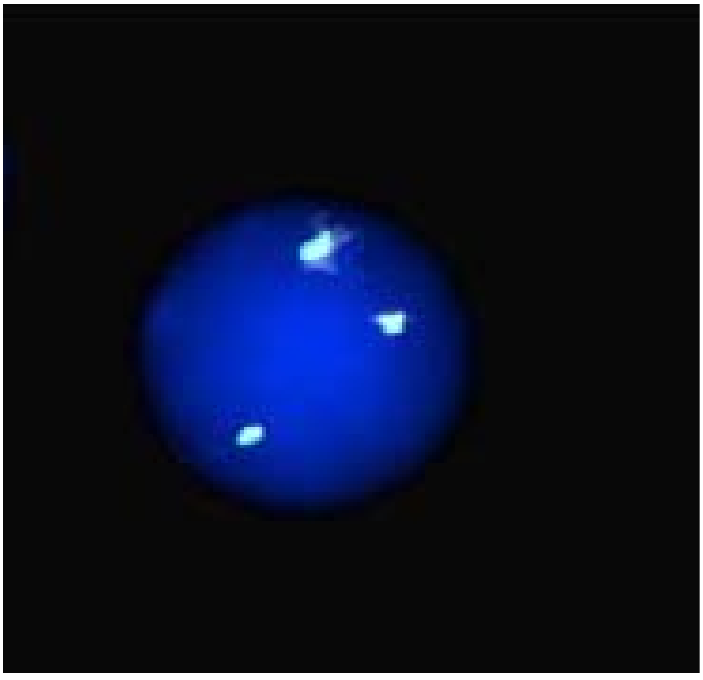
A: Absence of fetal nucleated red blood cells on Wright stain in normal newborns at birth (40 x magnifications)
B: Fetal nucleated red blood cells on Wright stain in trisomy 18 syndrome newborns at birth (40 x magnifications)

Figure 3.7 ε-globin staining (vector blue) of primitive fetal nucleated erythroblasts by alkaline phosphatase immunocytochemistry after positive selection with Glycophorin A following first step density gradient centrifugation with Ficoll 1077 on cord blood of trisomy 18 newborns at birth (n = 3)



A-H: Epsilon-globin positive primitive fetal nucleated erythroblasts in trisomy 18 newborns at birth (20 x magnifications) (Range = 6 - 23)

Figure 3.8 cFISH demonstrating three copies of chromosome 18 in fetal nucleated red blood cells



Blue colour depicts the DAPI staining of the nucleus (60 x magnifications)

Interpretation: Haematopoiesis in an embryo and fetus occurs at the mesoblastic, hepatic and myeloid period which corresponds to the yolk sac, liver and spleen and bone marrow stage, respectively. In a normal pregnancy, from the 16^{th} week onward, there is a rapid decrease in extramedullary and a rapid increase in medullary erythropoiesis. This declining contribution from the liver with an exponential decrease in fetal blood erythroblast count as gestation advances could be due to the consequence of the switch from hepatic to medullary erythropoiesis and maturation of the haematopoietic tissues (Nicolaides et al., 1989). The data in this chapter demonstrates an increase in proportion of nucleated erythroblasts in trisomy 18 fetus and in neonates at birth compared to normal euploid fetuses. This finding is consistent with that observed by Thilaganathan et al. (1995) who reported erythroblast count in aneuploidy fetuses to be significantly higher than the appropriate normal mean for gestation.

Similarly, the proportion of fetal nucleated erythroblasts was observed to be decreased with increase in gestation in chromosomally normal fetuses as compared to abnormal fetuses (Al-Mufti et al., 2000). What actually triggers the onset of increased erythropoiesis in aneuploidy fetuses is still unclear. However, it could be due to a developmental delay in maturation of fetal haematopoietic system in such aneuploidy fetuses (Thilaganathan et al., 1993; Thilaganathan et al., 1994) and therefore, likely to result in persistence of yolk sac and hepatic haematopoiesis. Further, it could be possible that when the bone marrow takes over the haematopoiesis, nucleated erythroblasts in such aneuploidy fetuses are deformed in their shape and subsequently are able to traverse the marrow sinusoids. Such cells have already been demonstrated to have increased mean cell volume (Fisk et al., 1989; Sipes et al., 1991). The data also shows the presence of embryonic ε-globin nucleated erythroblasts not only in the mid-trimester trisomy 18 fetus (n = 1) but also in neonates (n = 20) at birth, indicating that the ε-embryonic globin

gene is not completely turned off during early development but instead continues to be expressed up to term. Few groups have demonstrated that ε-globin positive nucleated erythroblasts persists in the fetal blood of aneuploidy fetuses up to 20 weeks (Christensen et al., 2003) and 28 weeks gestation (trisomy 21,18,13 and triploidy) (Al-Mufti et al., 2000) respectively. However, in the former (Christensen et al., 2003) the authors have not mentioned the type of aneupolidy present in the fetuses.

On the other hand, Huehns et al (1964a) using starch gel electrophoresis on haemolysates of day 4 and day 5 old trisomy 13 infants, observed embryonic haemoglobin Gower II, the proportion of which was 0.7% of the total haemoglobin, while Jansen and Murken (1976) observed the likely presence of embryonic haemoglobin Gower I and II in blood sample haemolysate of trisomy 13 infant at day 8 of birth using isoelectric focussing gel. However, persistence of ε-embryonic globin at single cell level in trisomy 18 neonates has not been reported. Such an observation cannot be explained by a gene dosage effect as proposed by Huehns et al. (1964b) because the genes for the hemoglobin chains are situated on chromosome 11 (Gusella et al., 1979) and 16 (Diesseroth et al., 1977). On the contrary, no ε-globin positive nucleated erythroblast cells or anucleated erythrocytes could be observed in the cord blood of 20 normal neonates at birth. This finding is similar to that observed by other groups (Al-Mufti et al., 2000; Christensen et al., 2003) who also could not demonstrate ε-globin positive nucleated erythroblast in cord blood of term karyotypically normal neonates.

3.3 Conclusion

The data presented in this chapter imply that ε-embryonic globin positive primitive fetal erythroblasts are not only detectable in trisomy 18 fetus in the mid second trimester, but

are also found to be present in affected newborns at birth. This information could be used for non-invasive prenatal diagnosis in group of women who are identified at particularly high-risk for chromosomal aneuploidy in the second trimester and beyond.

Chapter 4: Termination of pregnancy as an *in-vitro* model system to study first-trimester non-invasive prenatal diagnosis

4.1 Introduction

To-date, enrichment of fetal nucleated erythroblasts from maternal blood remains a challenge. This is because of their rarity in maternal circulation (there may be 1 fetal cell per millilitre of maternal blood; 10^{-7} nucleated cells) (Bianchi et al., 1997). As little can be done to alter the frequency of fetal erythroblasts in maternal blood, researchers have focussed on using various methods that could help them in enriching these cells from maternal blood (section 1.6.4.3). However, the enrichment protocols used by them for this rare cell isolation are limited by two issues:

(i) The protocols have been used without their efficiency being tested in an appropriate *in-vitro* model system.

(ii) The protocols have been designed on model mixture experiments comprising NRBCs obtained from cord blood (Ganshirt et al., 1992; 1998; Bhat et al., 1993; Troeger et al., 1999; Smits et al., 2000).

This chapter examines the hypothesis (2a) that surgical termination of pregnancy can be used to evaluate efficiency of a new fetal cell enrichment protocol in *in-vitro* model system.

The new fetal cell enrichment protocol comprised of Percoll 1118/anti-CD45/anti-GPA and has been adopted from Choolani et al. (2003). Before testing the protocol, it was considered whether a third-step enrichment process of selective lysis using ammonium chloride buffer could be incorporated. Earlier study by Choolani et al. (2003) was not in favour of incorporating it as the first-step enrichment process (section 1.6.4.3, Enzymes). If proven successful as a third-step method, it could help in reducing the number of background anucleated erythrocytes after the initial two-step protocol.

4.2 Effect of ammonium chloride lysis buffer on first-trimester primitive fetal nucleated erythroblasts and on adult anucleate erythrocytes (section 2.2.17)

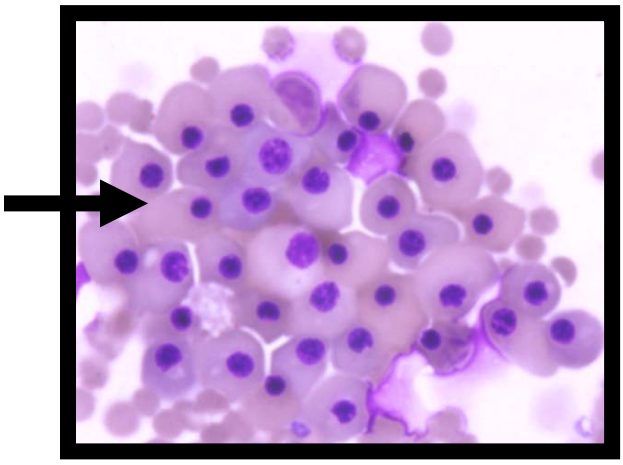
Adult erythrocytes are more susceptible to ammonium chloride lysis than fetal erythroblasts, as carbonic anhydrase activity is at least five-fold greater and acetazolamide permeability about ten-fold less in adult compared with FNRBCs. When lysis procedure was used as the first-step enrichment method, no FNRBCs were recovered in the pre-CVS samples, though there was 70% recovery in the model system (Voullaire et al., 2003) (section 1.6.4.3 Enzymes). Choolani et al. (2003) in their model mixture experiment were able to show that ammonium chloride/acetazolamide cocktail buffer could retrieve 100% of primitive FNRBCs. The authors did not favour its use as the first-step method as significant clumping between the primitive fetal erythroblasts and the remaining adult erythrocytes made it difficult to recognise the morphology of the target cells. However, it could be useful after the initial two-step enrichment when majority of adult anucleate red blood cells are already depleted at the first-step density gradient centrifugation method. If successful, inclusion of this technique could help in

reducing the number of background adult erythrocytes and could minimise the number of slides processed for analysis by Wright stain.

Aim: To identify the time period at which adult anucleate erythrocytes start to lyse and fetal primitive erythroblasts remain intact.

Results: The effect of the lysis buffer on FNRBCs and adult anucleated erythrocytes was observed across 5 to 40 minutes time interval. The experiment was repeated four times and average percentages of cells lysed were calculated. It was observed that 100% of fetal nucleated erythroblasts remained intact and no cells lysed on exposure to ammonium chloride up to 20 minutes time interval (95% CI, 0% - 3.5%). (Table 4.1) The cells started to lyse only after 20 minutes. In contrast, 93% of adult anucleated red blood cells were lysed at 5 minutes exposure to ammonium chloride (95% CI, 88.8% - 97.2%) and increased to approximately 100% at 40 minutes of time interval (95% CI, 97.9% - 100%). (Table 4.2)

Figure 4.1 Enrichment of primitive fetal nucleated erythroblasts from trophoblast tissue for model mixture experiments



Arrow depicts population of primitive fetal nucleated red blood cells on Wright stain (40 x magnifications)

Table 4.1 Effect of ammonium chloride lysis buffer on fetal nucleated erythroblasts across 5 to 40 minutes time interval (n = 4)

Time interval (minutes)	PFNRBCs lysed (%)	95% Confidence Interval (%)
5	0	0 – 0.01
10	0	0 – 0.05
15	0	0 – 3.5
20	0	0 – 3.5
25	25.0	0 – 53.5
30	37.5	9.0 – 66.0
35	75	46.5 – 100
40	87.5	59.0 – 100

Table 4.2 Effect of ammonium chloride lysis buffer on adult anucelated red blood cells across 5 to 40 minutes time interval (n = 4)

Time interval (minutes)	Percentage of adult erythrocytes lysed	95% Confidence Interval
5	93.0	88.8 - 97.2
10	93.2	88.9 - 97.5
15	94.3	85.7 - 90.1
20	95.1	91.0 - 99.4
25	97.2	93.0 - 100
30	97.2	93.0 - 100
35	97.3	95.6 - 100
40	99.8	97.9 - 100

Interpretation: The above data demonstrate 93% of selective adult erythrocyte lysis on exposure to ammonium chloride at 5 minutes time interval. 100% of adult anucleated erythrocytes are lysed only at 40 minutes. Although, fetal primitive erythroblasts remain intact up to 20 minutes time interval, adult RBCs lysis increase only marginally by 2%. Therefore, 5 mins lysis interval would be optimal for adult cell lysis while preserving FNRBC integrity which was then chosen as the cut-off point for subsequent model mixture experiments. The ratio of one volume of cell suspension to 2 volumes of lysis buffer was chosen so as to allow minimum volume exposure of the lysis buffer to the

target cells. This is because the sensitivity of FNRBCs to ammonium chloride lysis in this *in-vitro* model system may differ from the *in-vivo* situations, which are actually enriched from the maternal blood.

4.3 Effect of ammonium chloride alone and ammonium chloride/1mM acetazolamide on a model mixture of primitive fetal nucleated erythroblasts and adult anucleated red blood cells (section 2.2.18)

Aim: To study the effect of ammonium chloride lysis buffer alone and its combination with 1mM acetazolamide on the mixture of first trimester fetal erythroblasts and adult anucleated erythrocytes at a ratio of 1:2 at 5 minutes time interval. Acetazolamide was added to determine whether its enzyme (carbonic anhydrase) inhibition activity protects the fetal erythroblasts from lysis by ammonium chloride.

Result: In the first experiment, FNRBCs were spiked with adult erythrocytes and treated with ammonium chloride alone. In the second experiment, the mixture was treated with combination of ammonium chloride and acetazolamide. Significant number of FNRBCs (35%; $p = 0.01$) and adult erythrocytes (94%; $p = 0.001$) were lysed when exposed to ammonium chloride alone at 5 minutes of time interval (Table 4.3). When acetazolamide was added, significant number of adult erythrocytes were lysed (96%; $p = 0.001$), while there was no significant difference in the percentage of fetal cells remaining intact (93%) from the original number of cells introduced before the exposure ($p = 0.60$) (Table 4.4).

Table 4.3 Effect of ammonium chloride on cell lysis in a model mixture of PFNRBCs and adult erythrocytes (n = 4)

Cell type	Mean	Standard deviation	Cells lysed (%) (95% CI)	p - value*
PFNRBCs (Test value = 10^4)	6500	1290.9	35 (22 - 48)	0.01
Adult erythrocytes (Test value = 5×10^6)	275,000	134,783.7	94 (91.8 – 97.2)	0.001

* paired t-test

Table 4.4 Effect of ammonium chloride/1mM acetazolamide on a model mixture of PFNRBCs and adult erythrocytes (n = 4)

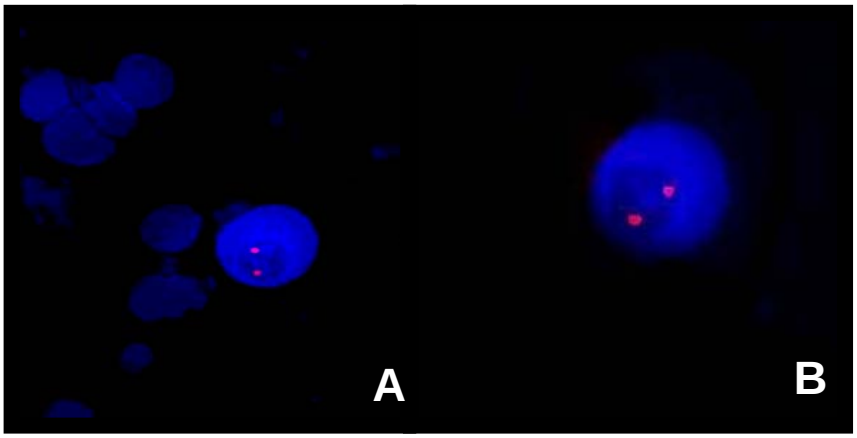
Cell type	Mean	Standard deviation	Cells lysed (%) (95% CI)	p - value*
PFNRBCs (Test value = 10^4)	9250	2629.9	7 (0 – 18.8)	0.60
Adult erythrocytes (Test value = 5×10^6)	182,500	66,017.6	96 (95.0 – 97.6)	0.001

* paired t-test

Choolani et al. (2003) had reported significant clumping between the primitive fetal nucleated erythroblasts and unlysed erythrocytes, which made it difficult to recognise the morphology of the target cells. A combined ε-globin fluorescence immunocytochemistry and cFISH was performed after cytocentrifuging the cell mixture on the glass slides. No significant clumping between unlysed erythrocytes and the target cells was observed.

The morphology of fetal nucleated erythroblasts were visualised clearly on fluorescence immunocytochemistry and cFISH (Figure 4.2).

Figure 4.2 Immunofluorescence staining and cFISH on primitive fetal nucleated erythroblasts after selective erythrocyte lysis



A,B: Epsilon-globin positive XX primitive fetal nucleated erythroblasts. The red signals are X-chromosome probes within the nucleus. Blue colour staining of the intracytoplasmic epsilon-globin by accumulation of AMCA, depicting the morphology of the entire cell (40 x magnifications)

Interpretation: In the model mixture experiment, 35% of fetal erythroblasts and 94.0% of adult anucleated erythrocytes were lysed with ammonium chloride alone at 5 minutes. This result is different than when fetal nucleated erythroblasts alone were treated with ammonium chloride, where at 5 minutes, 100% of cells remained intact (section 4.2). Unnexplained changes in the FNRBCs membrane morphology altered by adult red blood cells could have lead to its susceptibility to ammonium chloride lysis buffer.

By exposing cell mixtures to ammonium chloride/1mM acetazolamide for 5 minutes, 93% of primitive fetal nucleated erythroblasts were observed to be intact in the mixture. The addition of acetazolamide was found to have protective effect on primitive fetal nucleated erythroblasts. Thus, this buffer combination was found to be suitable for the selective lysis of adult erythrocytes. Hence, selective erythrocyte lysis with ammonium

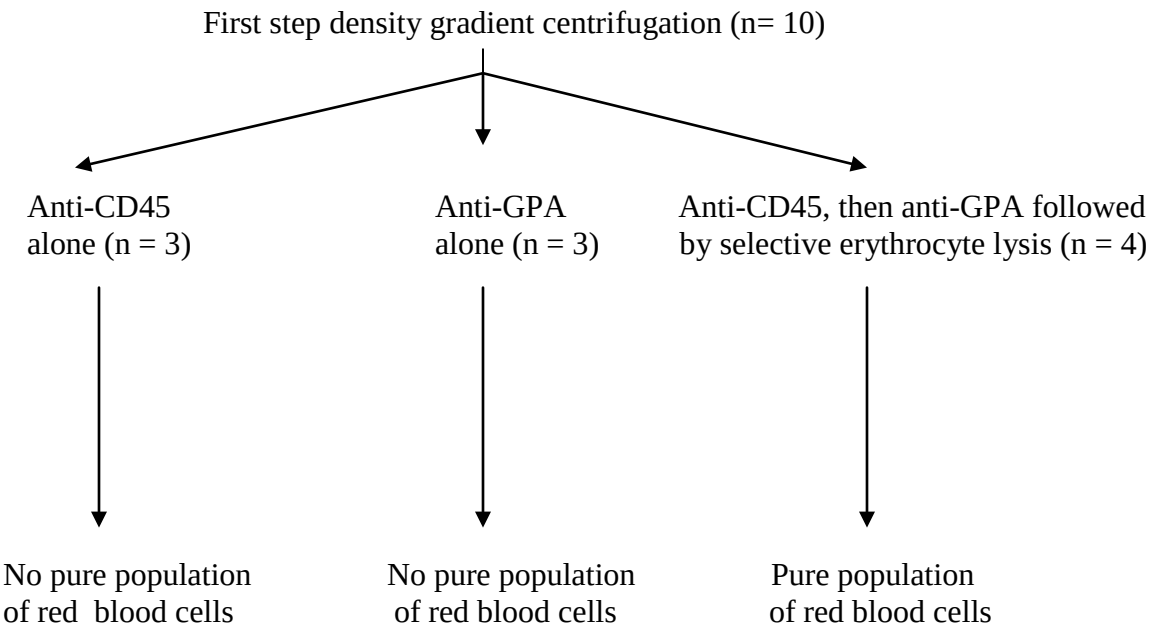
chloride/acetazolamide can be incorporated as a third-step for fetal erythroblasts enrichment.

4.4 Optimisation of second-step enrichment method on maternal blood samples (section 2.2.19)

This section investigates whether the use of anti-GPA or anti-CD45 antibody as the second-step enrichment method after the first step density gradient centrifugation with Percoll 1118 produces pure population of red blood cells. The choice for either antibody was made because CD45 is present on the cell surface of all mature human leukocytes and is not expressed on immature and mature erythrocytes (Zheng et al., 1993). Anti-GPA is an appropriate antibody to separate both anculeated red blood cells and NRBCs from nucleated white cell population after most of the maternal erythrocytes and white blood cells have been depleted by the first-step density gradient centrifugation method. At the end of the experiment, a pure population of red blood cells is expected. If obtained, this could minimise extra steps of washing and the possible loss of these rare fetal cells.

Aim: To demonstrate if a pure population of red blood cells can be obtained after second-step enrichment with anti-GPA or anti-CD45 antibody on MACS as compared to using anti-CD45 followed by anti-GPA after first step density gradient centrifugation with Percoll 1118.

Flow chart of three different groups subjected to different regime of selection/purification on MACS after first step density gradient centrifugation with Percoll 1118

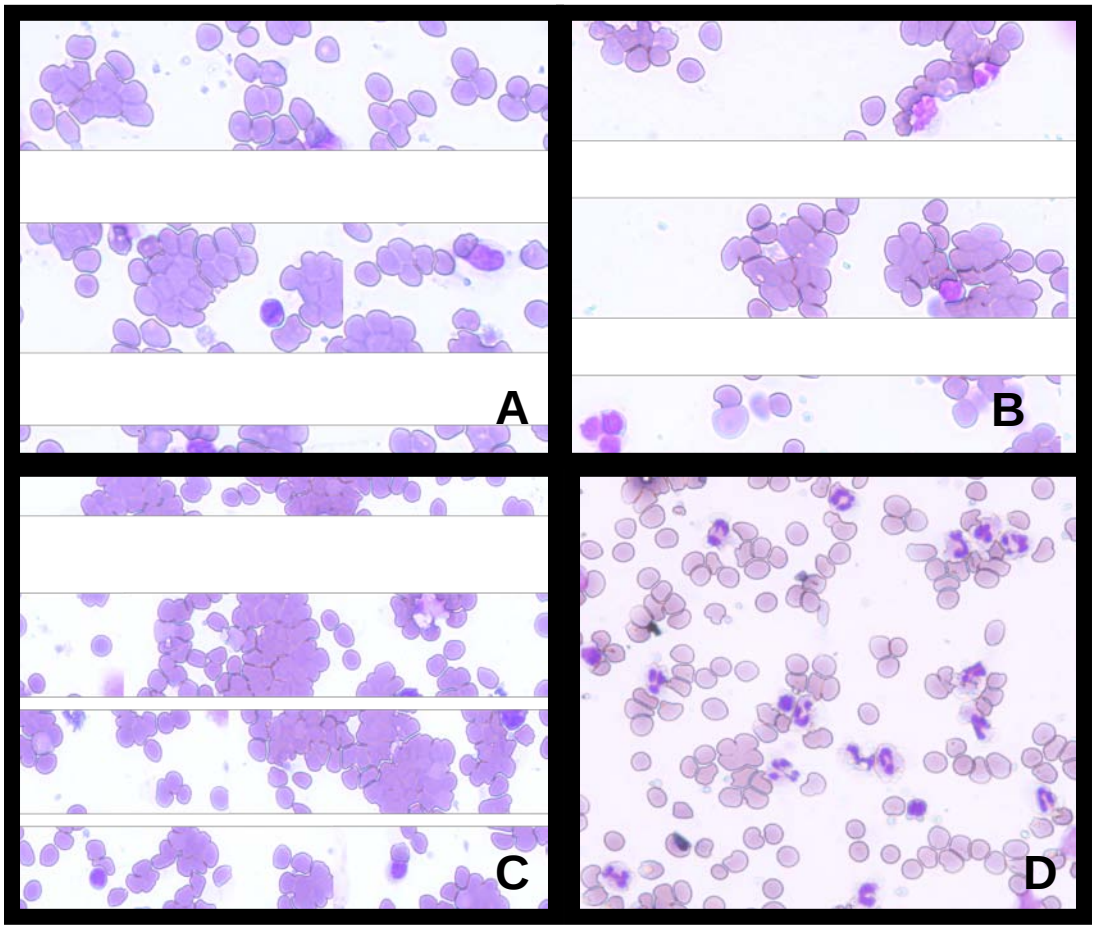


Results: Contamination from maternal white cells was noted on all the slides that were made using either anti-GPA or anti-CD45 alone during the second step (Figure 4.3). Using anti-CD45 to deplete maternal white cells followed by positive selection with anti-GPA as a second-step enrichment method gave pure population of red blood cells as compared to none on the samples where single antibody was applied (Fischer exact test; p-value = 0.02) (Figure 4.4).

Interpretation: Anti-CD45 or anti-GPA antibody alone on MACS after the first-step Percoll 1118 density gradient on maternal blood samples did not produce pure population of red blood cells.

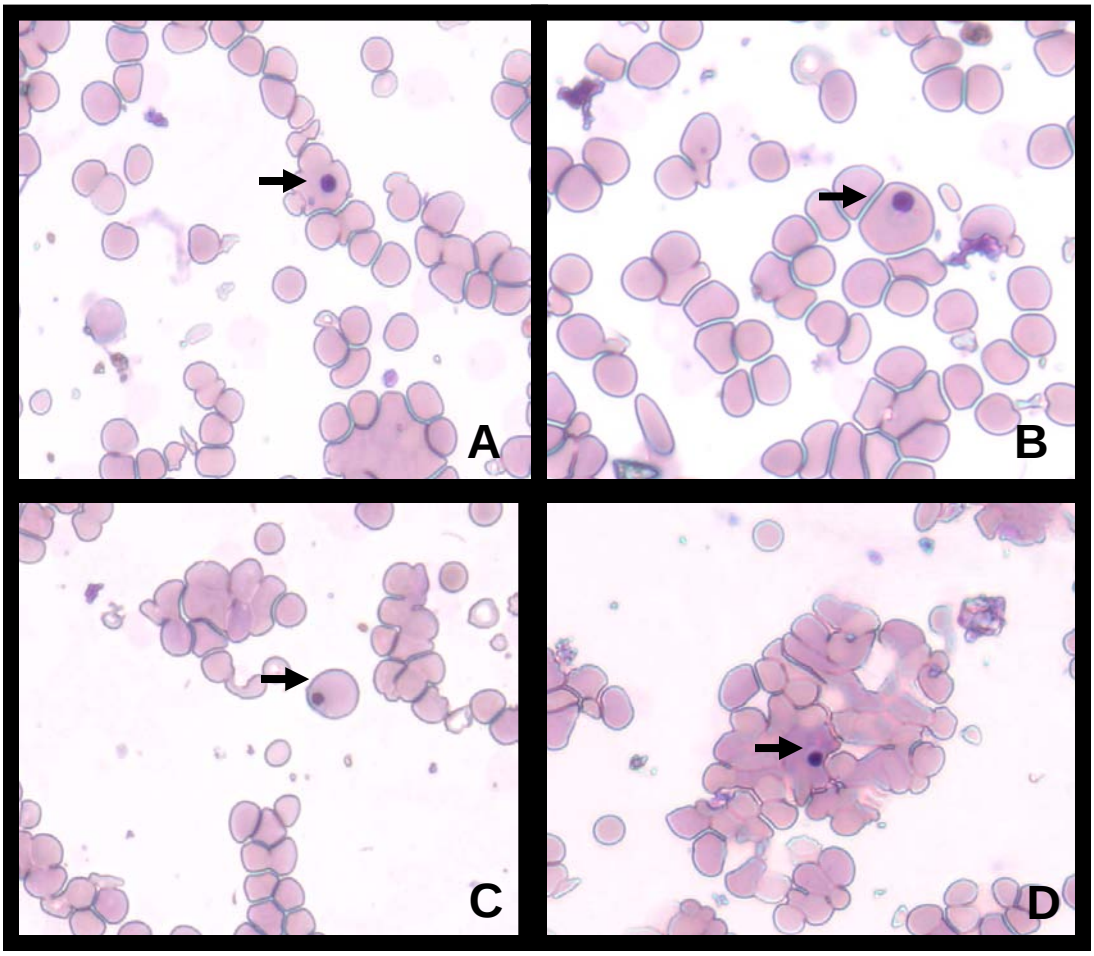
On a spiked model mixture system, Choolani et al. (2003) obtained similar results while using anti-CD45 antibody alone. However, they observed 100% purity with both anti-GPA and combined antiCD45/anti-GPA. The result from this experiment does not support the use of anti-CD45 or anti-GPA alone as a second-step enrichment method. We therefore decided to use Percoll 1118 followed by anti-CD45/anti-GPA and selective erythrocyte lysis as a three-step enrichment protocol to test its efficiency in *in-vitro* model for first-trimester non-invasive prenatal diagnosis.

Figure 4.3 Contamination with maternal polymorphonuclear leukocytes in enriched fraction using anti-GPA or anti-CD45 alone on MACS after first step Percoll 1118 density gradient centrifugation



A-D: Wright staining of enriched fraction showing contamination of maternal red blood cells with maternal polymorphonuclear leukocytes from four different patient samples after first step Percoll 1118 density gradient centrifugation and followed by antiGPA or antiCD45 on MACS (10 x magnifications)

Figure 4.4 Pure population of red blood cells in enriched fraction after first step density gradient centrifugation using Percoll 1118 followed by anti-CD45 then anti-GPA on MACS and selective erythrocyte lysis



A-D: Wright staining of pure population of red blood cells after three step enrichment protocol. Arrows indicate enriched NRBCs from maternal circulation from four different patient samples (40 x magnifications). Figure B and C is likely to be primitive fetal nucleated eryhtoblasts as they are large and have a high cytoplasmic:nuclear ratio. In contrast, figure A and D are likely to be definitive erythroblasts as they are smaller with a lower cytoplasmic:nuclear ratio

4.5 Development of combined immunocytochemical, immunoflourescence staining of ε-globin and cFISH for first-trimester non-invasive prenatal diagnosis

Aim: Embryonic ε-globin has been identified as a fetal cell specific marker (section 1.6.4.1). Simultaneous immunofluorescence cytochemistry in which the specificity of cell of fetal origin is confirmed using ε-globin and cFISH technique for gender analysis was recently reported by Choolani et al. (2001). In this section, we examine whether immunohistochemistry using vector blue substrate can be used to screen for cytospun slides which contain embryonic epsilon-globin before subjecting the slides directly for AMCA- ε-globin/cFISH after the two step enrichment protocol which is more expensive and labour intensive. Such a technique, if proven could help in reducing the number of slides being processed.

Results: Of the ten maternal peripheral blood samples processed (section 4.4), only four cases demonstrated 4, 24, 27 and 64 NRBCs across 4, 16, 2 and 21 slides, respectively. These 43 slides were processed for immunoenzymatic staining with anti-ε antibody for fetal primitive erythroblasts, using vector blue as a substrate (section 2.2.10). FNRBCs isolated from surgical termination of pregnancies served as internal positive controls whereas FNRBCs stained with the secondary antibody only were used as internal negative controls (section 2.2.3). Mononuclear cells enriched from adult peripheral blood were used as external negative controls (section 2.2.4). The frequency of ε-globin positive NRBCs in our enriched sample before cytospin was assumed to be in the region of 1-2 per 100,000 mononuclear cells given that one-third of the slides were positive for ε-globin cells and that each cytospin included 50,000 cells. There were 4, 5, 27 and 18

embryonic ε-globin positive primitive fetal nucleated erythroblasts identified (total = 54) across 4, 1, 2, and 7 slides (total = 14) respectively, under light microscopy. The area where these cells were located on the glass slide was noted using the scale on the X- and the Y-axis on the microscope. Only 14 out of 43 slides were then processed for simultaneous fluorescence immunocytochemistry using streptavidin AMCA identification of ε-globin and cFISH for determination of gender (section 2.2.14). All enriched fetal primitive nucleated erythroblasts could be clearly identified by their morphology and ε-globin status on immunofluorescence AMCA staining.

The fetal origin could be simultaneously confirmed by XY cFISH analysis and all cells clearly depicted the gender signals (95% CI, 95%-100%). Figure 4.5 shows nucleated red blood cells confirmed to be of male fetus in origin on combined immunocytochemical, immunofluorescence staining and cFISH (A-D). Figure 4.6 demonstrates primitive FNRBCs from a female fetus after combined immunocytochemical, immunofluorescence staining and cFISH. These were concordant with gender identified on trophoblast tissues (two female and two male fetuses) (Figure 4.7 a,b). Primitive erythroblasts stained in the absence of primary antibody and adult lymphocytes stained negative for ε-globin.

Interpretation: This data demonstrate that firstly the combined immunoenzymatic staining followed by subsequent fluorescence immunocytochemistry and cFISH technique is effective in proving NRBCs to be of fetal origin. The cell morphology was well preserved, which allowed visualisation of the fetal cell identifier and genetic signals at the same time. Secondly, the low numbers of ε-globin positive NRBCs enriched meant that not all the slides contained these cells. The method helped in reducing the number of slides being processed for AMCA and cFISH from 43 to 14. Such a strategy to screen for

ε-globin positive NRBCs using colorimetric immunostaining technique after the two step enrichment protocol would be cheaper and faster and would be considerable cost savings in performing AMCA- ε-globin/cFISH in only slides which contain such cells.

Figure 4.5 Combined immunocytochemical, immunofluorescence staining and cFISH on primitive fetal nucleated erythroblasts enriched from maternal blood: XY fetus

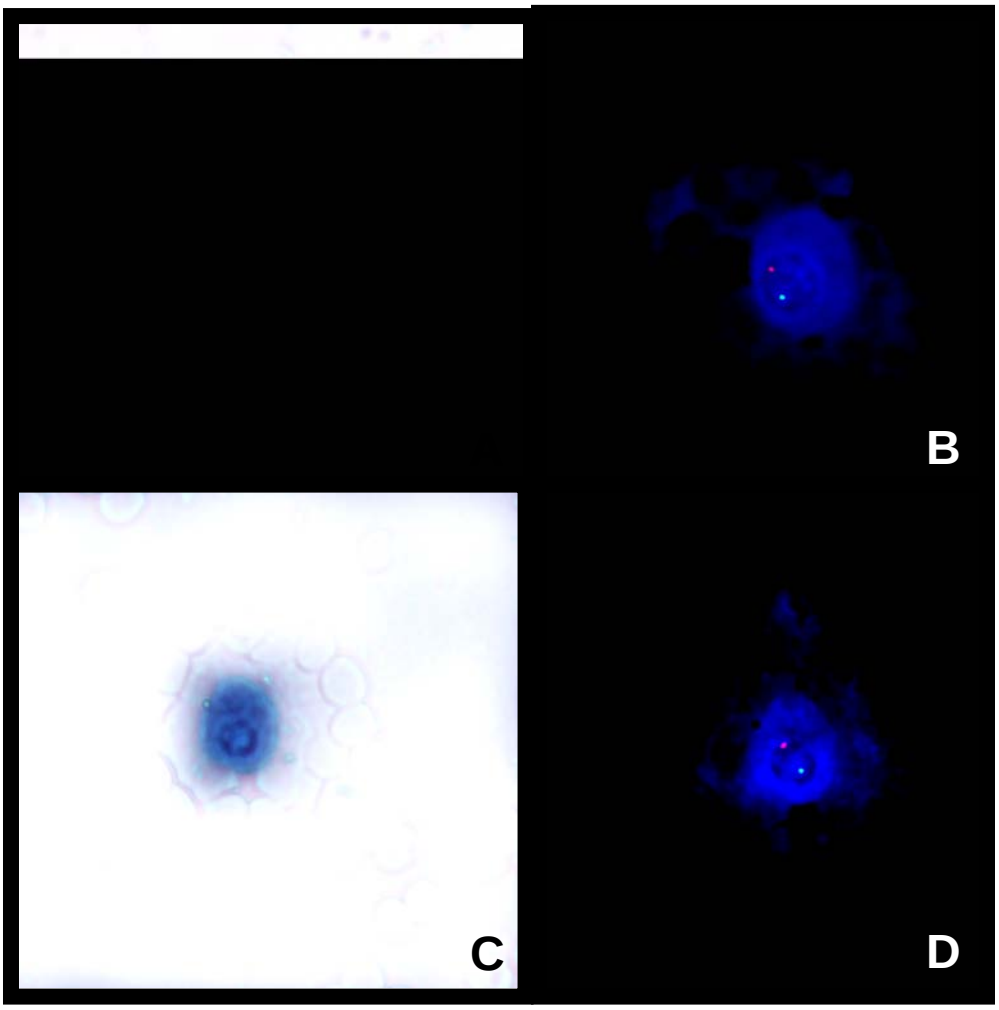


Figure A,C shows epsilon-globin positive primitive fetal nucleated erythoblasts on immunocytochemical staining. The figures show intact cell membrane and intact nucleus with vector blue staining of the cytoplasm containing epsilon-globin. Figure B,D shows immunofluorescence staining with cFISH (red signal X; green signal: Y; Male fetus) within the nucleus and accumulation of AMCA around the nucleus and staining of intracytoplasmic epsilon-globin positive primitive fetal nucleated erythroblasts, depicting the morphology of the entire cell (40 x magnifications)

Figure 4.6 Combined immunocytochemical, immunofluorescence staining and cFISH on primitive fetal nucleated erythroblasts enriched from maternal blood: XX fetus

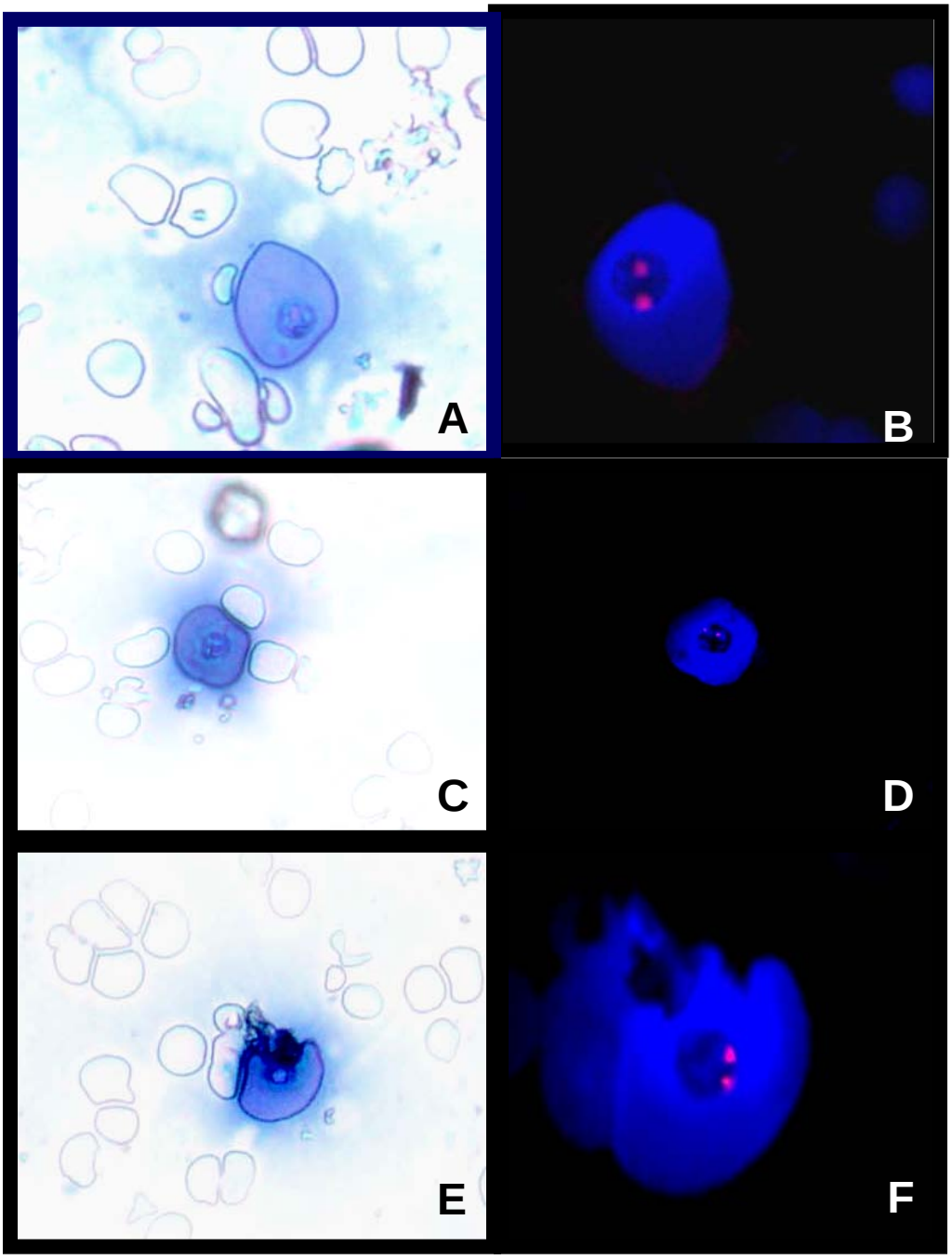
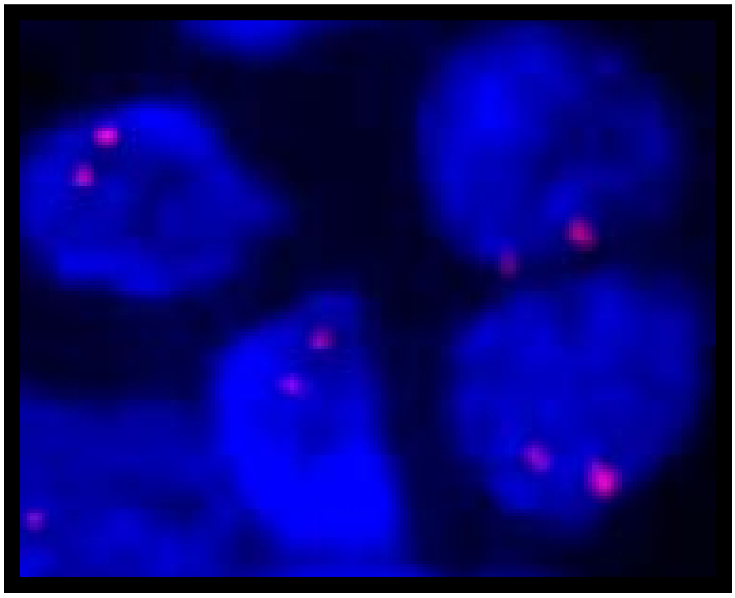


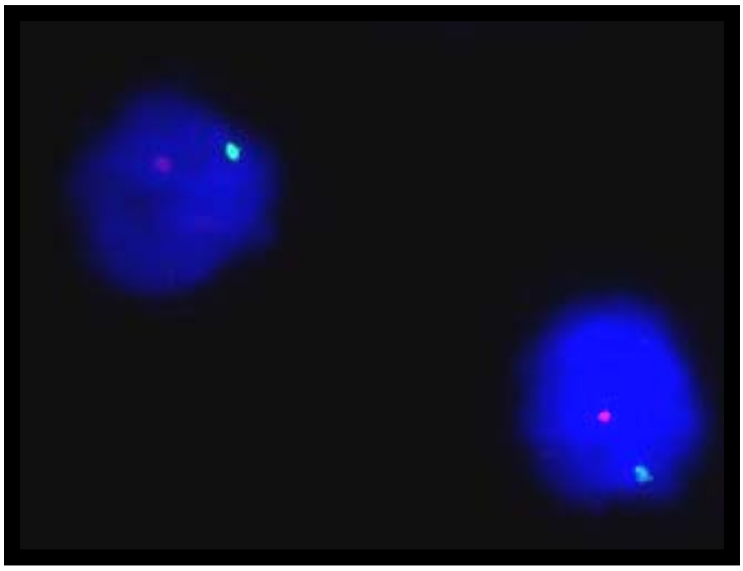
Figure A,C,E shows epsilon-globin positive primitive fetal nucleated erythoblasts on immunocytochemical staining. The figures show intact cell membrane and intact nucleus with vector blue staining of the cytoplasm containing epsilon-globin. Figure B,D,F shows immunofluorescence staining with cFISH (red signal XX chromosome; Female fetus) within the nucleus and accumulation of AMCA around the nucleus and staining of intracytoplasmic epsilon-globin positive primitive fetal nucleated erythroblasts depicting the morphology of the entire cell (40 x magnifications)

Figure 4.7a cFISH on fetal trophoblasts cells enriched from products of conception: Immunofluorescence staining of nucleus with DAPI (Blue colour). Red signal shows XX chromosome: Female fetus



(40 x magnifications)

Figure 4.7b cFISH on fetal trophoblasts cells enriched from products of conception: Immunofluorescence staining of nucleus with DAPI (Blue colour). Red signal shows X chromosome and green signal shows Y chromosome: Male fetus



(40 x magnifications)

4.6 Examination of the efficiency of a novel three-step enrichment protocol for first-trimester non-invasive prenatal diagnosis in *in-vitro* model system (2.2.20)

This section examines the efficiency of a three-step enrichment protocol comprising of Percoll 1118, anti-CD45/anti-GPA and ammonium chloride/acetazolamide in an *in-vitro* model system using termination of pregnancy derived FNRBCs. Following steps were integrated:

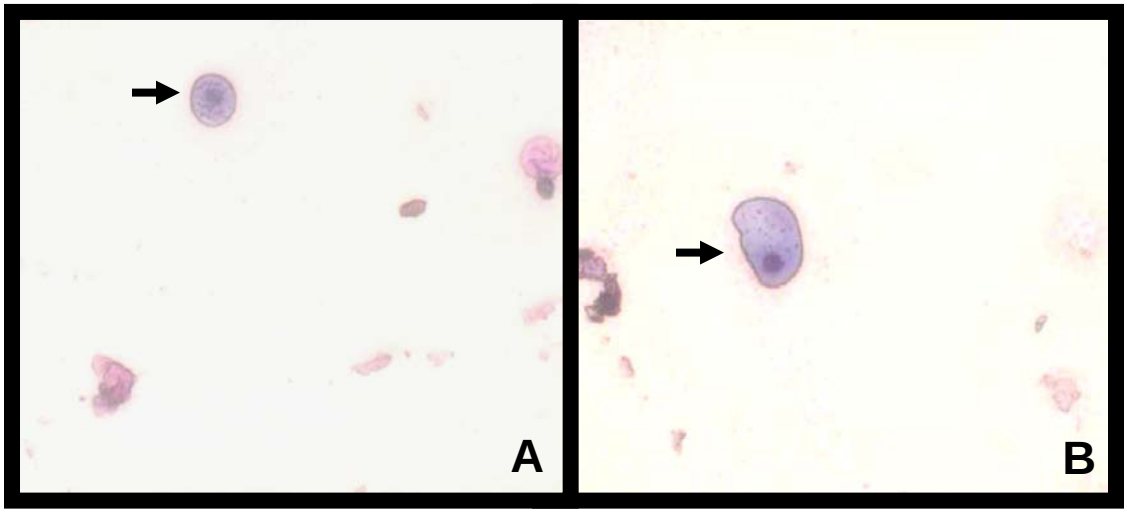
1. Red cell bulk was depleted using Percoll 1118.
2. Both the upper and the lower layer obtained after the first enrichment step were processed separately for subsequent MACS and erythrocytes lysis.
3. The supernatant obtained from the washings of both the layers were centrifuged at 3000 rpm (2060 g) for 10 minutes. Any cell pellet obtained was added to the cell suspension of their respective layers (Figure 4.8).
4. White cells were depleted using antibodies directed against lymphocytes, monocytes and granulocytes using anti-CD45, and red blood cells were positively-selected using anti-GPA
5. Ammonium chloride/acetazolamide was used for selective erythrocyte lysis as the third-step enrichment process.

Aim: To evaluate the efficiency of a new fetal cell enrichment protocol in *in-vitro* model system.

Results: The mean recovery of primitive fetal erythroblasts in *in-vitro* model system after the three-step enrichment method was 37% (95% CI, 28.5% - 45.6%; n = 8). There was

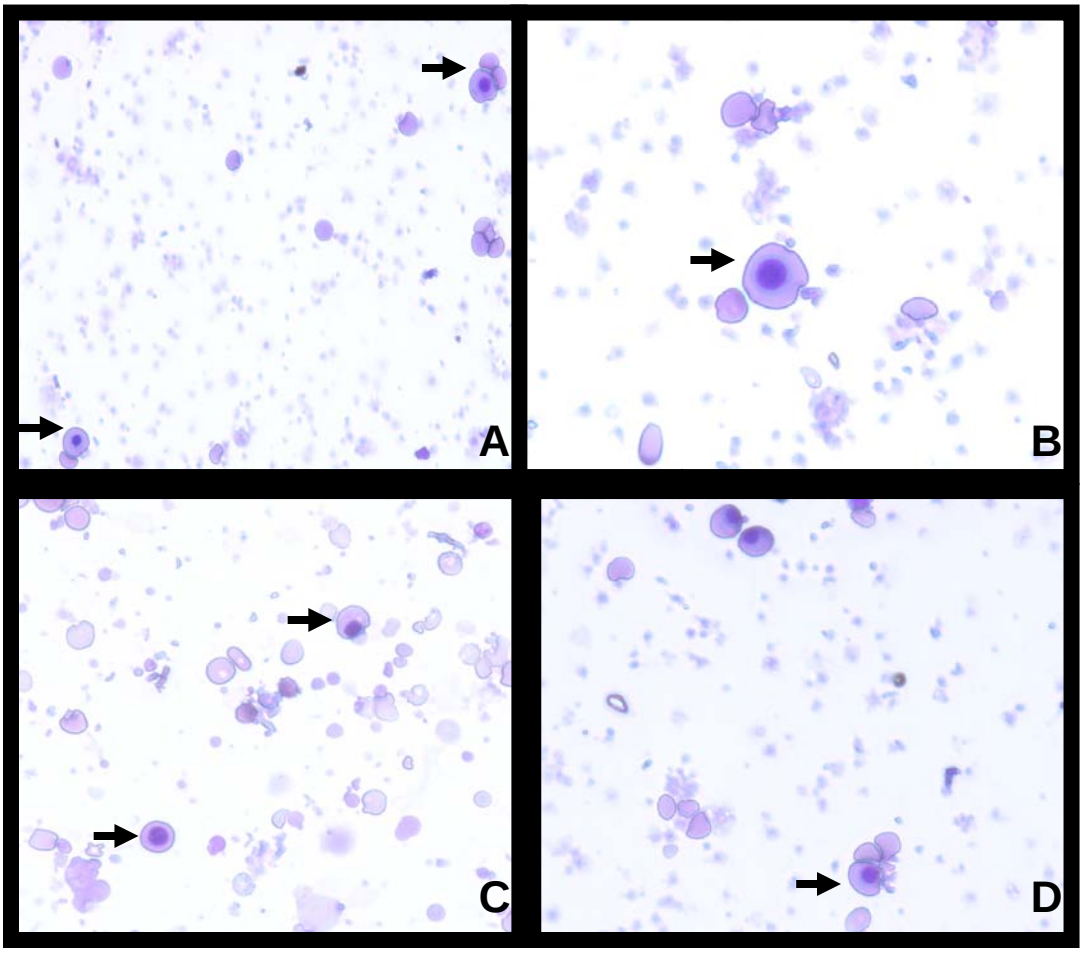
no statistically significant difference in the distribution of of FNRBCs between the upper and lower layer after the three step enrichment protocol (wilcoxon signed-rank test: p value = 0.063; two-sided test). Figure 4.9 demonstrate enriched FNRBCs in *in-vitro* model system. Figure 4.10 depicts a female fetus correctly identified on simultaneous ε-globin AMCA staining and cFISH which matched to that identified on trophoblast tissue (Figure 4.11). Figures 4.12, 4.13, and 4.14 shows the loss of primitive fetal erythroblasts in the Percoll 1118 cushion, within the depleted fraction of the LD column and the negative fraction of the MS column, respectively.

Figure 4.8 Loss of fetal nucleated red blood cells in supernatant after first step Percoll 1118 density gradient centrifugation



A, B: Wright staining of nucleated red blood cells. Arrows indicate fetal nucleated red blood cells in supernatant after the first-step density gradient centrifugation (20 x magnifications)

Figure 4.9 Enrichment of fetal nucleated red blood cells after three-step method in *in-vitro* model system



A-D: Arrow indicates enrichment of fetal nucleated erythroblasts after three-step enrichment process in *in-vitro* model (20 x magnifications)

Figure 4.10 Immunofluorescence staining and cFISH on primitive fetal nucleated erythroblasts after three-step enrichment method in *in-vitro* model system

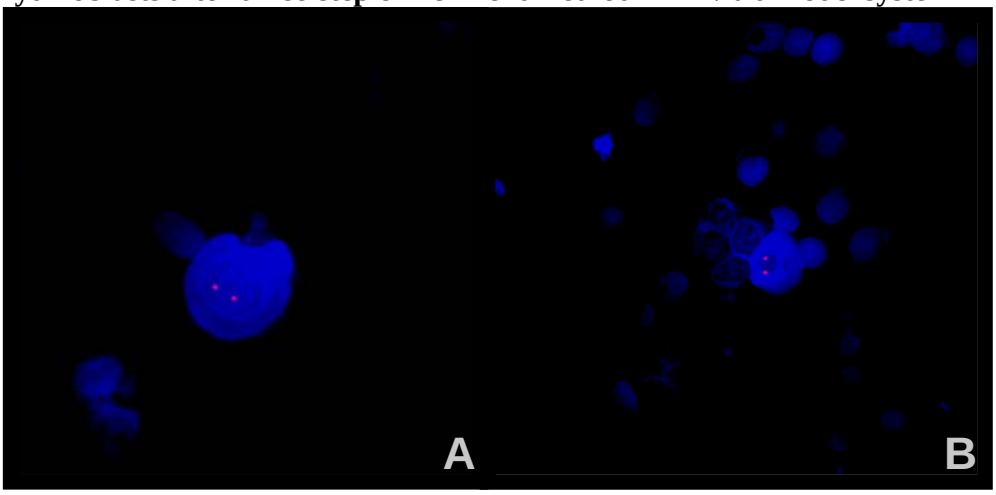
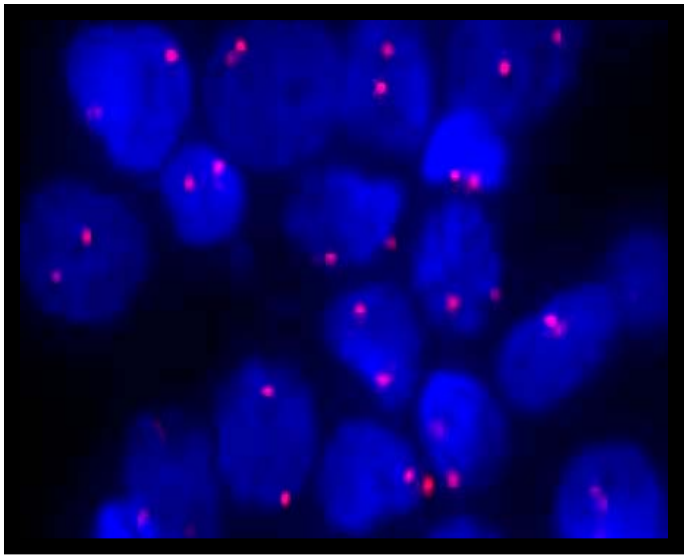


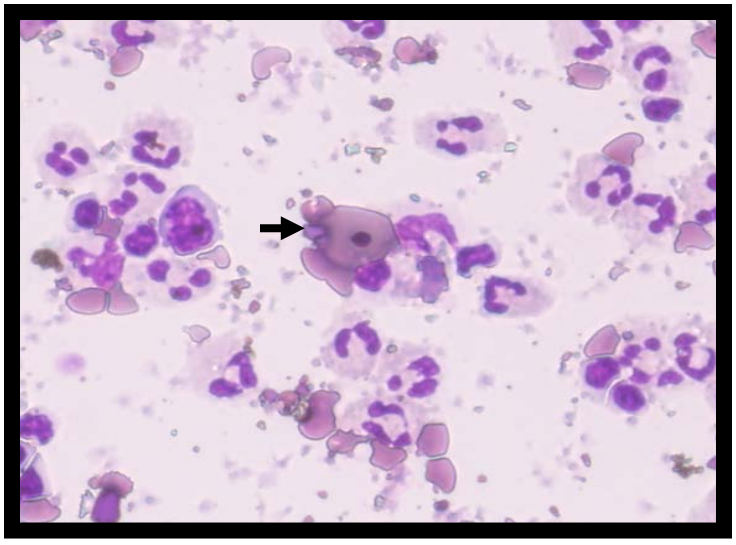
Figure A,B shows immunofluorescence staining with cFISH (red signal XX chromsome; Female fetus) within the nucleus and accumulation of AMCA around the nucleus and staining intracytoplasmic ε-globin positive primitive fetal nucleated erythroblasts. (20 x magnifications)

Figure 4.11 cFISH on fetal trophoblasts cells enriched from products of conception: Immunofluorescence staining of nucleus with DAPI (Blue colour). Red signal shows XX chromosome: Female fetus



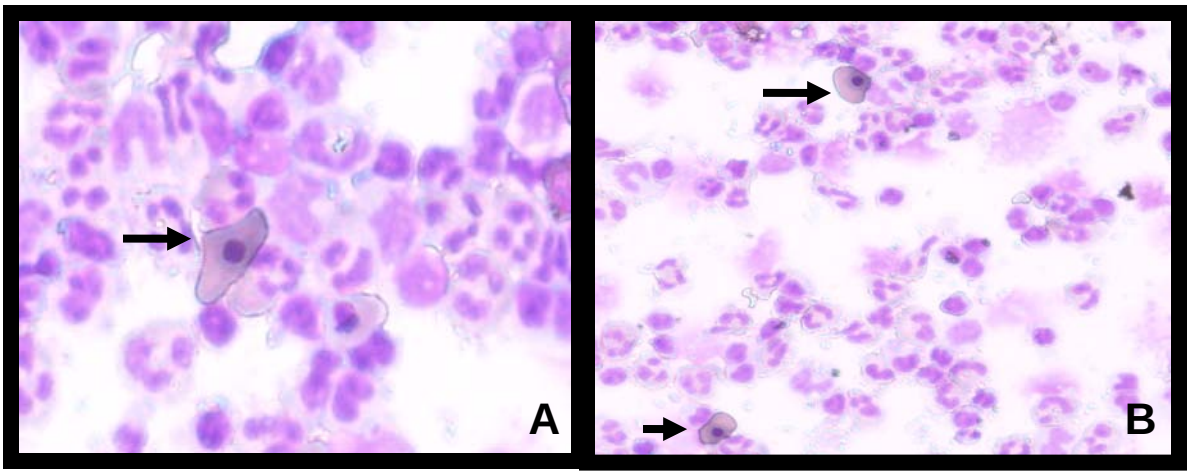
(20 x magnifications)

Figure 4.12 Loss of fetal primitive erythroblast in Percoll 1118 cushion after the first step density gradient centrifugation



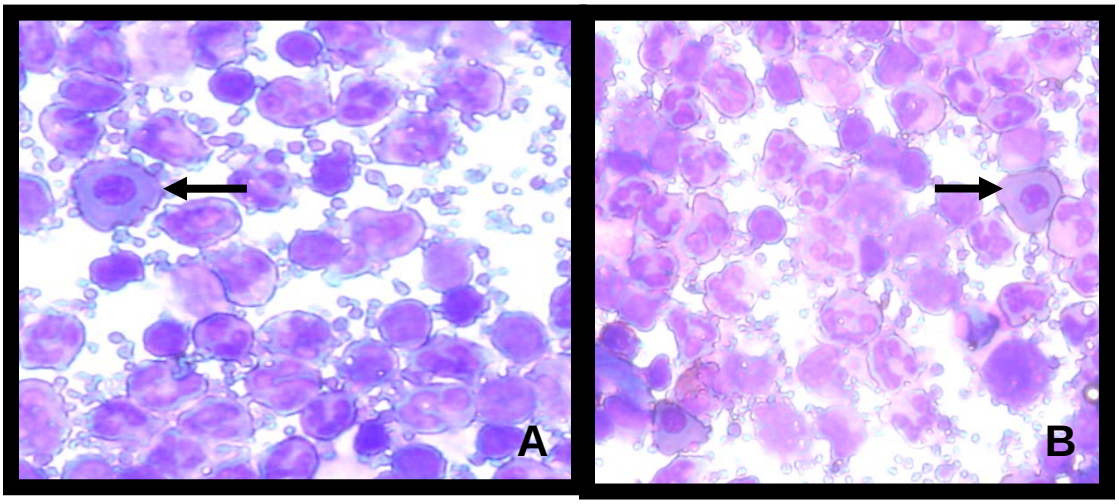
Wright stain of cells lost in the Percoll 1118 cushion. Arrow indicates fetal nucleated red blood cell lost in Percoll 1118 cushion. The cell is likely to be fetal primitive erythroblast as it is large in size and has a high cytoplasmic:nuclear ratio. Surrounding cells are maternal polymorphonuclear leukocytes (20 x magnifications)

Figure 4.13 Loss of fetal primitive erythroblasts within LD column during the second-step enrichment process on MACS



A,B shows wright stain of nucleated cells depleted in the LD column. Arrow indicates fetal nucleated red blood cells lost. Surrounding cells are maternal polymorphonuclear leukocytes (20 x magnifications)

Figure 4.14 Loss of fetal primitive erythroblasts in negative fraction of MS column during the second-step enrichment process on MACS



A,B shows wright stain of nucleated cells depleted in the MS column. Arrow indicates fetal nucleated red blood cells lost in the negative fraction of MS column during the second step enrichment process. Surrounding cells are maternal polymorphonuclear leukocytes (20 x magnifications)

Interpretation: The upper and lower layer obtained after the first step density gradient centrifugation and processed separately for anti-CD45/anti-GPA cell sorting on MACS and treated finally with ammonium chloride/1mM acetazolamide cocktail lysis buffer comprised of pure population of nucleated and anucelated red blood cells. Using three-step enrichment method, more than one-third of fetal primitive nucleated erythroblasts were recovered in *in-vitro* model. Assuming that 36% of target cells are lost after the first step enrichment process (Choolani et al., 2003) and 7% of target cells are expected to be lost during the lysis step (section 4.2), 20% of primitive fetal erythroblasts are lost in the second step enrichment process. This finding is similar to that reported by Choolani et al. (2003) who observed 24% loss of target cells after cell sorting with anti-CD45/anti-GPA enrichment process in their model mixture experiments.

4.7 Conclusion

It is important to evaluate the intrinsic protocol efficiency *in-vitro* before studying its enrichment efficiency in an *in-vivo* model. Choolani et al. (2003) had previously developed an enrichment protocol comprising percoll density gradient centrifugation and MACS. Here, we tested, and added, the selective lysis of adult anucleate erythrocytes using ammonium chloride and 1mM acetazolamide, an inhibitor of carbonic anhydrase (Boyer et al., 1976; De Graaf *et al.*, 1999; Volluaire *et al.*, 2001). This strategy selectively lysed 96% of adult anucleate red blood cells while keeping 93% of fetal NRBCs intact. Our *in-vitro* experiments were designed to model the enrichment of first trimester FNRBCs. Fetal primitive NRBCs extracted from placental tissues were mixed with adult blood to mimic their enrichment from maternal blood; we could recover 37% of spiked primitive NRBCs.

Hence, the results in this chapter demonstrate that:

(1) a combination of ammonium chloride/acetazolamide could be used as a third-step enrichment method;

(2) a combined immunoenzymatic staining, immunofluorescence cytochemistry and cFISH could be used to prove NRBCs of fetal origin; and immunohistochemistry using vector blue substrate can be used to screen for cytospun slides which contain PFNRBCs, that can subsequently be processed for AMCA- ε-globin/cFISH.

(3) surgical termination of pregnancy can be used to evaluate efficiency of a new fetal cell enrichment protocol in *in-vitro* model system.

(4) approximately one-third of target cells are likely to be enriched using the three-step enrichment system on maternal blood.

Chapter 5: Termination of pregnancy as an *in-vivo* model to study enrichment efficiency of a novel non-invasive prenatal diagnosis method in the first-trimester using cell-based strategy

5.1 Introduction

Two main strategies have emerged as a risk-free method for non-invasive prenatal diagnosis of genetic disorders: the enrichment of fetal cells from the maternal blood and recently, the analysis of cell-free fetal DNA in maternal plasma or serum. For cell-based strategy, the target cell that has been most sought for in the maternal blood for more than a decade is the fetal nucleated erythroblasts. Recently, primitive fetal nucleated erythroblast has been considered as an ideal target fetal cell type for first-trimester non-invasive prenatal diagnosis in contrast to definitive fetal nucleated erythroblasts. This is because (1) its characteristics differ significantly from that of definitive fetal nucleated erythroblasts and (2) it contains embryonic ε-globin as a fetal cell specific marker. This chapter examines termination of pregnancy as an *in-vivo* model system to test the efficiency of the three-step enrichment method for first-trimester non-invasive prenatal diagnosis. Target cells are likely to be obtained before and after the termination of pregnancy. To be certain that the numbers obtained indeed reflect 37% efficiency of the three-step enrichment method identified earlier in *in-vitro* model system, a mathematical model would be constructed. This would determine how accurate the efficiency of the three-step enrichment system in *in-vivo* model is to the one identified earlier in *in-vitro* model (section 4.6). If termination of pregnancy is proven as a model system for the

study of first-trimester non-invasive prenatal diagnosis, prediction mathematical model can then be postulated to the scientific community where the number of fetal cells present in the maternal blood in the first-trimester can be predicted based on the numbers enriched in the post-termination samples.

Recently, it has been speculated that termination of pregnancy is a biological model for fetomaternal haemorrhage (Bianchi et al., 1997; Bianchi et al., 2001). However, these studies suffered following limitations: (1) fetal cells instead of FNRBCs were targeted to prove the hypothesis. Fetal cells include erythroid or lymphoid progenitor cells as well, which tend to persist post-partum from previous pregnancies and could confound the numbers in the current pregnancy (2) no enrichment protocol was used (3) pregnant women were enrolled in the study either after the invasive procedure or those known to be carrying aneuploid pregnancy, and therefore the number of fetal cells were influenced by the invasive nature of the procedure or aneuploidy status of the fetus, respectively and (4) pre- and post-procedure samples belonged to two different groups of women enrolled at two different time periods (1997; 2001). This chapter also addresses whether first-trimester termination of pregnancy is a biological model for fetomaternal haemorrhage. It was hypothesised that a three-fold increase in embryonic ε-globin positive fetal primitive erythroblasts could be achieved after first-trimester termination of pregnancy.

5.2 Enrichment of primitive fetal nucleated erythroblasts from first-trimester maternal blood before and after termination of pregnancy

Aim: (1) To develop an *in-vivo* model that could be used to evaluate efficiency of new fetal cell enrichment protocol for non-invasive prenatal diagnosis in the first trimester and (2) to determine the termination of pregnancy as a model for fetomaternal haemorrhage.

Sample Size: A sample size of 10 subjects was calculated based on following assumptions:

- 2 sided test of 5%
- Power of 80%
- Three-fold increase in FNRBCs following CVS procedure, based on study by Al-Mufti et al. (2003).

Investigation: Paired blood samples (23 ml) were obtained 2 hours before and within 5 minutes of elective first-trimester surgical termination of pregnancy from 10 patients. Gestational age was established by ultrasonography. Those in whom cervical priming was to be carried out, blood was drawn prior to the insertion of pessary. Products of conception were collected and placed in phosphate buffered saline (PBS) for subsequent fetal gender confirmation only after cell sorting, staining for ε-globin and cFISH, so that the gender was predicted prospectively in a blinded fashion. A three-step enrichment method for sorting first-trimester fetal primitive erythroblasts from maternal blood was carried out (section 4.6). Subsequently, the cells from the upper and the lower layer were

separately cytospun on the glass slides and underwent Wright staining (section 2.2.5) and were viewed under light microscope for morphological visualisation and count of nucleated erythroblasts. Slides which showed the presence of NRBCs, were subsequently processed for combined immunohistochemistry, AMCA and cFISH using ε-globin as a fetal cell specific marker (section 2.2.14).

Data Analysis: The data was analysed by SPSS (Statistical Package for Social Sciences) version 11.5. Non-parametric Wilcoxon signed rank test for paired samples was applied for the comparison of the number of NRBCs isolated from each layer after the three-step enrichment method as the samples did not have a Gaussian distribution. A *p* value of <0.05 was considered to be statistically significant. Results are reported as means, median and ranges, though in a non-Gaussian distribution, the median is the best representative of all value in the series. The significance of the correlation was analysed using Spearman Rank analysis.

Results: The mean maternal age was 24 years (SD 3.92) (Table 5.1). The mean gestational age at the time of termination of pregnancy was 9^{+3} weeks (SD 1.53). After first-step density gradient centrifugation in the pre-termination samples, significantly greater number of mononuclear cells were found in the upper layer compared to the lower layer ($p = 0.007$). On the other hand, significant contamination with red blood cells was noted in the lower layer ($p = 0.009$) compared to the upper (Table 5.2). Although, a three-fold increase in the number of NRBCs and ε-globin positive nucleated cells was observed in the upper compared to the lower layer after the three-step enrichment method, the distribution of NRBCs ($p = 0.31$) and ε-globin positive nucleated cells ($p = 0.53$) in the upper layer were not significantly different from that of the lower layer (Table 5.1).

In the post-termination samples, the number of mononuclear cells was also significantly higher in the upper layer ($p = 0.005$) with no significant difference in the distribution of red blood cells ($p = 0.06$) across the two layers (Table 5.2). Despite a 1.3- and 1.4-fold increase in NRBCs and ε-positive nucleated cells in the upper compared to the lower layer, the number of NRBCs and the primitive fetal nucleated erythroblasts retrieved in the upper layer were not significantly different ($p = 0.64$) and ($p = 0.53$) from that of the lower layer after the three-step enrichment protocol (Table 5.1).

An average of 3 and 10 ε-globin positive fetal primitive erythroblasts were recovered from the three-step enrichment method per 20 ml maternal blood before and after the first-trimester termination of pregnancy, respectively. A significant increase in NRBCs ($p = 0.005$) and ε-globin positive nucleated erythroblasts ($p = 0.005$) were observed after termination of pregnancy. A 3- and 2.8-fold increase in NRBCs and ε-globin positive fetal nucleated cells were noted after the invasive procedure, respectively, which were statistically significant ($p = 0.005$) (Table 5.2). Fold increase in NRBCs after termination of pregnancy was associated with an increase in ε-globin positive nucleated cells and a significant positive correlation was noted between the two ($r = 0.82$; $p = 0.004$) (Figure 5.1). A significant positive correlation was also observed in the increase of NRBCs ($r = 0.66$; $p = 0.03$) and ε-globin positive cells ($r = 0.65$; $p = 0.04$) as gestational age advanced in pre-termination maternal blood samples (Figure 5.2 and 5.3)

Table 5.1: Enrichment of NRBCs and ε-globin positive primitive fetal erythroblasts after three-step protocol

Patients No	Maternal age (years)	Gestation at blood collection day (weeks/days)	Pre-TOP NRBCs 1st layer/2nd layer		Pre-TOP ε+ cells 1st layer/2nd layer		Post-TOP NRBCs 1st layer/2nd layer		Post-TOP ε+ cells 1st layer/2nd layer		Fetal karyotype	AMCA/ cFISH
1.	22	8^{+5}	6	0	6	0	7	0	7	0	XY	XY
2.	24	9^{+4}	2	0	2	0	6	0	4	0	XY	XY
3.	27	7^{+3}	0	2	0	1	1	4	0	2	XY	XY
4.	18	10^{+4}	3	3	3	1	0	12	0	10	XX	XX
5.	23	12	4	7	0	7	22	3	15	3	XY	XY
6.	20	8^{+2}	0	3	0	3	9	0	9	0	XY	XY
7.	25	11^{+5}	7	0	7	0	15	6	15	6	XY	XY
8.	32	8^{+5}	3	0	3	0	17	7	6	5	XX	XX
9.	24	9^{+1}	13	0	1	0	2	39	0	4	XY	XY
10.	21	8^{+2}	0	2	0	2	2	10	1	10	XY	XY
Total			38	17	22	14	81	81	57	40		
Minimum	18	7^{+3}	0	0	0	0	0	0	0	0		
Maximum	32	12	13	7	7	7	22	39	15	10		
Mean	23.6	9^{+3}	3.80	1.70	2.20	1.40	8.10	8.10	5.70	4.00		
Median			3.00	1.00	1.50	0.50	6.50	5.00	5.00	3.50		
SD	3.92	1.53	4.05	2.26	2.57	2.22	7.58	11.64	5.85	3.80		
p-value			0.31		0.53		0.64		0.53			

Table 5.2: Enrichment of NRBCs and ε-globin positive primitive fetal erythroblasts after three-step protocol

	Minimum	Maximum	Mean	Median	Standard Deviation	p-value
Pre-top upper layer MNCs	1.90×10^7	6.09×10^7	3.57×10^7	3.41×10^7	1.25×10^7	**0.007**
Pre-top lower layer MNCs	1.22×10^7	5.04×10^7	2.29×10^7	2.28 x 10	1.10×10^7	
Pre-top upper layer RBCs	0	1.05×10^7	3.72×10^6	2.76×10^6	4.04×10^6	
Pre-top lower layer RBCs	3.96×10^6	2.88×10^8	9.00×10^7	7.44×10^7	8.77×10^7	**0.009**
Post-top upper layer MNCs	1.92×10^7	6.60×10^7	4.22×10^7	3.87×10^7	1.26×10^7	**0.005**
Post-top lower layer MNCs	6.0×10^6	3.45×10^7	2.17×10^7	2.50×10^7	1.21×10^7	
Post-top upper layer RBCs	6.0×10^5	2.40×10^7	6.04×10^6	3.0×10^6	7.24×10^6	0.06
Post-top lower layer RBCs	6.0×10^5	2.75×10^7	1.34×10^7	1.44×10^7	9.58×10^6	
Pre-top NRBCs (total)	2	13	5.50	4.50	3.92	
Post-top NRBCs (total)	5	41	16.20	12	11.44	**0.005**
Fold increase in NRBCs	1.17	8	3.41	3	2.04	**0.005**
Pre-top ε-globin+ cells	1	7	3.60	3	2.32	
Post-top ε-globin+ cells	2	21	9.70	9.50	6.07	**0.005**
Fold increase in ε-globin+ cells	1.17	5.50	2.94	2.79	1.22	**0.005**

Top: Termination of pregnancy; MNCs: Mononuclear cells; RBCs: Red blood cells; NRBCs: Nucleated red blood cells

Figure 5.1 Correlation between fold increase in nucleated red blood cells and ε-globin positive primitive fetal nucleated erythroblasts after termination of pregnancy

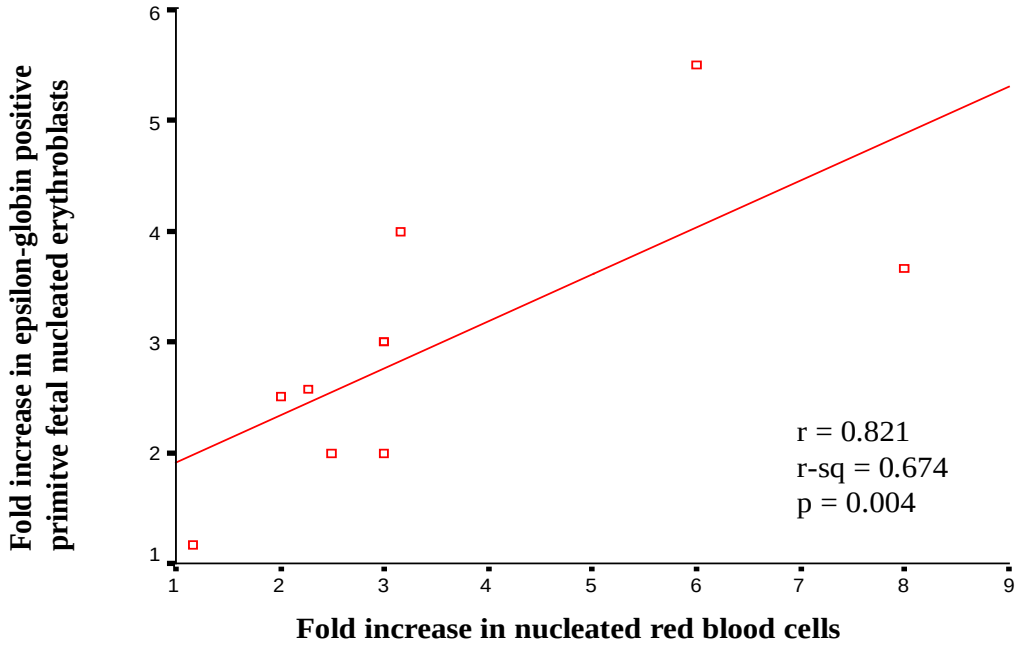


Figure 5.2 Correlation between pre-termination enriched NRBCs and gestational age

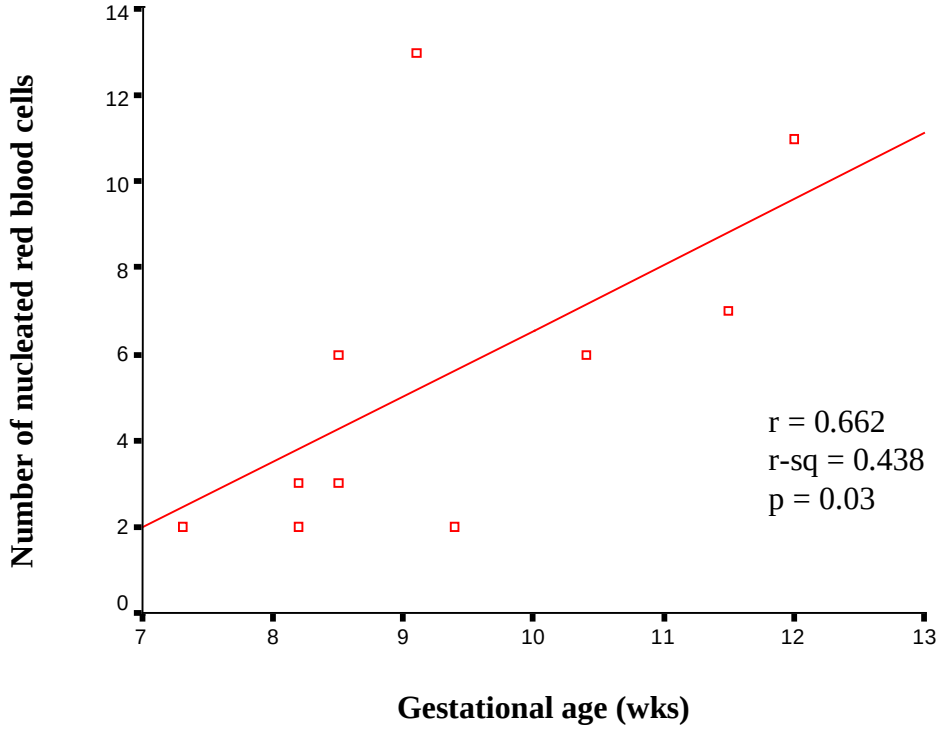
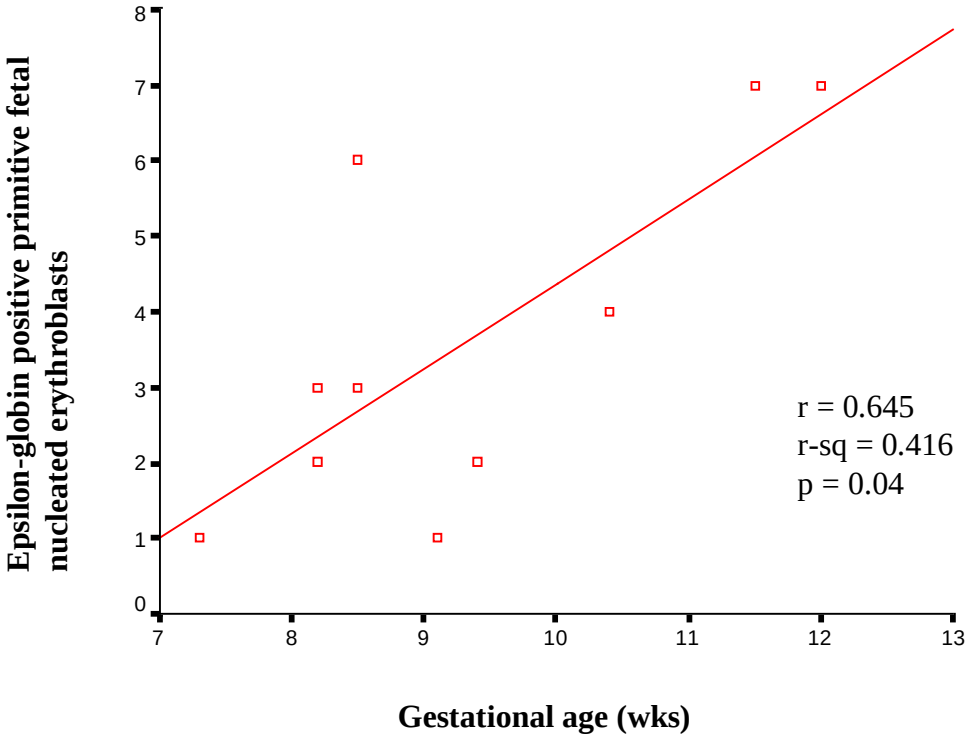


Figure 5.3 Correlation between pre-termination enriched ε-globin positive primitive fetal nucleated erythroblasts and gestational age



The predicted fetal gender on AMCA and cFISH matched that confirmed by cFISH on samples of trophoblast tissue in each case: (two females and eight male fetuses). The morphology of enriched cells was clearly visible on AMCA and the gender signals could be clearly visualised on cFISH analysis (Figure 5.4 and 5.5). No ε-globin positive XX cells were noted among male fetuses. Similarly, no ε-Hb-NRBCs were observed in the blood of healthy male volunteer.

Figure 5.4 Immunofluorescence staining and cFISH on male fetal primitive nucleated erythroblasts enriched from first-trimester maternal blood

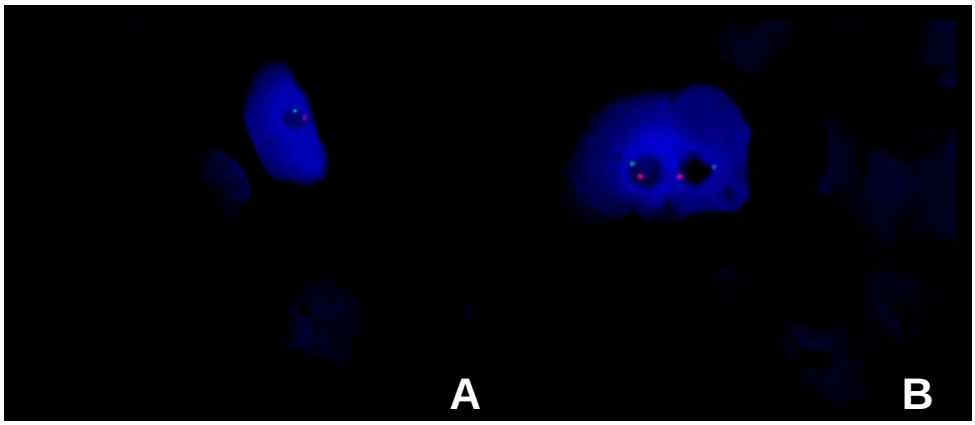


Figure A-D shows immunofluorescence staining with cFISH (red signal:X chromosome; green signal:Y chromosome: Male fetus) within the nucleus and accumulation of AMCA around the nucleus and staining of intracytoplasmic ε-globin positive primitive fetal nucleated erythroblasts, depicting the morphology of the entire cell (20 x magnifications)

Figure 5.5 Immunofluorescence staining and cFISH on female fetal primitive nucleated erythroblasts enriched from first-trimester maternal blood

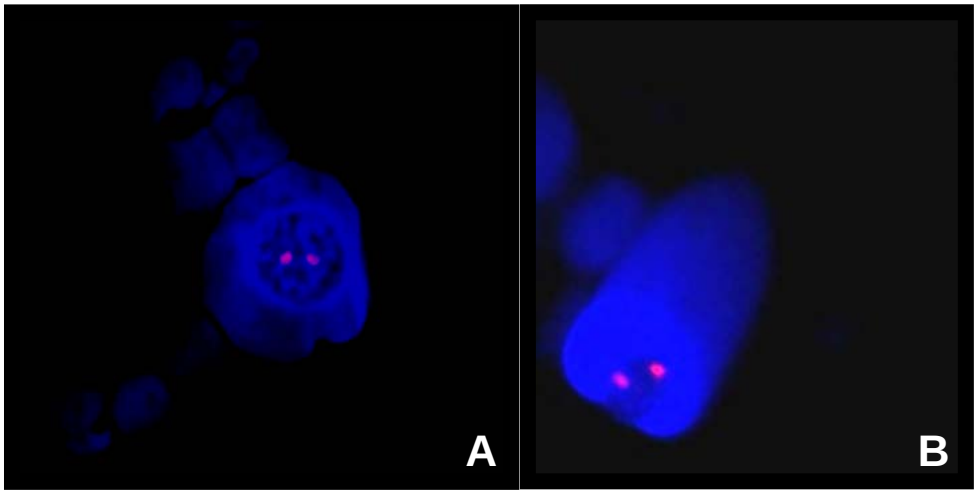


Figure A,B shows immunofluorescence staining with cFISH (red signal:XX chromosome: Female fetus) within the nucleus and accumulation of AMCA around the nucleus and staining of intracytoplasmic ε-globin positive primitive fetal nucleated erythroblasts, depicting the morphology of the entire cell (40 x magnifications)

Interpretation: The data show that application of the three-step enrichment protocol recovered on an average 3 fetal primitive nucleated erythroblasts cells before and 10 after termination of pregnancy per 20 ml maternal blood, respectively. The procedure also led to an approximately three-fold increase in ε-globin positive primitive fetal nucleated erythroblasts following termination. A significant increase in mononuclear cells was obtained in the upper layer as compared to the lower layer after first-step Percoll 1118 density gradient centrifugation implying more recovery of NRBCs and primitive fetal nucleated erythroblasts was to be expected in the upper layer. Although, a three-fold increase was noted in the recovery of both NRBCs and primitive fetal nucleated erythroblasts from the upper layer, no significant difference was observed in their distribution in the upper as compared to the lower layer after the three-step enrichment method.

In the post-termination samples, despite a significant increase in mononuclear cells in the upper as compared to the lower layer, the number of NRBCs and primitive fetal nucleated erythroblasts retrieved were not significantly increased compared with that of the lower layer. The frequency of NRBCs and primitive fetal erythroblasts in maternal blood increased progressively as the gestational age advanced from 7^{+3} weeks to 12 weeks gestation.

Based on the above results, a mathematical model was constructed. The relationship between the observed number of pre- and post-termination enriched primitive fetal nucleated erythroblasts determined by linear regression is:

Observed number of pre-termination primitive FNRBCs = co-efficient between pre and post-termination primitive FNRBCs x observed number of post-termination primitive FNRBCs

The co-efficient for our model = 0.354 (95% CI, 0.264% - 0.444%)

The 89.8% of the variability in the number of fetal primitive nucleated erythroblasts observed in the pre-termination samples is determined by the number of cells obtained in the post-termination samples (R^2 =0.898). Therefore, the accuracy of our *in-vivo* model is approximately 90%. With 37% efficiency, we could enrich 3 and 10 cells before and after termination of pregnancy, respectively. Therefore, the actual number of primitive fetal nucleated erythroblasts likely to be circulating before and after termination of pregnancy would be 8 and 27 per 20 ml maternal blood, respectively.

Thus, the following mathematical relationship for the prediction of number of pre-termination primitive FNRBCs to be obtained through an enrichment system is postulated:

Expected number of enriched fetal nucleated erythroblast in pre-termination sample = efficiency of the enrichment system x enriched fetal nucleated erythroblast in post-termination sample / 100

5.3 Conclusion

This chapter examines the use of maternal blood obtained post-surgical termination of pregnancy as a suitable *in-vivo* model to test the efficiency of the three-step enrichment method for first-trimester non-invasive prenatal diagnosis. The protocol was able to enrich 3 and 10 ε-globin positive primitive fetal nucleated erythroblasts per 20 ml maternal blood before and after termination of pregnancy, respectively. The data also suggest that termination of pregnancy can lead to an approximately three-fold increase in the trafficking of embryonic ε-globin positive fetal primitive nucleated erythroblasts cells into maternal blood and hence, is a biological model for fetomaternal haemorrhage as that not only FRNBCs is raised but so as our target cell types.

The results demonstrate that surgical termination of pregnancy can be used as a model, both *in-vitro* and *in-vivo* for the study of first-trimester non-invasive prenatal diagnosis. We show that post-termination of pregnancy enrichment of ε-globin positive primitive fetal erythroblasts can estimate the efficiency of that particular enrichment system in on-going pregnancies. Earlier studies have used post-termination maternal blood to evaluate the usefulness of fetal cell enrichment methods, but none have been able to indicate how this correlates with on-going pregnancies (Samura et al., 2000; Wang et al., 2000; Choolani et al., 2003). Our study shows that an enrichment protocol can expect three-fold fewer fetal cells in an ongoing first trimester pregnancy than the number of cells enriched from first trimester maternal blood obtained after surgical termination of pregnancy.

This study is unique as the factors such as the timing of drawing of the blood before and after invasive procedure, recruiting only those pregnant women who had no episode of

bleeding, and drawing blood before the insertion of pessary were taken into account during the design phase of the study. Further, the blood samples were taken from the same women before and after the invasive procedure.

There is no study in the current literature which has used termination of pregnancy as a model for first-trimester non-invasive prenatal diagnosis. This is the first time such a procedure has been used and proven as a model for the study of non-invasive prenatal diagnosis. Voullaire et al. (2001) tried to use first-trimester CVS as a model to study non-invasive prenatal diagnosis. The efficiency of their enrichment protocol was 70% in *in-vitro* model. However, when applied on the patient samples before and after CVS, no first-trimester FNRBCs could be enriched from the pre-CVS samples (Voullaire et al., 2003). This could be because the numbers of first-trimester FNRBCs obtained from the CVS washings were too small to test the efficiency of an enrichment system in an *in-vitro* model.

The three-step enrichment system can be applied on patient samples in an ongoing pregnancy in the first-trimester to calculate the number of epsilon-globin positive primitive fetal nucleated erythroblasts present. Surgical termination of pregnancy can be used to evaluate efficiency of an enrichment method in *in-vitro* model by other investigators to test the efficiency of their protocol for first-trimester non-invasive prenatal diagnosis. Once the efficiency of their enrichment system is determined, the procedure can be used as an *in-vivo* model where the enrichment method could be applied only to post-termination maternal blood samples. The number of cells circulating in the post-termination samples can be calculated. The mathematical model mentioned above can then be used to predict the number of pre-termination target cells present in the maternal blood. The predicted numbers can help the investigators decide, whether the

enrichment system is efficient enough to be used on patient samples in an ongoing pregnancy in the first trimester for non-invasive prenatal diagnosis.

Chapter 6: Termination of pregnancy as a model system to study first-trimester non-invasive prenatal diagnosis using cell-free fetal DNA

6.1 Introduction

The recent demonstration of relatively large amounts of circulating cell-free fetal DNA in maternal blood (Lo et al., 1997) has revealed another source of fetal genetic material that could be used for non-invasive prenatal diagnosis. Recently, few investigators have tried to determine whether cell-free fetal DNA can be used as a marker of fetomaternal haemorrhage using invasive procedures such as second-trimester amniocentesis (Samura et al., 2003) or first-trimester termination of pregnancy (Wataganara et al., 2004; Wataganara et al., 2005) as a biological model. Samura et al. (2003) were able to quantify and show a significant increase in the cell-free fetal DNA levels after amniocentesis. However, the result could have been influenced by (1) the inclusion of a single outlier which should have been excluded from final analysis so as to help understand the data distribution and (2) mean has been reported as a measure of central tendency instead of median. Wataganara et al. reported conflicting results. In their earlier study (2004) the authors demonstrated an increase in post-procedure cell-free fetal DNA levels while in their later report (2005), they did not observe significant difference in the post-termination DNA levels over the pre-termination in the first trimester.

It was demonstrated in chapter 5, that termination of pregnancy is a biological model for fetomaternal haemorrhage. A post-procedure three-fold increase in fetal primitive nucleated erythroblasts trafficking was observed. This chapter examines whether first-

trimester termination of pregnancy can be used to quantify cell-free fetal DNA as a marker of fetomaternal haemorrhage. If proven, the procedure can be used as a model system to study non-invasive prenatal diagnosis using cell-free fetal DNA as a biological marker.

6.2 Effect of first-trimester termination of pregnancy on cell-free fetal DNA levels in maternal blood

Aim: To determine whether an increase in cell-free fetal DNA levels in maternal blood can be obtained after first-trimester termination of pregnancy.

Investigation: Paired blood samples (3 ml) were obtained 2 hours before and within 5 minutes of elective first-trimester surgical termination of pregnancy from 10 patients for quantitative analysis of cell-free fetal DNA (section 2.2.15). Twenty millilitre of blood sample both before and after the procedure had been obtained from the same group of patients for fetal cell enrichment and identification (Chapter 5, section 5.2). The data were analysed by SPSS (Statistical Package for Social Sciences) version 11.5. Non-parametric Wilcoxon signed rank test for paired samples was applied for the comparison of the concentration of cell-free fetal DNA levels before and after the procedure. A p value of <0.05 was considered to be statistically significant. Results are reported as means, median and ranges, though in a non-Gaussian distribution, the median is the best representative of all value in the series.

Result: There were 8 male fetuses as determined by the amplification curve using SRY sequence (Figure 6.1). The gender was confirmed with cFISH on trophoblast cells.

Among these pregnancies with male fetus (n = 8), a significant increase in NRBCs (p = 0.01) and ε-globin positive fetal nucleated erythroblasts (p = 0.01) were observed after the termination of pregnancy (Table 6.1). A significantly three-fold and a 2.79 fold increase in NRBCs (p = 0.01) and ε-globin positive primitive fetal nucleated erythroblasts (p = 0.01) respectively, were also observed after the invasive procedure (Table 6.2). A significant positive correlation exist between the fold increase in post-termination NRBCs and ε-globin positive primitive fetal nucleated erythroblasts (r = 0.864; p = 0.006) (Figure 6.2). No significant difference was noted in the concentration of cell-free fetal DNA levels after the termination of pregnancy compared to before the procedure (p = 0.52) (Table 6.1). Although a 1.16 fold increase in cell-free fetal DNA after the invasive procedure was observed, it was not statistically significant (p = 0.77) (Table 6.2).

Figure 6.1 Amplification curve of *SRY* on cell-free fetal DNA from maternal plasma

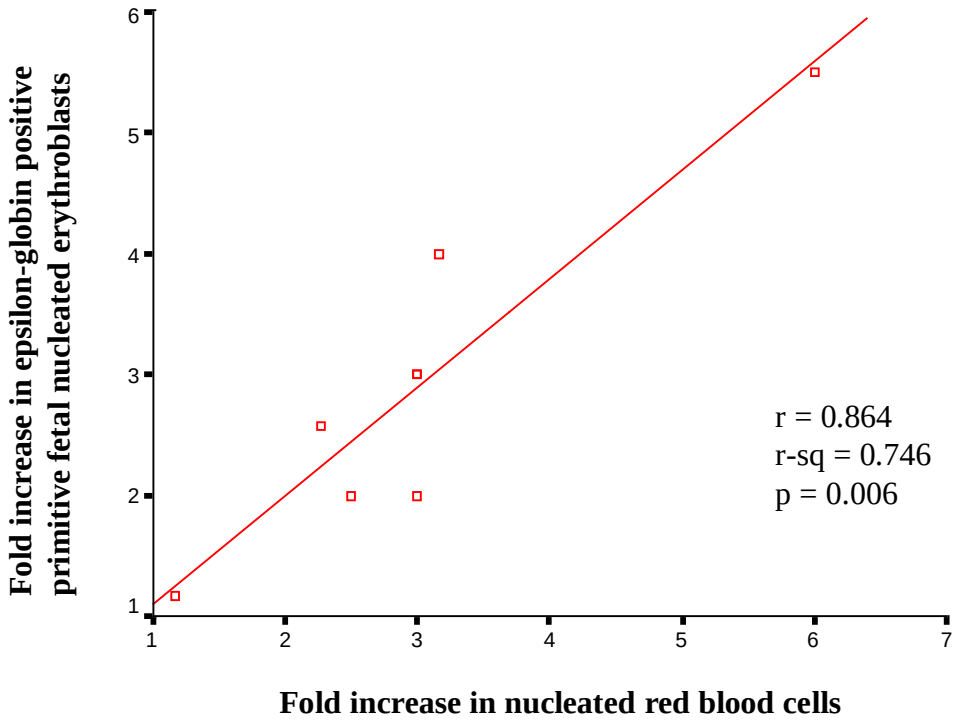
Table 6.1: Cell-free fetal DNA concentration before and after termination of pregnancy in the first-trimester

Patients No	Maternal age (years)	Gestation age (weeks/days)	Pre-TOP NRBCs	Post-TOP NRBCs	Pre-TOP ε+ cells	Post-TOP ε+ cells	Pre-TOP β-globin (total DNA) (GE/ml)	Post-TOP β-globin (total DNA) (GE/ml)	Pre-TOP DNA levels (GE/ml)	Post-TOP DNA levels (GE/ml)
1.	22	8^{+5}	6	7	6	7	2431.87	808.53	11.12	0.48
2.	24	9^{+4}	2	6	2	4	1820.60	3054.40	7.42	10.88
3.	27	7^{+3}	2	5	1	2	1321.20	3217.20	2.60	75.20
4.	23	12	11	25	7	18	1083.60	3263.40	8.62	6.22
5.	20	8^{+2}	3	9	3	9	1213.60	1797.40	23.60	20.80
6.	25	11^{+5}	7	21	7	21	1022.00	1097.80	1.55	0.01
7.	24	9^{+1}	13	41	1	4	4779.40	5077.60	19.10	27.40
8.	21	8^{+2}	2	12	2	11	1309.80	1536.00	5.94	8.74
Minimum	20	7^{+3}	2	5	1	2	1022	808.53	1.55	0.01
Maximum	27	12	13	41	7	21	4779.40	5077.60	23.60	75.20
Mean	23.25	9.27	5.75	15.75	3.63	9.50	1872.75	2481.54	9.99	18.71
Median	-	-	4.50	10.50	2.50	8.00	1315.50	2425.90	8.02	9.81
SD	2.25	1.65	4.33	12.52	2.62	6.87	1262.99	1429.08	7.74	24.69
p-value			**0.01**		**0.01**		0.09		0.52	

Table 6.2 Fold-increase in NRBCs, ε-globin positive primitive fetal nucleated erythroblasts and cell-free fetal DNA concentration after termination of pregnancy in the first-trimester

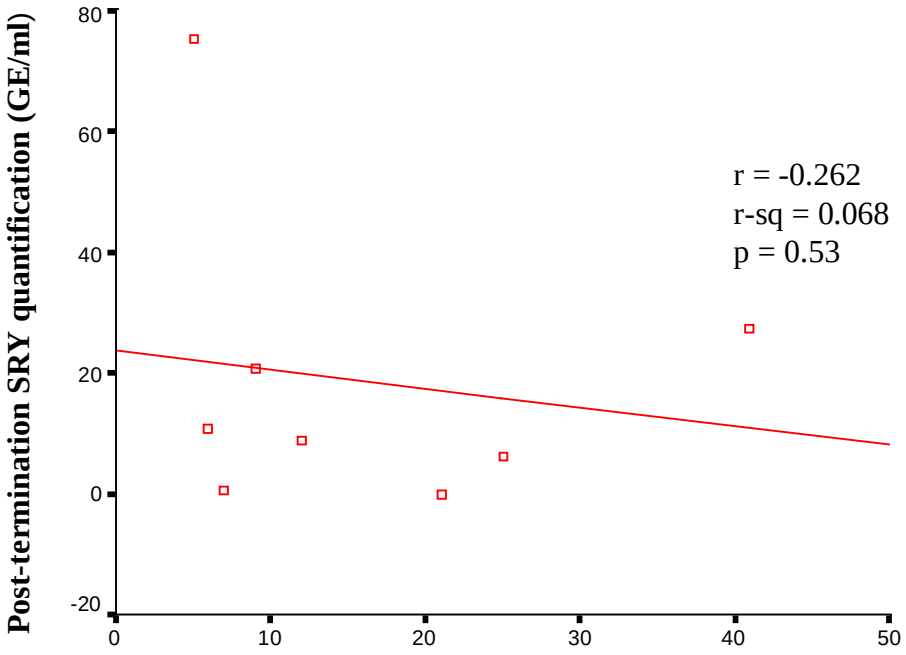
	Minimum	Maximum	Mean	Median	SD	p-value
Fold increase in NRBCs	1.17	6	3.01	3.00	1.37	**0.01**
Fold increase in ε-globin+ cells	1.17	5.50	2.90	2.79	1.35	**0.01**
Fold increase in β-globin	0.33	3.01	1.53	1.33	0.85	0.06
Fold increase in cell-free fetal DNA levels	0.01	28.83	4.36	1.16	9.91	0.77

Figure 6.2 Correlation between fold increase in NRBCs and ε-globin positive primitive fetal nucleated erythroblasts after termination of pregnancy (Male fetuses)



No significant correlation was found to exist between the number of NRBCs obtained in these fetuses and the corresponding concentration of cell-free fetal DNA after the termination of pregnancy (r = -0.262; p = 0.53) (Figure 6.3).

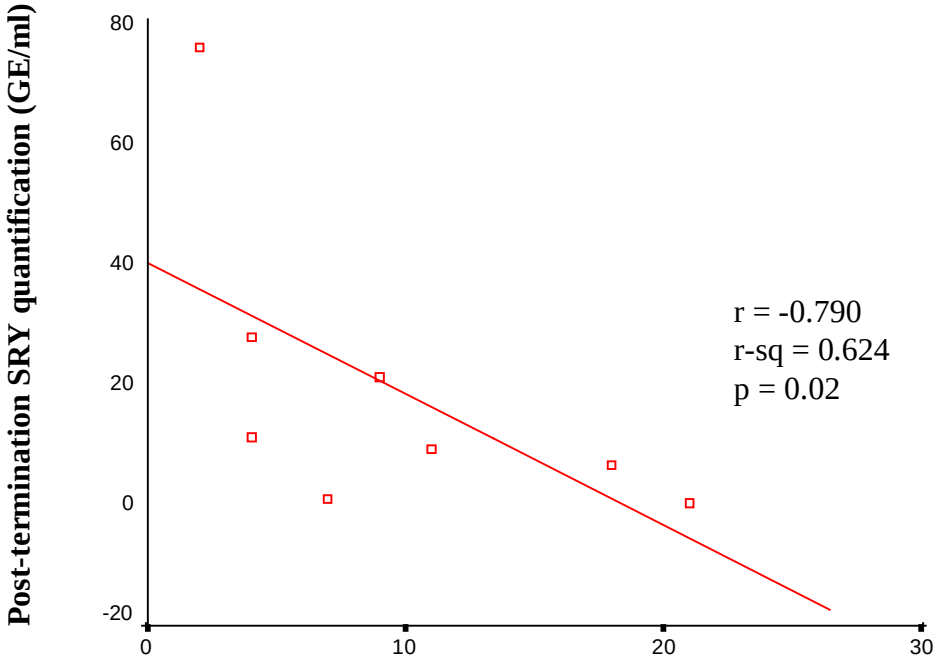
Figure 6.3 Lack of correlation between numbers of post-termination enriched NRBCs and cell-free fetal DNA concentration



However, a significantly strong negative correlation was observed between the number of primitive fetal nucleated erythroblasts recovered after the invasive procedure and the post-termination levels of cell-free fetal DNA ($r = -0.790$; $p = 0.02$) (Figure 6.4).

Interpretation: A significant increase in NRBCs and ε-globin positive primitive fetal nucleated erythroblasts were observed after the invasive procedure in women carrying eight male fetuses. Increases in post-procedure NRBCs were associated with significant concomitant increase in ε-globin positive primitive FNRBCs. No significant increase was observed in cell-free fetal DNA levels after the surgical termination of pregnancy.

Figure 6.4 Negative correlation between numbers of post-termination enriched ε-globin positive primitive fetal nucleated erythroblasts and cell-free fetal DNA concentration



No significant relationship could be demonstrated between the post-termination NRBCs number and levels of cell-free fetal DNA. On the other hand, after termination of pregnancy, the number of ε-globin positive fetal nucleated erythroblasts cells obtained had an inverse relationship with that of cell-free fetal DNA levels.

6.3 Conclusion

The data from this chapter show that cell-free fetal DNA levels cannot be used as a marker to demonstrate fetomaternal haemorrhage using first-trimester termination of pregnancy as a biological model. Although, an approximately three-fold increase in

NRBCs and epsilon-globin positive primitive fetal nucleated erythroblasts were observed following termination of pregnancy, no significant increase in cell-free fetal DNA levels were observed. Both fetal primitive nucleated red blood cells and cell-free fetal DNA are concurrently present in the circulation of all pregnant women in the first trimester. Fetomaternal haemorrhage after termination of pregnancy can be expected as early as 7 weeks of gestation, unlike the results reported by Wataganara et al. (2005), who suggested that fetomaternal haemorrhage is likely to occur only after 8 weeks gestation. Despite an increase in the trafficking of fetal primitive nucleated erythroblasts following termination of pregnancy, and assuming that many of these cells traversing the fetomaternal interface are readily undergoing programmed cell death and are likely to deliver fetal genetic material into maternal circulation, a significant decrease in the post-procedure fetal DNA levels was observed. The mechanism for such an increase in fetal cell trafficking following first-trimester termination of pregnancy with no significant increase in cell-free fetal DNA remains unknown. Therefore, termination of pregnancy cannot be used as a model system for the study of first-trimester non-invasive prenatal diagnosis using cell-free fetal DNA.

Chapter 7: General Discussion

This thesis aimed to determine (i) if embryonic ε-globin positive fetal nucleated erythroblasts persists beyond the first-trimester in trisomy 18 fetus and at birth in trisomy 18 neonates and (ii) if termination of pregnancy can be used as a model system to study efficiency of a three-step enrichment protocol for first-trimester non-invasive prenatal diagnosis.

In this chapter, I examine if the hypotheses put forward can be accepted or must be rejected, assess the clinical implications of these results within the field of non-invasive prenatal diagnosis, crticically evaluate the limitations of these data, and propose potential directions for future work.

7.1 Hypotheses

Epsilon-globin positive primitive fetal nucleated erythroblast was found to persist not only in the second trimester in trisomy 18 fetus but also at term in these neonates. Therefore hypothesis 1 which states that: *"Embryonic epsilon-globin positive nucleated red blood cells persists beyond the second trimester using trisomy 18 as a model"* can be accepted.

Most investigators attempted fetal cell recovery directly from maternal blood without first demonstrating the efficiency of their enrichment protocol in model mixtures. This assumes 100% recovery and no variation. We show this is not the case. Using one protocol we get only 37% recovery on a consistent basis, with some variation (95% CI, 28.5%-45.6%; n=8). In studies that have attempted model mixture evaluation, the spiked

cells did not always represent the exact target for non-invasive prenatal diagnosis, which in the first trimester would be fetal primitive NRBCs. Cells that have been used include cord blood NRBCs, cord blood derived expanded erythroid cells, and fetal liver cells (Gan-Shirt *et al*., 1998; Bhat *et al*., 1993; Troeger et al., 1999; Smits *et al*., 2000; Bianchi et al., 1996). We used our exact target cells (first trimester FNRBCs) in our model mixtures so that we could determine accurately how our protocol would perform when using first trimester maternal blood. Large numbers of primitive fetal nucleated erythroblasts was successfully enriched from termination of pregnancy tissue for this purpose (section 4.2). The target cells were spiked in model mixture and the efficiency of the three-step enrichment system, Percoll 1118, anti-CD45/anti-GPA and ammonium chloride/acetzolamide was effectively tested using termination of pregnancy as an *in-vitro* model (section 4.6).

To date, there is no biological model to determine the *in vivo* efficiency of any new fetal cell enrichment protocol. Ideally, a procedure that causes increased fetal cell trafficking could allow evaluation of the *in-vivo* enrichment efficiency of new such protocols by studying their enrichment in maternal blood obtained post-procedure. Furthermore, the model should closely reflect the average-risk maternal population, and not bias the enrichment efficiency by studying only high risk pregnancies. We show that maternal blood obtained post surgical termination of pregnancy is a suitable *in-vivo* biological model for new fetal cell enrichment protocols and methods. We have demonstrated that three fold fewer fetal cells would be enriched from maternal blood in ongoing pregnancies than those isolated from post termination maternal blood. Thus hypothesis 2 which states that *surgical termination of pregnancy can be used to evaluate efficiency of a new fetal cell enrichment protocol in in-vitro model system and it can be used as an in-*

vivo model to study enrichment efficiency of a novel non-invasive prenatal diagnosis method in the first trimester can be accepted.

Cell-free fetal DNA in maternal blood has been investigated as a marker of FMH (Lau et al., 2000; Bianchi et al., 2001; Wataganara et al., 2004; 2005). Surprisingly, we did not observe any change in cffDNA and total DNA levels in maternal plasma within 5 min after surgical TOP. Earlier studies had suggested an increase in these levels within maternal blood samples taken after surgical TOP, but the sampling times had varied (Bianchi et al., 2001; Wataganara et al., 2004; 2005). Recently, Jeong et al. (2007) reported a linear increase in both total DNA and cffDNA levels in maternal plasma at different time points after elective first trimester surgical TOP. DNA levels were significantly higher at 90 min after the procedure. It was suggested that this may be due to the slow release of cell-free nucleic acids from the fetal cells that had entered into the maternal circulation after the procedure. These data by Jeong et al. (2007) may explain the insignificant change in total DNA and cffDNA levels we observed in our study where maternal blood was collected immediately after surgical TOP.

7.2 Implication of results in the context of non-invasive prenatal diagnosis

New protocols and novel methods of non-invasive prenatal diagnosis are difficult to compare because the *in-vivo* model has not been standardized. We propose an ideal *in-vivo* model where new systems can be tested because a greater number of fetal cells are circulating in maternal blood after surgical termination of pregnancy. The results obtained from this model can be extrapolated to obtain likely efficiency of the system in both healthy and at-risk pregnancies. This model can be readily standardized within and

between research groups, so that the data can now be compared. We believe this *in-vivo* model is a useful addition that would bring non-invasive prenatal diagnosis closer to clinical service.

This thesis also shows that the three-step enrichment protocol is able to enrich 37% of the ε-globin positive primitive fetal nucleated erythroblasts circulating in the maternal blood in the first trimester and that such cells are likely to persists beyond the first-trimester and upto term in trisomy 18 newborns. The number of cells enriched may not allow the applicability of the technique as an independent diagnostic test for non-invasive prenatal diagnosis in the first-trimester in near future, as even the gold standard tests such as amniocentesis and chorionic villus sampling requires analysis of at least 20 cells from two different flask cultures (Guetta et al., 2004). However, it can be used as an additional screening marker and can represent a new approach to screening for Down syndrome. The current non-invasive screening test gives a risk estimate to the couples. At the same time an independent test can be carried out on maternal blood for the number of ε-globin positive nucleated cells using the three-step enrichment protocol. An increase in the number of cells in the first-trimester maternal blood and the persistence of such cells in the second-trimester could indicate the pregnant women at risk of carrying an aneuploidy fetus. The decision to go on to invasive procedure could then be based on the results of two independent non-invasive screening tests.

Embryonic ε-globin is a specific fetal cell identification marker and the three-step method gives a pure population of nucleated and anucleated red blood cells with no maternal contamination of white cells. Therefore, a combined immunohistochemistry together with AMCA and cFISH for chromosome-21 can be performed on ε-globin nucleated cells enriched from those women who are identified at high-risk after two non-invasive

independent screening tests. AMCA being a fetal cell identifier would help in depicting the morphology and therefore, would help in the identification of the fetal cells with three copies of chromosome 21.

7.3 Using epsilon-globin positive primitive fetal nucleated erythroblasts for diagnosis of genetic disorders at single cell level

The numbers of primitive fetal nucleated erythroblasts obtained from the three-step protocol may be low enough to preclude its usage for first-trimester non-invasive prenatal diagnosis of chromosomal disorders for the time being, micromanipulation is one strategy that could allow its utilisation for the diagnosis of genetic disorders. Sekizawa et al. (1996) demonstrated that it was possible to select single fetal NRBCs by micromanipulation and potentially diagnose genetic conditions such as Duchenne muscular dystrophy (DMD) by PCR. In one of six cases studied, DMD was suspected when four out of seven exons within the DMD gene were not amplified but the diagnosis was not confirmed since all seven exons were subsequently amplified from another sample obtained from the same slide. To show that their technique could have identified DMD if present, they tested one lymphocyte from a patient with DMD and demonstrated, by PCR, the correct deletion in exon 50. One limitation of the study was that erythroblast identified by morphology alone was presumed to be fetal in origin. Though Watanabe et al. (1998) demonstrated the prenatal diagnosis of the X-linked ornithine transcarbamylase deficiency syndrome, NRBCs were picked up based on their morphology rather then fetal in origin. Martel-Petit et al. (2001) identified NRBCs based on Kleihauer test and applied micromanipulation to a pregnancy at risk for cystic fibrosis. However, one cell

demonstrated a heterozygous genotype, which could have resulted from selection and analysis of a maternal cell. Therefore, using non-specific fetal cell marker could lead to the procedure to be labour intensive and time consuming as it is not clear whether the cells picked up are indeed fetal or maternal NRBCs. In contrast, Chueng et al. (1996) used micromanipulation to pick ζ-globin positive fetal NRBCs and diagnosed fetal haemoglobinopathy in two pregnancies between 10-12 weeks. However, ζ-chain is not absolutely fetal specific and has been reported in adult carriers of α-thalassaemia (Cheung et al., 1984; Chui et al., 1986, Tang et al., 1992). On the other hand, ε-globin is a highly specific fetal cell marker and under the current circumstance whereby low numbers of these cells are obtained from maternal blood, primitive fetal erythroblasts staining positive for ε-chain can be isolated by micromanipulation technique and utilised for PCR based diagnosis of genetic disorders. Figure 7.1 shows the usage of micromanipulation technique on first trimester fetal primitive nucleated erythroblasts enriched from termination of pregnancy tissue and Figure 7.2 demonstrates ε-globin fetal cell identification marker and cFISH signals on these cells which were fixed on the glass slides. It can be seen that the morphology of the cells are clearly intact after micromanipulation.

Figure 7.1 Application of micromanipulation technique on first-trimester primitive fetal nucleated erythroblasts enriched from products of conception

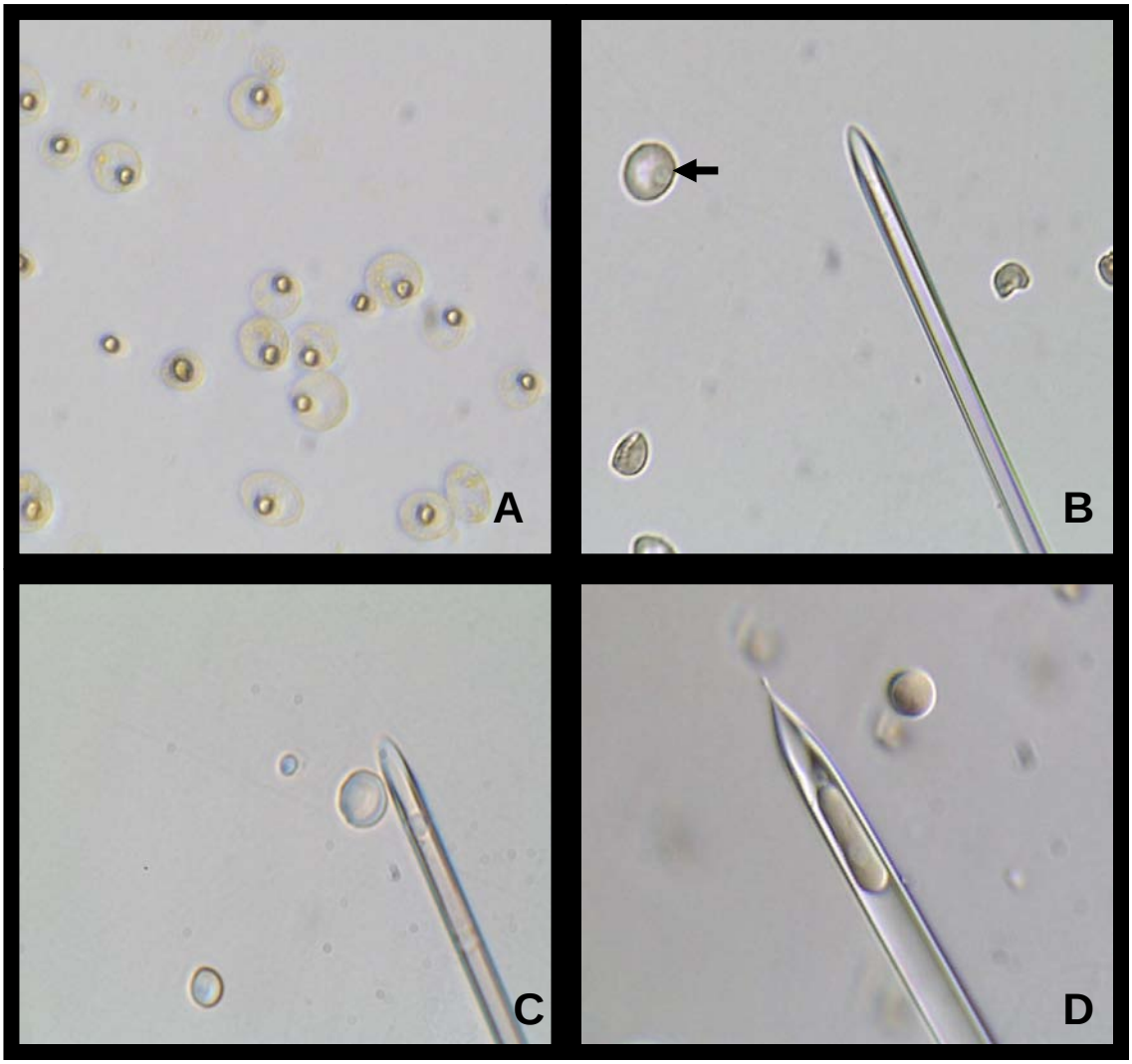


Figure A-D indicates primitive fetal nucleated erythroblasts being picked using micromanipulator at a single cell level (20 x magnifications)

Figure 7.2 Immunofluorescence staining of ε-globin and cFISH for gender identification on primitive fetal nucleated erythroblasts after micromanipulation

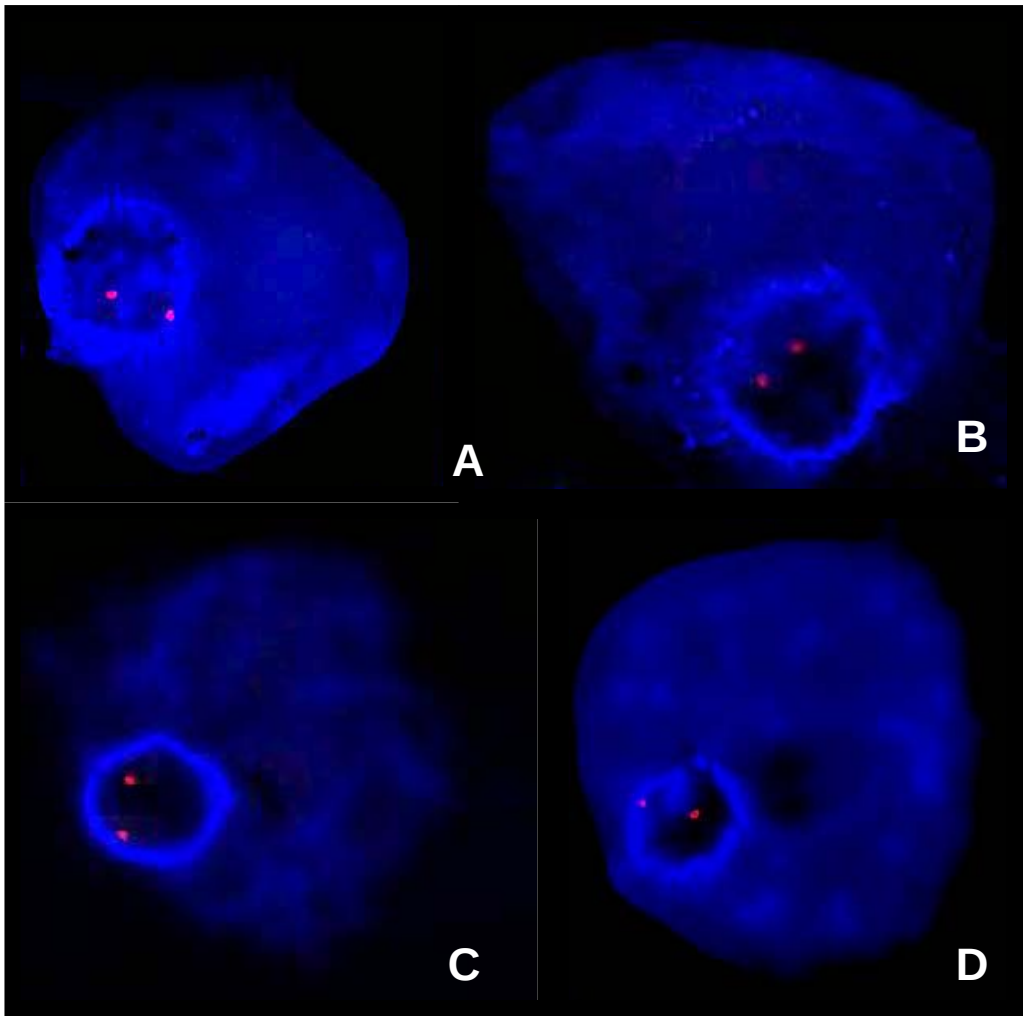


Figure A-D shows immunofluorescence staining with cFISH (red signal:XX chromosome: Female fetus) within the nucleus and accumulation of AMCA around the nucleus and staining of intracytoplasmic ε-globin positive primitive fetal nucleated erythroblasts, depicting the morphology of the entire cell (60 x magnifications). Morphology of the cells are well preserved after micromanipulation at the single cell level

7.4 Limitations of this research

The low number of cells retrieved using the three-step enrichment protocol could have been likely due to too many steps involved in the enrichment process. Sixty-three percent of the target cells are expected to be lost after the three-step enrichment process

(section 4.6). Besides, the enrichment procedure is time-consuming, as each of the two layers was processed separately after the first-step density gradient centrifugation.

Although, a very short exposure to ice-cold ammonium chloride/acetazolamide was incorporated into the enrichment protocol for *in-vitro* model system and clinical samples, it is expected that 7% of fetal cells may have been lost during this step after the first two step of enrichment process (section 4.3). If a surface antigen-based immunselection strategy were available, both the density gradient centrifugation and the lysis step would preferably be omitted.

7.5 Directions for future research

The three-step enrichment protocol using ε-globin as a fetal cell specific marker applied in this thesis, retrieves a small number of fetal primitive nucleated erythroblasts from pretermination maternal blood samples. The future work should aim to involve incorporation of ζ-globin together with the application of ε-globin as a fetal cell identification marker so as to increase the number of target cells.

The one step during the enrichment process that is associated with the greatest cell loss is density gradient centrifugation (Choolani et al., 2003). It is recognised that elimination of this mechanical step and replacement by an immunsorting strategy would enhance rare fetal cell recovery. Thus, identification of unique, differentially expressed surface membrane proteins and antigens is the key to improved enrichment protocols. A proteomic approach can be utilised to address this issue. Comparing membrane protein profiling of RBCs and FNRBCs using the complimentary techniques of one and two-dimensional electrophoresis and Surface Enhanced Laser Desorption and Ionisation mass

spectrometry (SELDI-TOF/MS) could be useful. Matrix Assisted Laser Desorption and Ionisation mass spectrometry (MALDI-TOF/MS) could also be used for peptide mapping and identification. Finding surface antigens that could be used to differentiate fetal erythroblasts from adult anucleate erythrocytes would eventually be ideal, so that the use of density gradient centrifugation and selective erythrocyte lysis can be avoided. This in turn could cut down the number of steps involved in the enrichment method and therefore the cell loss.

As adult anucleate erythrocytes are smaller and more deformable than fetal primitive erythroblasts, these properties could be exploited to deplete maternal erythrocytes and eliminate the need for density gradient centrifugation. One such technique that could be beneficial is the designing and development of microelectromechanical systems (MEMS) filters for size-based filtration of fetal and maternal cells.

Finally, for clinical applicability, once all aspects of the non-invasive prenatal diagnostic technique have been optimised, these can be automated including locating ε-globin positive erythroblasts on the microscope slides and capturing the images for evaluation by a technician at a convenient time. This could in future may cut down the number of steps involved in enrichment process, as purity would not be seen as an utmost criteria and therefore contamination with maternal nucleated cells would not interfere with the automated scanning system, as it would capture only AMCA positive embryonic ε-globin primitive fetal erythroblasts. However, such an automated diagnostic technique would need to be prospectively evaluated in a proper clinical trial before being offered as a diagnostic service.

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Appendix: Publications

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- S Ponnusamy, N Mohammed, SSY Ho, HM Zhang, YH Chan, YW Ng, LL Su, AP Mahyuddin, A Venkat, J Chan, M Rauff, A Biswas, M Choolani. *In vivo* model to determine fetal cell enrichment efficiency of novel non-invasive prenatal diagnosis methods. Prenat Diagn. 2008 (In press).