FETAL CELLS IN THE MATERNAL CIRCULATION

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To Mitchell and Eleanor, both of whom arrived during work on this thesis and made it so difficult to write up!

To Keith, who is in large part responsible for the arrival of Mitchell and Eleanor and who tried to make it easier for me to write up.
Abstract

Current practice in antenatal care aims to assess possible risk factors which may affect the mother, the fetus or both. These risks are evaluated in general (as for maternal age or parental ethnic background), and also specifically (for risks identified through family histories and the history of previous pregnancies). Once these data are ascertained and discussed between parents and doctors, choices involving further investigation of the fetus may need to be made. This may involve invasive procedures, such as amniocentesis or chorionic villus biopsy which may pose a risk (albeit usually small) to the pregnancy.

It has been known for many years that fetal cells escape into the maternal blood. However, it is only in the last few years that new molecular techniques have been developed which could utilise these cells for non-invasive prenatal diagnosis. This thesis investigates the possibility of gaining information about the pregnancy from fetal cells shed into the maternal circulation.

I first conducted a prospective study to determine the time of appearance of fetal cells in the maternal circulation. Using PCR analysis of maternal blood samples, fetal DNA was detected in all the pregnancies studied at seven weeks and the fetal sex correctly predicted. These data demonstrate that fetal DNA is present in all pregnancies at a time suitable for first trimester prenatal diagnosis, that it first appears within a two week window, and that (as DNA is no longer detected two months after delivery), contamination with DNA from previous pregnancies should not be a problem.
I next investigated the use of PCR to assess quantitatively the amount of fetal DNA directly on whole blood samples, since the routes of fetal cell dissemination suggest that the amount may increase during pregnancy and may be linked with the development of maternal raised blood pressure and intrauterine growth retardation. The PCR method chosen was found to be unsuitable for quantitation of the small amounts of fetal DNA present in the vast excess of maternal DNA.

Enrichment of cells from the maternal circulation will be necessary before DNA analysis or karyotyping can be carried out easily. Antibodies to the epidermal growth factor receptor were attached to magnetic beads which were then used in test systems of first trimester placenta and blood to attempt to separate trophoblast with successful enrichment of 150-fold. The system was then applied to maternal blood samples and the isolated cells analysed with dual fluorescence in situ hybridisation, to detect male (fetal) cells.

A system for the isolation of a single interphase nucleus was then developed, to permit single cell analysis of single gene disorders. The nuclear X and Y chromosomes were identified with dual fluorescence in situ hybridisation and the nucleus was removed from the slide with a micro-dissection needle. The DNA was co-amplified using two sets of PCR primers for the detection of amelogenin and a common mutation causing cystic fibrosis. Encouraging results were obtained, and further refinement of the technique for single nuclear isolation could provide non-invasive diagnosis for a single gene disorder. However, close attention to the avoidance of DNA contamination will be required to provide reliable results.
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Abbreviations

AC  alternating current
AEC  3-amino-9-ethyl-carbazole
AMG  amelogenin
AMGL  amelogenin-like
APS  ammonium persulphate
ATP  adenosine triphosphate
B  blank
βhCG  β sub-unit of human chorionic gonadotrophin
bp  base pairs
BPG  2,3-bisphosphoglycerate
BSA  bovine serum albumin
CAT  Cambridge Antibody Technology
CF  cystic fibrosis
CVS  chorionic villus sample
DAPI  4', 6-diamidino-2-phenylindole
dATP  deoxyadenine triphosphate
dCTP  deoxycytidine triphosphate
DMEM  Dulbecco's modified Eagle's media
DMSO  dimethyl sulphoxide
dNTP  deoxynucleotide triphosphates
DNA  deoxyribonucleic acid
EDTA  ethylene diamine tetracetic acid
EGFR  epidermal growth factor receptor
α-EGFR  Epidermal growth factor receptor antibodies
pg, ng, μg, mg, g  pico, nano, micro, milli, gram
FACS  fluorescence activated cell sorting
FCS  fetal calf serum
FISH  fluorescent in situ hybridization
FITC  fluorescein isothiocyanate
H₂O₂  hydrogen peroxide
HLA  human leucocyte antigen
ICRF  Imperial Cancer Research Foundation
IgG  immunoglobulin
ISH  in situ hybridization
IUGR  intra-uterine growth retardation
IUUI  intra-uterine insemination
IVF  in vitro fertilisation
kb  kilo base pairs
kD  kilo dalton
LMP  last menstrual period
MACS  magnetic activated cell sorting
MW  molecular weight
nM, µM, mM, M  nano, micro, milli, molar
NISH  non-isotopic hybridization
PBS  phosphate buffered saline
PBS-CMF  phosphate buffered saline (calcium and magnesium free)
PCR  polymerase chain reaction
PIH  pregnancy induced hypertension
RCOG  Royal college of obstetricians and gynaecologists
rpm  revolutions per minute
SLE  systemic lupus erythymatosis
TEMED  N,N,N′N′-tetramethylethylenediamine
Tris  trizma base (2-amino-2-[hydroxymethyl]-propane-1,3-diol)
U  units
µl, ml, l  micro, milli, litre
UV  ultra-violet
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Nichtinvasive pranataldiagnostik an fetalen zellen im mutterlichen blut (Gynakologe)
CHAPTER 1
INTRODUCTION

Expecting a baby is a very exciting event during which we transfer our dreams and aspirations to the next generation. To me, the best thing about obstetrics is that unlike any other medical speciality, patients and partners are usually looking forward to having a child and are healthy and keen to participate in antenatal care.

Being pregnant can also be an anxious time, especially for those mothers in high risk groups, such as the older mother, and critical decisions regarding screening and prenatal diagnosis may need to be made.

It is the nature of the practice of obstetrics that the joy of the delivery of a healthy child is balanced by the tragedy of the delivery of a severely handicapped child and anything that can be done to tip that balance in favour of health is worth pursuing.

We all want healthy babies, and with ever increasing expectations, many feel this is a right - but no matter how science and medicine advance there will always be some abnormal babies born. We would not wish to eradicate minor defects by genetic selection, but there is increasing knowledge of major handicap arising during pregnancy. Choices face those expecting a baby, and obtaining clinical information about the pregnancy may place it at risk, if it involves invasive procedures.

This project is aimed at the further understanding of the presence of fetal cells in the maternal circulation, which may in the future offer non-invasive prenatal diagnosis and widen the choice for screening for those people who wish to have such information.
Further understanding of the pathophysiology of the entry of fetal cells into the maternal blood may also lead to early prediction of life threatening conditions such as pre-eclampsia and intrauterine growth retardation.

1.1 Risks of Pregnancy

Congenital malformations, often genetically determined, occur in 2-3% of all infants and lethal malformations account for 25% of all perinatal deaths (Ash et al 1977, Chitty and Winter 1989, McKusick,1990). A review of several studies over the last 20 years shows that the contribution of congenital malformations to perinatal mortality is increasing (Winter et al 1988). In addition, there are many congenital malformations which cause serious morbidity in infancy and childhood (morbidity and mortality weekly report 1989). The cost to the NHS of neonatal intensive care and rehabilitation programmes for the severely handicapped is huge and the emotional and financial impact on families immeasurable.

The risks to the fetus which may lead to congenital abnormalities may range from the relative simplicity of a single gene defect, to complex multifactorial situations, where genetic and environmental factors must be present at a specific stage of development for an abnormality to arise. In single gene disorders, a change in the DNA sequence can lead to production of a dysfunctional, or no protein. This can cause abnormalities such as those seen in Tay-Sachs disease, thalassaemia, and cystic fibrosis.

Where larger amounts of genetic material are involved, an associated group of clinical abnormalities lead to recognisable syndromes. The commonest defects of this type are aneuploides.
where an entire chromosome is missing or extra copies are present as a result of non-dysjunction at meiosis. The major aneuploidies diagnosed prenatally involve the autosomes 13, 18 and 21 and the sex chromosomes. Translocations of smaller amounts of genetic material can occur, with transfer from one chromosome to another. When these are balanced, they may not result in any phenotypic abnormality, but when unbalanced translocation occurs abnormalities are often seen.

Other risks to the fetus may be environmental, most commonly maternal smoking or alcohol ingestion, or risks the mother may be unaware of, such as exposure to radiation, or iatrogenic as in folate antagonist antibiotics.

With the growing recognition of the frequency and importance of congenital disorders and with current social trends towards smaller families and delaying childbearing, prenatal diagnosis has an important role in the management of many pregnancies. It may aid decisions regarding the place, gestation and method of delivery so that appropriate care is immediately available, as well as helping parental psychological adjustment to potential neonatal problems. Prenatal diagnosis is of paramount importance to parents in cases where elective termination is being considered.

1.1.1 Current Screening and Diagnosis

The identification of pregnancies in which there is an increased chance of diagnosable fetal disorders involves a search for general and specific risk factors. Prepregnancy counselling is appropriate in some cases where a genetic problem has already been identified in a family, allowing genetic counselling, planning of possible prenatal
tests and preventative treatment such as folic acid supplements in women who have had a child with a neural tube defect (MRC vitamin study 1991). Women with pre-existing disease such as diabetes benefit from strict control of blood sugars at and prior to the time of conception and increased early antenatal surveillance. Those with a previous unfavourable obstetric outcome and those taking potentially teratogenic medications for epilepsy or clotting disorders are other targets for pre-pregnancy counselling.

For most women, thorough history taking in the antenatal clinic should elicit the required genetic information. The task is then to consider, with the prospective parents, the risk of the birth of an abnormal child versus the risk of an investigative procedure. A person's age, previous experience, ethnic and cultural background and religious beliefs will affect the acceptability of these choices and therefore what kind and whether screening or diagnosis is appropriate for an individual.

1.1.2 Predictive Testing by Group (General Risk Factors)

Numerical chromosomal abnormalities occur with increasing frequency with advancing maternal age in particular, 47+21 associated with Down syndrome (Hook 1981, Hook et al 1983). Down syndrome is the commonest cause of severe mental retardation, with a birth prevalence of 1.3/1000 in England and Wales and a life expectation of 60 years (Baird and Sadovnick 1988). Apart from mental handicap, it is associated with malformations of the heart, digestive system, eyes and ears.

Prenatal diagnosis through amniocentesis or CVS is generally available through the NHS to all women who will be age 36 or over at
their expected delivery date and in these cases the relative risk of handicap versus loss of the pregnancy through an invasive procedure must be individually evaluated. Approximately two thirds of pregnancies associated with fetal Down syndrome end in miscarriage, with most of these losses in the first trimester, the prevalence at 18 weeks' gestation being 25% higher than it is at birth (Creasey and Crolla 1974).

Only 5% to 10% of pregnancies occur to women in this age group and they account for 20% of all Down syndrome births. Only 40% of Down syndrome births will be detected if women over 34 are offered amniocentesis and in practice it is less than this because some women are not offered the test and others decline it (Knott et al 1986). As a result, few screening programmes based on maternal age have achieved a reduction in the birth prevalence of Down syndrome of more than 15%. Targeting women over 35 for amniocentesis has shifted the maternal age relation of Down syndrome births, with women under 30 giving birth to more babies with Down syndrome than those over 30.

Currently, many hospitals are offering screening based on biochemical markers in maternal serum. The test is non-invasive, can be offered regardless of age and used to screen patients at risk for certain structural and cytogenetic abnormalities. Down syndrome and trisomy 18 are associated with raised maternal hCG, low maternal serum unconjugated oestriol and low maternal serum AFP (Merkatz et al 1984). The levels rise steadily throughout the second trimester and cannot be evaluated until 16 weeks' gestation. The markers are also affected by factors other than Down syndrome, making interpretation difficult in multiple pregnancies, extremes of maternal weight, diabetics and cigarette smokers. Accurate dating of the
pregnancy is required in order to interpret correctly the relative risk which may assist decisions regarding invasive diagnosis.

Using a calculated risk value of 1:250 as a cut off, the detection rate is 61%, with 5% false positives. These are clearly important, since they are the unaffected pregnancies which would be exposed to the (unnecessary) risk of an invasive procedure. By adding an ultrasound scan for accurate dating (this is now routine in most pregnancies) the detection rate can be increased by 10% and the false positive rate reduced to 3.8% (Haddow et al 1992). In borderline cases, fetal nuchal translucency measured on ultrasound scan may indicate skin oedema and is associated with Down syndrome. Combining the maternal age, maternal serum hCG and fetal nuchal translucency in the first trimester (11-13 weeks' gestation) has provided an improved estimate of risk for fetal trisomies (Brizot et al 1995) although the difference in nuchal translucency thickness alone between the chromosomally normal or abnormal groups was not significant. Poor reproducibility of the measurement of nuchal translucency in the first trimester may diminish its usefulness as a screening test for chromosomal abnormalities (Roberts et al 1995).

AFP, the major circulating protein in early fetal life, is synthesised in the fetal liver and yolk sac. Open neural tube and ventral wall defects are associated with exposed fetal membrane and blood vessel surfaces that increase the levels of AFP in both amniotic fluid and maternal serum (UK collaborative study 1977). A screening programme based on maternal serum AFP levels can identify 80-90% of fetuses with neural tube defects, almost all cases of gastroschisis, and 70-80% of cases of omphalocele (UK collaborative study 1977, Palomaki et al 1988) and women are routinely offered such screening antenatally. The addition of skilled ultrasound scanning (in particular
vaginal scanning) can detect minor degrees of neural tube defects and increase the accuracy of diagnosis and prognosis in the future.

1.1.3 Predictive Testing by Group (Specific Risk Factors)

Specific risk factors may be identified in the family history, the history of previous pregnancies, or the mother's medical history. After the birth of one child with trisomy 21, the likelihood of recurrence is 1% (Stene 1970). The rate of recurrence of neural tube defects is 2-5% compared with 0.1-0.2% in the general population (Cowchock et al 1980). The rate of recurrence of a cardiac defect is 2-4% as compared with 0.4-0.8% of live births in the general population (Lin et al 1988, Nora et al 1988). If a parent has spina bifida, congenital heart disease, or a known chromosomal translocation or inversion, there is an increased chance that a child will have a related defect. Prenatal diagnosis is possible for many inborn errors of metabolism, almost all of which are transmitted as autosomal recessives. Diabetes mellitus and phenylketonuria in the mother are associated with an increased risk of fetal malformations and where the opportunity for pre-pregnancy counselling has been missed, increased antenatal surveillance must be maintained. Any history of working with or exposure to known teratogens such as ionising radiation, therapeutic and illegal drugs and infections should be elicited as part of the personal history, preferably pre-pregnancy.

Gene frequencies differ among population groups defined geographically or ethnically. Programmes exist to detect carrier status for Tay-Sachs disease, haemoglobin S and thalassaemia genes for the at risk ethnic groups prior to pregnancy, although testing is
often undertaken for the first time in the antenatal clinic, adding to parental anxiety.

1.2 Prenatal Diagnostic Procedures

With an increasing number of older mothers and the advances in understanding and diagnosis of genetic disorders, more couples are having to decide whether to undergo prenatal diagnosis, and with advances in molecular genetics, this number will increase. It is therefore important to discuss the benefits, risks and limitations of the currently available diagnostic procedures.

1.2.1 Mid-Trimester Amniocentesis

Mid-trimester amniocentesis is a procedure performed under ultrasound guidance at 16 weeks of gestation onwards. A narrow gauge needle (less than 18 gauge) is introduced transabdominally through the uterine wall and into the amniotic fluid surrounding the fetus. The application of aseptic techniques with antiseptic solutions are important in minimising the risk of infection. 20 ml of the fluid is removed and aliquots sent in separate containers for analysis. The fluid may be assayed for other fetal products used for prenatal diagnosis, including AFP, bilirubin, acetylcholinesterase and pulmonary phospholipids. The cells contained in the fluid are shed from the fetal skin, respiratory, digestive and genitourinary tracts, amnion and umbilical cord. Only 20% of the cells obtained are viable, with the least common cell type, the fibroblast, having the greatest mitotic potential. Chromosomal studies are carried out by culturing
the cells (failure occurs in less than 1% of cases) and the results are available in 10-14 days.

Mid-trimester amniocentesis has been performed since the early 1960's and the safety and accuracy have been established in three prospective studies (UK collaborative study 1977, The NICHD National Registry for Amniocentesis Study Group 1976, Simpson et al 1976) and carries an estimated maximum risk of fetal loss of 0.5-1.0% (Working Party on Amniocentesis 1978, Tabor et al 1986).

Since amniocentesis of necessity involves rupturing the amniotic membrane, there is a risk that the rupture remains patent leading to leakage of the amniotic fluid, ascending infection and miscarriage. If the pregnancy does continue, the reduced liquor volume may lead to fetal lung hypoplasia.

Another major disadvantage of amniocentesis is that results are not available until 18 weeks gestation. At this time, fetal movements may have been felt and the pregnancy may be apparent to other people, making difficult decisions about termination of the pregnancy even more fraught. Dilatation and evacuation of the uterus is much less safe at this gestation, with increased risks of haemorrhage, infection and cervical trauma and most obstetricians would recommend induction of labour with prostaglandins as the safest method of termination. This can be a lengthy and painful procedure which adds to the emotional trauma of the situation.

Chromosomal mosaicism is the presence of two or more cell lines with different karyotypes in a single person. It most commonly occurs as a result of postzygotic nondisjunction. Because of the origin and variety of cells obtained by amniocentesis, failure to detect the true fetal karyotype is rare, with discordance rates being over 20 times less than those of CVS. The most common type of mosaicism detected by amniocentesis is pseudomosaicism. This does not reflect
the fetal karyotype since it arises during in vitro cultures and is suspected when an abnormality is present in only one of several cultures of an amniotic fluid specimen. True fetal mosaicism is rare (0.25%), clinically important (Hsu et al 1984) and is diagnosed when the same abnormality is present in more than one culture. Contamination by maternal cells can also be a problem, and can be minimised by discarding the first few drops of aspirated amniotic fluid.

Midtrimester amniocentesis may be undertaken in the case of multiple pregnancies, with fluid removed from each sac. The instillation of dye into the sampled sac helps to minimise the error of sampling the same sac twice. However, this requires at least two needle insertions and has a failure rate of 2-8%. The procedural loss rate of 3.2% is higher than for singleton pregnancy and there are theoretical concerns about creating amniotic band syndrome and mosaicism from cross-sac contamination (Librach et al 1987).

1.2.2 Early Amniocentesis

Experience has been accumulating with early amniocentesis performed before 15 weeks of gestation (Choo 1991). Although early amniocentesis is a technically easier procedure than CVS, the rates of success of the two procedures in obtaining samples are similar (Byrne et al 1991).

The safety of early amniocentesis cannot be assumed to be the same as for conventional amniocentesis. The volume of fluid removed constitutes a much greater proportion of the total fluid volume and its withdrawal may increase fetal loss and decrease fetal lung function. On occasion, aspiration of the fluid may be hampered
by the tenting of the membranes, since the amnion has not fused with the chorion early in gestation leading to multiple needle insertions, a factor known to increase the infection and miscarriage rates.

Crandall et al (1994), performed a large prospective study and found a spontaneous abortion rate of 1.5% in those undergoing early amniocentesis compared with 0.6% in the control group undergoing midtrimester amniocentesis. Some of the losses subsequent to early amniocentesis are independent of the procedure reflecting the higher spontaneous abortion rate in the first trimester, but the adjusted rate is still marginally higher for early than midtrimester amniocentesis. The success rate for cell culture in the two groups was the same and there was no difference in the rate of stillbirths and neonatal deaths. These data suggest that the theoretical risks of early amniocentesis may be less important than was thought and if performed at 10 weeks' gestation would bring the benefits of first trimester prenatal diagnosis to amniocentesis.

1.2.3 Chorionic Villus Sampling (CVS)

The main advantage of CVS over standard amniocentesis is the earlier availability of results. During the gestational period 9-12 weeks from LMP, the amniotic sac does not yet fill the uterine cavity and the chorion has begun to differentiate into the mitotically active chorion frondosum, which will become the placental site. This is an ideal time to sample the chorionic villi, either transabdominally or transcervically. A needle (transabdominally) or biopsy forceps (transcervically) is introduced under ultrasound guidance into the chorion frondosum where the villi float freely in the intervillus space and are only loosely attached to the underlying decidua. Some of
these villi are removed by aspiration or biopsy and the mitotically active cytотrophoblast is used for a rapid direct karyotype preparation (results in 24-48 hours), while the mesenchymal core is used as the source of chromosomes for culture (results in 10-14 days). An additional advantage of CVS is that more tissue is obtained than by amniocentesis. This is extremely useful when DNA or enzymatic analysis is required.

The ability to perform any invasive procedure is improved with practice and operator experience will influence the success and complication rates. The procedure of transabdominal sampling is technically easier than transcervical as a higher level of skill is required from the ultrasound operator to guide and view the biopsy. In centres with little experience, the infection rates for the transcervical route can be much higher, reflecting a prolonged and more traumatic procedure.

Two randomised trials have demonstrated that a woman assigned to undergo first trimester CVS had a 1.7%-4.6% lower chance of a successful pregnancy outcome than a woman assigned to a second trimester amniocentesis (Canadian Collaborative CVS-Amniocentesis Clinical Trial Group (1989), MRC Working Party on the Evaluation of CVS 1991). The background spontaneous loss rate increases with maternal age, is hard to ascertain and is of key importance if meaningful comparisons are to be made with mid-trimester amniocentesis. It is at its highest precisely in the age range of those most at risk for chromosomal abnormalities and thus in those most likely to present for prenatal diagnosis. Spontaneous losses occur most commonly from the 9th to the 16th weeks of gestation (Simpson 1990), coinciding with the post-CVS observation period. The Canadian Collaborative CVS-Amniocentesis Clinical Trial Group 1989 demonstrated an excess loss rate of 0.6% over amniocentesis which
was not statistically significant. Perhaps of more relevance (since it reflects the European population and the practice of CVS undertaken here) the MRC Working Party on the Evaluation of CVS (1991), reported a 4.6% increase in the CVS loss rate compared with amniocentesis, however, improved ultrasound scanners and operator experience may reduce this in the future.

Other rare, but potentially important complications following CVS have been reported. Vaginal bleeding is rare (<1%) following transabdominal procedures, but is not uncommon after transcervical sampling. Subchorionic haematoma formation may follow incorrect positioning of the forceps into the vascular decidua basalis layer and is minimised by experienced operators.

Chorioamnionitis, particularly through the introduction of vaginal flora into the uterus via the transcervical route has been a theoretical concern. (Hogge et al 1986) reported an incidence of 0.3% prior to 20 weeks’ gestation and this was confirmed by the Canadian collaborative group study 1989. Likewise, acute rupture of the membranes has proved a very rare event, with no cases seen in a review of 6000 procedures. Rupture of the membranes may be seen days or weeks following the procedure, due to exposure and subsequent damage to the amnion, or low grade chorioamnionitis. (Hogge et al 1986) and (Brambati et al 1987) reported an incidence of 0.3%.

(Firth et al 1991) reported limb reduction defects in five infants born to a group of 289 women undergoing CVS 56-66 days after the LMP. Oromandibular hypogenesis was present in four of the five infants. (Burton et al 1992) have also reported transverse limb defects after CVS, although a causal relationship has not been established and the data are still controversial with some groups disputing these effects. The crucial aspect of those studies reporting deformities
seems to be the timing of the CVS, with limb defects reported only in those who underwent sampling at less than 10 weeks gestational age. The NICHHD workshop (1993) concluded that there was a greater incidence of oromandibular-limb hypogenesis in CVS exposed infants, and that this appeared to correlate with, but may not be limited to CVS performed earlier than 7 weeks after fertilization.

Perinatal complications such as preterm labour, premature rupture of the membranes and small-for-dates infants have not shown any increase following CVS (Williams et al 1987).

The greater frequency of maternal cells contaminating the sample (reported by Ledbetter et al 1990 as 2%) and mosaicism in CVS samples contribute to the reduced cytogenetic accuracy of the procedure compared with amniocentesis. In confined placental mosaicism, there is a discordance in the karyotype between the fetus and the placenta, usually arising as a result of nondisjunction during embryogenesis which leads to the presence of aneuploid cells in the extraembryonic tissues that are not present in the fetus.

Evidence of mosaicism occurs in less than 1% of samples (Ledbetter et al 1990), is more common in direct preparations, and is usually due to confined placental mosaicism (Kalousek et al 1987). Further testing with amniocentesis is then warranted, with up to 10% of CVS samples requiring follow-up amniocentesis to ascertain the diagnosis in the Canadian collaborative CVS-amniocentesis trial group (1989). The possibility of the 'vanishing twin' has been proposed to explain the discordance between chorionic villus karyotype and fetal phenotype in some cases (Reddy et al 1991).

Confined placental mosaicism cannot be dismissed as unimportant, since there is an associated increase in perinatal complications in these pregnancies, in particular growth retardation (Kalousek and Dill 1983). The fetal loss rate in this group extended
well into the second and third trimesters and was 24% when mosaicism was confined exclusively to the cytotrophoblast (Johnson et al 1990).

The clinical disadvantages of increased risk of pregnancy loss and potential diagnostic error with CVS must be individually weighed against the disadvantage of the later timing of amniocentesis.

1.2.4 Percutaneous Umbilical Blood Sampling

Fetal blood can be obtained from 18 weeks gestation through a 20-22 gauge needle inserted into the umbilical cord under ultrasound guidance. It was developed in 1980 for the diagnosis of thalassaemia and later used to diagnose toxoplasmosis by Daffos et al 1985. Access to the fetal circulation permits evaluation of haematological disorders and allows the prenatal diagnosis of some inborn errors of metabolism and assessment of viral, bacterial and parasitic infections. Analysis of fetal blood gasses and pH may reveal acute hypoxia necessitating early delivery. Fetal blood sampling may be used to clarify chromosomal mosaicism identified by CVS or amniocentesis and cytogenetic results from short term fetal lymphocyte cultures are usually available in 48-72 hours.

The rate of fetal loss following percutaneous umbilical blood sampling is about 2% more than the background risk to the particular fetus (Daffos et al 1985, Shulman et al 1990). Because of the substantially greater risk of pregnancy loss, umbilical cord sampling should be reserved for cases where rapid diagnosis is essential or where diagnostic information cannot be obtained by safer means.

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1.2.5 Fetal Biopsy

Fetal biopsy is performed under ultrasound guidance for certain genetic disorders, such as epidermolysis bullosa which cannot currently be diagnosed by DNA analysis. Fetal muscle biopsy has been used to diagnose Duchenne's muscular dystrophy in a family in which DNA studies were uninformative (Evans et al 1991). Experience with fetal biopsy is limited, which makes the safety and accuracy of the procedure hard to assess. Rapid advances in DNA technology can be expected to elucidate the molecular basis of many diseases that now require fetal biopsy so that it is likely to become an obsolete procedure.

1.2.6 Preimplantation Diagnosis

In very specific cases, the technique of preimplantation diagnosis may be undertaken, so that affected embryos are screened prior to implantation. The technique involves in vitro fertilization, embryo biopsy and DNA diagnosis in order to select the unaffected embryos which will be placed in the uterus. In the case of X linked disorders, preimplantation sexing and selection of female embryos for transfer has been undertaken to avoid transmission of the disorder. Sexing has been performed on one to two cells, which are biopsied at the eight cell stage without adverse effects (Hardy et al 1990). The cells were analysed by PCR amplification of the DNA which identified any X and Y sequence DNA present within 6 hours (Chong et al 1993). Pregnancy rates comparable to non biopsied embryos are achieved, provided the embryo is transferred on the same day (Handyside 1992). Several normal girls have been born to couples at risk of X-
linked disorders following preimplantation sex identification (Handyside et al 1990).

The same technique of embryo biopsy has allowed FISH analysis of the embryos prior to transfer and prevented implantation of an embryo with a mitotic nondisjunction and an X monosomy (Delhanty et al 1993). The accuracy of such highly specialised techniques is being evaluated at specialist centres.

1.2.7 Ultrasonography

Ultrasonography is an important aid in assessing gestation, fetal growth, placental site, multiple gestation, and the diagnosis of major fetal abnormalities, 90% of which occur in fetuses born to parents with no recognisable risk factors. Accurate dating through early ultrasound measurements has significantly reduced the rate of induction of labour in the screened group (Eik-Nes et al 1984, Waldenstrom et al 1988), while other studies have demonstrated a significantly higher rate of hospital admission in the screened group without any improvement in neonatal outcome. Individual centres report impressive achievements in the ultrasound diagnosis of renal and bladder abnormalities, hydrocephaly, neural tube and ventral wall defects (D'Alton et al 1986, Mercer et al 1988, Van den Hof et al 1990). Ultrasound can also be useful in the diagnosis of Mendelian disorders characterised by anatomical defects, such as skeletal dysplasias.

In the UK, as in Germany and France, ultrasound screening is undertaken in almost all pregnancies, with most women undergoing at least one scan during their pregnancy. Current RCOG guidelines recommend a 19 week anomaly scan in all pregnancies as this is the
time when fetal structures are large enough to be scrutinised in
detail. Trials conducted in the 1980's found that 40% of fetal
abnormalities could be detected by routine ultrasound screening in
the low risk population, but only 21% of these were identified prior to
Saari-Kemppainen et al (1990) found that perinatal mortality was
significantly lower in women who underwent routine fetal ultrasound
assessment, with 49% of this due to detection of major malformations
of which 41% were detected. Two studies have reported a significant
reduction in the rate of induction of labour in the screened group
(Eik-Nes et al 1984, Waldenstrom et al 1988). Data on the use of
ultrasound for screening for the diagnosis of congenital abnormalities
is currently being assimilated, with much depending on the skill of
the operator, the timing of the screening and the development of
improved resolution through the use of vaginal as well as abdominal
probes.

In addition to the potential effect on perinatal mortality and
infant handicap, ultrasound diagnosis is increasingly raising options
which go beyond elective abortion. There is certainly scope in the
future for ultrasound-guided intrauterine therapy, as well as helping
to plan appropriate timing, geographical location and method of
delivery so that any necessary care is immediately available.

The most common congenital abnormalities are cardiovascular
malformations, which are among the major malformations most
commonly missed in prenatal ultrasound examinations. Experienced
personnel examining a high risk referred population were reported to
detect 92% of congenital heart defects (Copel et al 1987). Fetuses
with a cardiac defect have a 25-45% risk of having another
anatomical anomaly (Copel et al 1986). Where cardiac and extra-
cardiac malformations are identified, chromosomal analysis should be

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offered, since approximately one third will have a chromosomal disorder (Copel et al 1988, Platt et al 1986).

1.2.8 Endocervical Retrieval of Fetal Cells

A number of conflicting reports on the retrieval of fetal cells from the endocervical canal have been published since 1970 (Adinolfi et al 1994). In 1977, Rhine et al documented the presence of fetal cells in mucous flushed from the endocervical canals of pregnant women. 5 ml of sterile saline was introduced into the endocervical canal through a syringe and the solution aspirated. The cells contained in this fluid, when cultured in vitro, had the characteristic morphology of trophoblast cells and some metaphase spreads contained the Y chromosome (Rhine and Milunsky 1979). In 1971, Shettles was able to detect the Y chromosome with a fluorescent dye test in some cells present on cotton swabs used to wipe the endocervical canal in 30 women in the first, second and third trimesters of pregnancy. He was able to identify the sex of the fetus in 10 out of 18 pregnancies using this technique. However, Bobrow and Lewis (1971) were unable to confirm these results.

The idea was revived in 1992, when Griffith-Jones et al collected cervical flushings or cotton swab samples and were able to demonstrate Y-chromosome specific sequences using PCR and correctly predicted the fetal sex in 25 out of 26 cases. They further identified syncytiotrophoblast and mesenchymal elements with monoclonal antibodies. A similar study by Morris and Williamson in 1992 had difficulties with 'false positive' Y results, which may have been due to contamination with spermatozoa or seminal fluid cells in the collected samples. To avoid this problem, cells collected by trans-
cervical flushing were analysed with non-isotopic *in situ* hybridisation (NISH) and shown to contain Y sequence. 26% of cells retrieved endocervically from a mother with a fetus with trisomy 18 showed three signals when tested by NISH and a chromosome 18-derived repetitive probe (Adinolfi *et al* 1993). The procedure is simple, rapid and relatively inexpensive and may provide cells suitable for culture.

The technique of retrieving fetal cells from the endocervix and lower part of the uterus needs further evaluation. In particular, the question of the time when cells can be retrieved, whether placental localisation affects cell retrieval and whether they can be collected in all pregnancies. Monoclonal antibodies, specific for trophoblast may help avoid contamination with non-fetal cells, although the potential for confined placental mosaicism in these cells may be expected to occur with the same frequency as CVS samples. The evaluation of cervical flushing, aspiration of cervical mucous (Pertl *et al* 1993) and swabbing techniques need to be evaluated with regard to potential introduction of infection, artificially rupturing the membranes, provoking haemorrhage and differences in the number of cells obtained. For the present, it must be regarded as an invasive technique with the potential to harm the ongoing pregnancy, but it remains an exciting prospect for the future.

### 1.3 Improvements for Prenatal testing

The ideal prenatal test would be performed only on those parents who have been counselled and feel they could benefit from the information provided. It should not pose any risk to the mother
or fetus and should provide specific and unequivocal information regarding the pregnancy.

In the case of single gene disorders, an accurate family history and informative testing will remain paramount, but as the genetic rearrangements responsible for disease become known and the speed of testing improves, *de novo* mutations which are responsible for many congenital abnormalities could be screened routinely in all pregnancies. Obtaining a sample of fetal cells for diagnosis should be a non-invasive procedure free from maternal contamination, and analysis of the sample should be available within 24 hours so that an unequivocal and accurate diagnosis can be given to the prospective parents.

In the case of aneuploides, a non-invasive screening test should ideally be available to all mothers regardless of age. This should provide a sample of fetal cells free from maternal contamination suitable for FISH analysis of the five commonest aneuploides within 24 hours.

### 1.3.1 Isolation of Fetal Cells from the Maternal Peripheral blood

A major improvement in prenatal diagnosis and screening would be to offer a method of testing which is non-invasive and therefore poses no risk to the mother or fetus and would provide specific results about the pregnancy. Fetal cells retrieved from the maternal blood could provide the basis for such a test, which could either be a screening test (to determine a group of mothers at high risk and to be followed by a definitive test such as amniocentesis) or as a definitive test itself if it has sufficient accuracy.
Theoretically, two types of cell may cross from the conceptus into the maternal blood, trophoblast and fetal blood cells. Since up to 90% of fetal red cells are nucleated erythroblasts at early gestations, either cell type would be suitable for prenatal analysis of aneuploides, chromosomal rearrangements and single gene disorders.

Cells from the conceptus present in a blood sample drawn from an antecubital vein could be identified and isolated using antibody technology. The genetic material in the cells could then be analysed using FISH to detect aneuploides or translocations, or PCR to detect specific gene mutations.

In order to use fetal cells routinely, there must be a sufficient number (in a suitable condition) present in 20 ml of maternal blood in early pregnancy. The number of fetal cells available in early pregnancy is not known, but it is certainly very small compared to the number of maternal cells, and is probably in the order of one fetal cell to $10^6$ maternal cells (Holz greve et al 1992). This number may vary between pregnancies and also within the same pregnancy at different gestations. The small number of fetal cells would need to be enriched and methods developed to distinguish them from maternal cells unequivocally before testing would be safe and accurate.

Recovering fetal cells was first reported by Walknowska et al (1969) who identified XY metaphases using Y-chromatin staining in the blood of pregnant women carrying a male fetus. This work was confirmed in 1971 by De Grouchy and Trubuchet and Y-chromatin positive cells were identified in the blood of pregnant women with a male fetus by several other groups (Schroder et al 1972, 1974, Zilliacus et al 1975). Y-chromatin staining was based on fluorescence of the distal two thirds of the long arm of the Y chromosome, but its specificity and sensitivity was highly questionable, since some males lack distal Yq (which may give false negatives) and autosomal
fluorescence was also found, particularly satellites of acrocentric chromosomes (which may give false positives) (Sargent et al 1994). These technical and interpretative difficulties led to conflicting data, including the predictable findings that not all individuals carrying male fetuses showed XY metaphases, and some women carrying female fetuses appeared to show XY metaphases. Despite the great interest generated by these data, their relevance was brought into question.

Herzenberg and colleagues (1979, 1981) used flow sorting techniques to enrich for lymphocytes using HLA-A2, where the mother was negative and the father was HLA-A2 positive. The sorted cells were therefore presumed to be of fetal origin. However confirmation of this could not be achieved by Y-chromatin analysis, a technique lacking the required specificity.

Since the technology was not available to advance our knowledge, there followed a period of relative inactivity, until the development of PCR (Mullis et al 1986), its refinement to the single cell level (Arnheim et al 1990) and advances in FISH technology allowed its application to non-cultured interphase cells (Cremer et al 1986, Julien et al 1986, Pinkel et al 1988).

Lo et al (1989, 1990) used nested primer PCR to amplify a Y-specific sequence from unsorted maternal blood and demonstrated the presence of fetal DNA in maternal blood. Since that time, several groups have confirmed their results and it has become a recognised method of demonstrating the presence of fetal cells. FISH technology applied to sorted cells from maternal blood in the case of an abnormal fetus has been able to identify the chromosomal abnormality (Cacheux at al 1992, Bianchi et al 1992). The reliability of results obtained where maternal peripheral blood has been collected following an invasive procedure should always be examined critically,
since it may liberate fetal cells into the maternal blood leading to the
detection of iatrogenic cells which may not be present in undisturbed
pregnancies. Nevertheless, such results continue to be published
(Elias and Simpson 1994, Zheng et al 1995) and are all too often
interpreted as being representative of all pregnancies.

Trophoblast is an obvious candidate cell type which might be
retrieved from maternal blood (see section 1.4.1.6). Their use for
prenatal diagnosis and screening could potentially be limited by
mosaicism and difficulties applying FISH techniques to pyknotic
nuclei with condensed chromatin. However, in practice the single
most important limiting factor to the successful detection of
trophoblast in maternal blood has been the poor specificity of
antibodies to trophoblast cell surface markers. These antibodies may
in vivo identify only a subset of trophoblast cells (e.g.
syncytiotrophoblast) and in vitro label maternal as well as fetal cells.
This has been confounded by poor methods of identification of
trophoblast elements in blood and has led to the possibly erroneous
finding that trophoblast cells are extremely rare in peripheral
maternal blood.

Monoclonal antibodies directed against alkaline phosphatase
have been used (Covone et al 1984, Kozma et al 1986), and although
these were apparently specific for syncytiotrophoblast, FISH analysis
of the flow sorted cells revealed that most of them were of maternal
origin. This was later found to be the result of adsorption of the fetal
antigens onto maternal blood cells which led to confounding maternal
cell contamination (Bertero et al 1988). Other groups have failed to
demonstrate trophoblast cells in the peripheral maternal blood using
monoclonal antibodies (Pool et al 1987, Bertero et al 1988, Chua et al
In contrast, Mueller et al (1990) and Hawes et al (1994) have claimed success in isolating up to 100 syncytio and cytotrophoblast elements from peripheral maternal blood in the first trimester in almost all pregnancies studied using hybridoma generated monoclonal antibodies specific for trophoblast. The presence of fetal cells was confirmed using Y-specific PCR amplification. Such a technique confirms the presence of small numbers of fetal cells in the cells selected (if adequate controls to detect contamination have been undertaken), but it certainly does not imply that all, or even the majority of the cells isolated were of fetal origin. Detailed histological and immunocytochemical technology would be required to ascertain the true origin of each of the selected cells. Only small numbers of pregnancies have been analysed to date (27 in total). This number would undoubtedly have been larger if the antibodies had been made available to the general scientific community.

Bruch et al (1991), used a battery of monoclonal antibodies to flow sort fetal cells from maternal peripheral blood and while obtaining a number of cells observed that morphologically they appeared to be leukocytes and were only able to demonstrate PCR amplified Y-specific sequence in two of the sorted samples. This study illustrates the classical difficulty of morphological identification of trophoblasts and marked maternal cell contamination in the sorted samples when antibodies of poor specificity are used for sorting.

Nucleated erythroblasts are another promising candidate cell to isolate from maternal peripheral blood (see section 1.4.2.2). The potential destruction of fetal nucleated erythroblasts in cases of ABO or Rhesus incompatible mothers may limit their use in some pregnancies. Unlike trophoblast, maternal blood also contains a small population of nucleated erythroblasts, so that any method of selection for such cells will select maternal cells and fetal cells indiscriminately.
The use of CD71 antigen to select nucleated erythroblasts (Bianchi et al. 1990, Watchel et al. 1991, Price et al. 1991, Ganshirt-Ahlert et al. 1992) allowed the isolation of large numbers of cells, indeed so many that the contamination problem was very apparent when magnetic cell sorting was applied to maternal blood (Ganshirt-Ahlert et al. 1992). Other, more specific antibodies for nucleated erythroblasts were identified: CD36 (Bianchi et al. 1993) glycophorin A (Price et al. 1991 Bianchi et al. 1993) however the predictable contamination problems remained.

The technology required to identify, isolate and analyse fetal cells is now available. To achieve reliable prenatal diagnosis will require adequate controls, good experimental design (including patient sampling) defined isolation and detection techniques and a determination to push the current technology to its limits.

1.4 Candidate Fetal Cells

The cell types potentially available for prenatal screening and diagnosis are trophoblast and fetal blood elements.

1.4.1 Trophoblast

Trophoblast is an obvious candidate for a cell that might be found in the maternal blood. It is the interface tissue between mother and fetus and is continually bathed in maternal blood and would theoretically provide multinucleate syncyiotrophoblast cells for prenatal diagnosis. As with CVS, placental mosaicism is a
potential problem, and syncytiotrophoblast cells shed into blood would be postmitotic and may have pyknotic nuclei unsuitable for FISH analysis.

An understanding of the development and pathophysiology of the placenta is essential to identify the type(s) of trophoblast which may be present, how the quantity of trophoblast deportation may vary at different gestations, and the pathophysiology that may alter the quantity and type(s) of cell found in maternal blood.

1.4.1.1 Development of the Placenta

By 7-8 days after ovulation, the embryo is embedded in the superficial layers of the endometrium. At this time, the embryonic trophoblast begins to differentiate into syncytiotrophoblast and cytotrophoblast, with the syncytiotrophoblast forming the outermost layer, between the endometrium and the embryo. The syncytial tissues next project into the surrounding endometrium, in an invasive growth phase. The outer syncytiotrophoblast is derived from the cytotrophoblast, with DNA synthesis and mitotic activity confined to the cytotrophoblastic cells. The syncytiotrophoblast is a postmitotic, terminally differentiated tissue and is formed by a breaking down of the limiting membrane of the cytotrophoblast cells (Enders 1965). The syncytial cells produce hormones such as hCG, progesterone and oestrogen and have specialised populations which facilitate gas transfer across the placenta (Dempsey and Luse 1971, Fox and Agrofojo-Blanco 1974). Intact maternal capillaries can be traced to and from the syncytiotrophoblast, and within these areas the red cells occupy a series of irregularly shaped spaces (Harris et al 1966).
By the 9th day after ovulation, maternal endometrial vessels form communications with the numerous lacunae that develop within the syncytiotrophoblast, as a prelude to the placental circulation. At 11-12 days after ovulation, cells forming the extraembryonic mesoderm which is lined by cytotrophoblast begin to aggregate as precursors to chorionic villus formation. Ultrastructural studies demonstrate the syncytiotrophoblast invading the maternal vessels, with the syncytium having many microvilli and pinocytotic vesicles at its surface (Knoth et al 1972).

From the 14th to the 21st post ovulatory days, the columns of syncytiotrophoblast become radially orientated with a central core of cytotrophoblast, forming primary villus stems (Boyd 1970). By the 23rd day after fertilization, the villus stems begin to become vascularised, with vessels developing in the mesenchyme of the core and establish functional continuity with others differentiating in the body stalk and inner chorionic mesenchyme. The establishment of a cytotrophoblast shell allows rapid circumferential growth with continued formation of mesenchymal cores and vascularisation. The primary stem villi grow and divide to form secondary and tertiary stem villi, with eventual formation of the terminal villus tree. Figure 1 demonstrates the macroscopic appearance of first trimester placental tissue.

In terminal villi, the syncytial nuclei are irregularly distributed and often form aggregates which protrude into the intervillous space (Fox 1978) (see Figure 2). Ultrastructurally, the nuclei within these knots show features of senescence and appear to be shed into the maternal circulation, with their loss being made up by the division and differentiation of the cytotrophoblast (Fox 1978). As the number of terminal villi increases up to 60% in the third trimester, it follows that syncytial knot formation and their shedding
into the maternal circulation would increase with advancing gestation. The placental septa appear during the third month of gestation, which divide the maternal surface into 15-20 lobes. By the end of the fourth month, the placenta has achieved its definitive form with growth continuing through branching of the villus tree and formation of fresh villi. Figure 2 demonstrates the histology of a first trimester placenta.

Figure 1.

First trimester human trophoblast washed and immersed in saline.
Figure 2.

Light micrograph of human first trimester trophoblast stained with haematoxylin and eosin, magnification X50.

C - cytотrophoblast
S - syncytiotrophoblast
S K - syncytial knot
M C - mesenchymal core
M B - maternal blood space
1.4.1.2 Adaption of the Placental Vessels in Early Pregnancy

By the sixth week of gestation, cytotrophoblastic cells penetrate the trophoblastic shell, colonise the decidua and adjacent myometrium, and become known as extravillus cytotrophoblast. They also invade the lumines of the intradecidual part of the spiral arterioles in the placental bed. Here they form intraluminal plugs and constitute the intravascular extravillus cytotrophoblast. These cells destroy and replace the endothelium of the maternal vessels and invade the media, destroying the medial elastic and muscle tissue (Brosens 1967). The arterial wall becomes replaced by fibrinoid material, derived partly from maternal blood cells and partly from the invading trophoblast cells (De Wolf et al 1973). The process is completed by the end of the first trimester and is followed by a period with no further invasion of the vessels. At the 14th to 16th week of gestation there is a resurgence of endovascular trophoblastic migration with a second wave of cells moving into the myometrial segments of the spiral arteries (Robertson et al 1975, Pijnenborg et al 1983). Once there, the trophoblast cells continue replacing the endothelium, invading and destroying the elastic and muscular layers resulting in a 'non-contractile aneurysm'. These vessels can passively dilate to accommodate the increased blood volume, but cannot constrict in response to hormonal influences. The invading trophoblast plays a key role in establishing a low pressure, high conductance vascular system to ensure an uninterrupted supply of oxygen and nutrients for the developing fetus. The key elements of this process are shown diagrammatically in figure 3.
1.4.1.3 Pathophysiology of the Placenta

The importance of the remodelling of the maternal spiral arterioles by the invasion of cytотrophoblast has already been described. There is good evidence from haemodynamic studies (Johnson et al 1957, Dixon et al 1963, Trudinger et al 1985) that the blood supply to the fetoplacental unit is impaired in PIH and IUGR. These two conditions constitute a major source of morbidity for both mother and fetus in obstetrics today. PIH is estimated to affect 3-8% of pregnancies, and although only a small proportion of these will go on to develop serious problems, it is responsible for 25% of all antenatal admissions imposing a major burden on health service resources in terms of manpower and bed costs. The most recent report on confidential enquiries into maternal deaths has shown that hypertensive disorders of pregnancy are the main direct cause of maternal death in the United Kingdom, accounting for 18.6% of such deaths. It is classically diagnosed by the triad of findings; high blood pressure (greater than 90 diastolic), oedema and proteinuria, although serious problems may occur in women who do not exhibit all three findings. It is a multisystem disorder affecting the kidneys, liver, coagulation system and nervous system of the mother and can result in IUGR for the fetus and increased morbidity through prematurity.
Figure 3.
Placentation and pre-eclampsia at a) 6 weeks; B) 12 weeks; C) 18 weeks.
Trophoblast (T) from the anchoring villus (AV) invades the decidua (D) and the distal end of the spiral arteriole (SA). By 18 weeks the decidual and myometrial portions (M) of the arterioles have been invaded by cytotrophoblast and remodelled by enzymatic action. The resultant dilated, thin walled vessel allows an increased blood supply to the intervillus space (IVS). A chorionic villus is also shown (CV). In pre-eclampsia, this process fails to extend beyond the normal stage for 12 weeks so that the ability of the spiral artery to deliver an adequate blood supply is impaired.

IUGR can threaten the fetus in the absence of PIH and is diagnosed by serial ultrasound scans which show the fetal growth
dropping down through the centiles. Premature delivery may complicate IUGR and increases fetal morbidity and mortality.

The factors which control and limit intravascular invasion by the extravillus cytotrophoblast are unknown, but in women destined to develop PIH in the later stages of their pregnancy there is a failure of placental arterial adaptation which markedly restricts blood flow to the placenta. This consists of a failure to invade all the spiral arteries with a significant proportion of the placental bed arteries showing a complete absence of physiological change (Khong et al 1986, Meekins et al 1994). Also, although the first stage of the arterial invasion process occurs to some degree, with alteration of approximately half the interdecidual segments, there is minimal (18%) invasion of the intramyometrial portion of the arterioles (Robertson et al 1967, 1975, Meekins et al 1994). As a result of the incomplete transformation of the arterioles, the blood supply is restricted, which may account for the placental abnormalities and fetal complications seen in PIH.

Defective invasion of the spiral arterioles by extravillus cytotrophoblast is also a feature of many cases of IUGR in normotensive women, the IUGR being the result of reduced maternal supply of oxygen and nutrients (Robertson et al 1981). Acute atherosclerosis, with the deposition of fibrin, platelets and macrophages is also seen, which may partially or completely occlude the arterioles (Robertson et al 1967). The placental ischaemia resulting from atherosclerosis and incomplete placentation of the arterioles can result in hypoxia which is known to stimulate trophoblast proliferation and to form syncytial knots (Tominaga and Page 1966), and these changes are marked in PIH (Burstein et al 1957, Wentworth 1967).
1.4.1.4 Immunobiology of the Placenta

The placenta and in particular trophoblast presents the interface between mother and fetus, the conceptus being an allograft of paternal and maternal origin. Because of extensive genetic polymorphism, nearly all human pregnancies involve feto-maternal major histocompatibility complex (MHC) incompatibility. Therefore to avoid rejection, the trophoblast must have modified expression of MHC genes. It has been consistently established that villus cytotrophoblast and syncytiotrophoblast lack MHC class I and class II HLA expression (Faulk and McIntyre 1983, Bulmer and Johnson 1985, Hunt et al 1987). This is clearly an important mechanism which protects the conceptus from effective maternal immune recognition and cytotoxic cell attack. Trophoblast cells break away from the implantation site into the maternal circulation throughout pregnancy and lodge in the lung where most degrade (Thomas et al 1959, Attwood and Park 1961, Benirschke 1994). The degradation does not provoke any inflammatory or immunological rejection response. It has been postulated that the continuous deportation may induce immunological tolerance to fetal trophoblast antigens in the mother.

Although trophoblast does not express class I MHC antigens, extravillus cytotrophoblast populations do express an antigenically related molecule that is neither HLA-A or HLA-B antigen (Redman 1984). An HLA-G antigen has been identified on trophoblast, which is abundant on the cells during the first trimester and decreases in term placenta (Kovats et al 1990). HLA-G lacks the polymorphisms of HLA-A, B, and C and hence may not be recognised as foreign. These adaptations have made it difficult to raise monoclonal antibodies specific to trophoblast and has necessitated testing thousands of hybridomas in the search for such an antibody.
1.4.1.5 Possible Variations in Trophoblast Deportation at Different Gestations and in Abnormal Pregnancies

From the preceding review, the importance of trophoblast as the anatomical and immunological interface between mother and fetus is apparent. Two types of trophoblast may be expected to be retrieved from the maternal circulation, syncytial knots and invading cytotrophoblast, the latter being more likely in early pregnancy. The syncytial cells may be shed in increasing amounts as the pregnancy progresses and as their formation is stimulated by hypoxia, may be even more abundant in pregnancies with PIH or IUGR. The underlying cytotrophoblast may be shed with the syncytial cells providing an alternate source of cytotrophoblast in later pregnancy. Cytotrophoblast cells may fail to invade and remodel the placental bed in early pregnancy and so may be less numerous in pregnancies with PIH or IUGR. If the failure to remodel is not a lack of invasion, but a failure to adhere to the appropriate placental bed arteries, more cytotrophoblast may escape into the maternal circulation in pregnancies with PIH or IUGR. The two types of trophoblast could be distinguished with monoclonal antibodies, and a combination of monoclonal antibodies specific for both cell types may offer the best chance of retrieving any trophoblast in the peripheral circulation. Chua et al (1991) studied trophoblast deportation in the uterine vein in pregnancies with PIH and noted an increase in both cytotrophoblast and syncytiotrophoblast compared with normal pregnancies. There was no apparent correlation with the severity of the PIH although an acute event precipitating delivery was associated with increased deportation in all cases.
1.4.1.6 A Review of Trophoblast Retrieval

One hundred years ago, in 1893, a German pathologist named Schmorl identified multinucleate syncytiotrophoblast cells in the lung capillaries of fourteen out of seventeen women who had died from eclampsia, so the idea that cells from the conceptus pass into the maternal circulation is not a new one. Since then, syncytiotrophoblastic elements have been identified by many groups in the uterine veins during pregnancy (Douglas et al 1959, Thomas et al 1959, Attwood and Park 1961, Ikle 1961, Boyd and Hamilton 1970, Chua et al 1991) and trapping in the maternal lung has been observed (Jaameri et al 1965). A small number of cells have been identified in the peripheral circulation, both histologically (Goodfellow et al 1982) and with monoclonal antibodies and flow cytometry (Covone et al 1984, 1988, Kozma et al 1986).

This research has built up a picture of trophoblast being continually shed into the surrounding maternal blood, transported via the uterine veins and trapped in the maternal lungs, with very few cells reaching the peripheral veins where they are accessible for sampling and diagnosis. Even this may be a relatively optimistic scenario, since those identifying multinucleate cells histologically simply presumed them to be of trophoblastic origin. It is extremely difficult to identify trophoblast in an excess of blood cells by histological methods alone, and at least some of the cells may have been megakaryocytes, which though a rare blood cell, would still be expected to be more numerous than the sought-for syncytiotrophoblast cells.

Even monoclonal antibodies do not guarantee identification of the desired cell type. Covone and his group had very promising data
to present in 1988, when they used H315 and flow cytometry to isolate syncytiotrophoblast, but when the very few cells isolated from women with male fetuses were tested with Y-specific probes, no hybridisation was observed and they were found to be maternal cells which had adsorbed the fetal antigen onto their surface.

Mueller et al (1990) laboriously searched through 8000 candidate monoclonal antibodies generated by hybridoma technology to obtain one to sort trophoblast cells in early pregnancy. They were able to isolate between 10 and 1000 cells from each pregnancy which gave a Y-specific signal in the case of a male fetus when analysed with PCR. They also recorded false positive results, and as the antibody has not been released to other investigators the findings have not been confirmed.

More recently, the same antibody has been used to isolate syncytiotrophoblast cells in first and second trimester pregnancies from peripheral blood in 12 of 14 samples (Hawes et al 1994). Subsequent morphological evaluation showed that they were identical to syncytiotrophoblast sprouts, and PCR analysis in cases where the paternal and maternal mutations for B-thalassemia differed has detected the paternal allele (Hawes et al 1994a).

In 1991 Chua et al showed convincing data that trophoblast can be found in uterine vein and maternal peripheral blood. They studied women with PIH and identified the cells in blood smears with cytokeratin antibodies. Only very small numbers of multinucleate and mononuclear cells (11-25µm size) were found in uterine vein blood of all the women and in the peripheral blood of only one of five women tested. However, these results were obtained in late pregnancy, and the numbers and proportions of syncytio and cytotrophoblast cells may be different during the first trimester when prenatal diagnosis is performed.
Bruch et al (1991) used multiple monoclonal antibodies and flow cytometry to sort trophoblast like cells and could detect a Y-specific signal in two thirds of the samples with male fetuses. Two of the antibodies were known to cross react with blood elements, resulting in heavy contamination of the sorted cells with maternal cells. Cacheux et al (1992) refined this method and used negative selection prior to sorting with several antibodies and flow cytometry on the blood of a woman pregnant with a known XYY fetus. After examining 1400 cells, 4.25% showed one or two Y-specific domains when analysed with a Y-chromosome centromeric FISH probe. Although the blood samples were not taken until 28 weeks' gestation, the data imply that the method had achieved an enrichment level at which aneuploidies could be diagnosed.

1.4.2 Nucleated Fetal Red Cells

The cell type that has been used most successfully for prenatal diagnosis is the fetal nucleated red cell. Fetal blood elements are thought to "leak" into the maternal circulation following minor trauma inducing haemorrhages which are said to occur in all pregnancies and allow all elements of fetal blood to enter the maternal circulation. Fetal nucleated red blood cells comprise 90% of red cells in the 11 week fetus and 30% in the 24 week fetus, dropping to 5-10% at term (Ganshirt et al 1994). Since nucleated red cells are rare in adult blood, confusion with maternal cells, it was thought, should be minimal. In addition, nucleated erythroblasts have the advantage that they could be cultured in special media to provide a larger sample of a pure population of cells for diagnosis.
A theoretical concern in the use of erythroblasts is that Rh or ABO incompatibilities between the mother and fetus may result in lysis of the rare fetal cells. This may not be a major factor because fetal red blood cells contain fewer active A or B sites than adults cells and consequently are thought to be less antigenic and less specific than adult antigens.

Usually, the fetal nucleated erythrocyte condenses and extrudes its nucleus so that it loses its mitotic capability and restricts its life span to 90 days. This is of importance since sequestering into the bone marrow and derived clones would seem unlikely to pose problems with contamination of cells from previous pregnancies.

1.4.2.1 Early Development of the Fetus and Cardiovascular systems

Implantation takes place between the sixth and eighth day after fertilization, at the blastocyst stage. This is based on the ability to flush blastocysts from the uterine cavity five days after ovulation (Buster et al 1985). The inner cell mass then forms a bilaminar embryonic disc, defining the endoderm and ectoderm, with the amniotic cavity between them. The whole of the remainder of the conceptus goes on to form the fetal membranes. Between 14 and 16 days after fertilization, the trilaminar embryonic disc is formed by the interposition of the notocord and mesoderm between embryonic ectoderm and endoderm. The cells of the upper layer proliferate and migrate backwards and medially to form the primitive streak. In the first trimester, differentiation and organogenesis is completed and the second and third trimesters are characterised by growth of the fetus. The blood vascular system is initially formed from endothelial tubes.
which develop in the mesenchyme both within the embryo and extraembryonic membranes. The scattered endothelial tubes link up to form a primitive circulatory system in which the first peristalsis-like heart beats begin on day 21 after fertilization. Blood islets first appear in the yolk sac at 4 weeks of gestation, although some authorities suggest that haemopoietic stem cells originate in the embryo itself (Dourain 1982). In the first trimester, the fetal blood constituents differ from the adult and indeed the neonate. 90% of the red blood cells are nucleated in the fetus 9 weeks and 5-10% at term (Simpson and Elias 1994) and the granulocyte and lymphocyte populations form less than 2% of the nucleated blood cells until stimulated after birth.

1.4.2.2 A Review of Fetal Erythroblast Retrieval

Bianchi *et al* (1990) were the first to work on identifying and isolating fetal erythroblasts. By taking advantage of the presence of the transferrin receptor (CD71) on the cells and using flow cytometry, the sorted cells were shown with PCR to have a Y-specific sequence in 8 cases, 6 derived from pregnancies with a male fetus. CD71 antibodies also bind some monocytes and activated lymphocytes leading to considerable contamination with maternal cells. Wachtel *et al* (1991), and Price *et al* (1991), sorted cells with the addition of glycophorin-A receptor positivity (which has the advantage of being absent from lymphocytes) and were able to use PCR to correctly identify 12 of 12 samples with male fetuses and 5 of 6 samples with female fetuses. However, the timing of blood sampling for use in isolating fetal erythroblasts may be critical, with best results being
achieved in the first trimester. Bianchi et al (1991), reported an absence of Y-specific sequence amplified by PCR in sorted cells from women at 16, 19 and 20 weeks' gestation, who had previously given positive results and were known to be carrying male fetuses, implying that second and third trimester diagnosis with fetal erythroblasts may not be possible.

Ganshirt-Ahlert et al (1992), applied a magnetic cell sorting approach to erythroblast isolation, hoping to avoid the use of expensive and cumbersome cell sorters. Although the magnetic sorter achieved good results when sorting rare event cells, it was felt that the use of the tranferrin receptor alone did not provide reliable separation of fetal erythroblasts as there was considerable contamination with maternal cells. Price et al (1991), were able to detect trisomy 21 in cells sorted one week after CVS and trisomy 18 just prior to CVS, and Bianchi et al (1992), detected trisomy 21 at 19 weeks' gestation, two weeks after amniocentesis. These encouraging results, achieved with FISH technology on interphase cells sorted by flow cytometry have prompted the initiation of clinical trials in America to evaluate erythroblast isolation against the standards of amniocentesis and CVS for the prenatal diagnosis of aneuploides.

Despite all the encouraging results, there is a feeling throughout all the research of a rush to achieve the lucrative goal of prenatal diagnosis without answering basic questions in a logical and systematic way. Answering such questions as when cells appear and disappear and variations throughout and between normal pregnancies prior to clinical trials could avoid disappointment and allow problems to be anticipated and dealt with before they arise.
1.4.3 A Review of Lymphocyte Retrieval

Some of the first work suggesting the existence of fetal cells in the maternal blood, by Walknowska et al (1969), was performed on lymphocytes. Although this cell type is only of historical interest at present, with other cell types proving more reliable, some of the pioneering and most elegant work has been with lymphocytes. The work undertaken by Herzenberg et al (1979) has already been described and Yeoh and colleagues (1991) studied couples in whom the HLA types were known. By obtaining maternal blood at 28 and 32 weeks' gestation and amplifying paternal HLA-DR sequences from the unsorted blood, they were able to demonstrate the presence of fetal cells.

A potential problem with the use of lymphocytes for prenatal diagnosis is their longevity, with reports of survival in the circulation of up to 15 years. Bianchi et al (1994) flow sorted with antibodies which bind B cells, T lymphocytes and haematopoietic stem cells from eight women who had male babies between six months and 27 years earlier. They detected no Y sequence in the B cells, Y sequence in one of seven for T cells and six of eight stem cells. This suggests that the earlier the lineage of cells isolated by the antibodies, the greater the potential for contamination with cells from previous pregnancies. The stem cells may be capable of populating the maternal bone marrow, thus perpetuating cells of fetal lineage. It also sounds a general note of warning for all cell types and highlights the importance of understanding the biology of fetal cells in maternal blood. For accurate prenatal diagnosis, a terminally differentiated fetal cell type would avoid diagnostic confusion by cells from a prior pregnancy.
1.4.4 Granulocytes

One group, Wessman et al (1992), have reported the isolation of fetal granulocytes from maternal blood. They used a Ficoll gradient separation followed by FISH with a Y specific probe. Y-specific cells were recovered from eight women, seven of whom gave birth to male babies. In the case of the eighth woman, the Y signal was found in lymphocyte like cells which it is suggested persisted from a previous pregnancy. Their group found a surprisingly high number of fetal granulocytes, even prior to fetal cell enrichment, given that the percentage of granulocytes present in fetal blood is very small.

1.5 Isolation Procedures

The correct identification and isolation of fetal cells from the maternal peripheral blood, free of maternal contamination, is the goal to be achieved for accurate prenatal diagnosis. In practice, this is not easy because of the small number of fetal cells and the vast number of contaminating maternal cells. Estimates range from 1 in 50,000 to 1 in 10 million (Lo et al 1989, Lo et al 1990, Kao et al 1992, Hamada et al 1993, Wessman et al 1992, Wachtel et al 1991 Zheng et al 1993). Thus it is apparent that some degree of enrichment of the fetal cells will be required for accurate prenatal diagnosis. To date, studies have achieved enrichment, not purification, since fetal-specific antibodies which do not cross react with adult cells have not yet been demonstrated. A prenatal test using fetal cells from the maternal circulation will be constrained by the necessity of recovering as many
of the rare fetal cells as possible, while eliminating as many maternal cells as possible.

1.5.1 Density Centrifugation Gradients

An initial enrichment step used by many groups to eliminate maternal red cells is to centrifuge maternal blood through a ficoll/plasma interface, with Holzgreve et al (1992) developing a triple density technique for separating nucleated erythroblasts. The technique utilises the different density of cells in the blood which migrate at different speeds through a medium according to cell size, granularity and nuclear/cytoplasmic ratio with red cells settling to the bottom because of their high iron and haem content (see figure 21).

1.5.2 Monoclonal Antibodies

The ability to positively and negatively select cells using monoclonal antibodies which have been generated by hybridoma technology is the basis of all the current enrichment strategies.

Monoclonal antibodies, first isolated by Kohler and Milstein (1975), are powerful biological tools. The antibody (IgG) is a Y shaped molecule, in which the domains forming the tips of the arms bind to antigen, and those on the stem trigger effector functions that eliminate antigens. The domains carrying antigen binding activities (Fv, Fab fragments) or effector functions (Fc fragments) can be used separately as fragments or swapped between antibodies. Generating monoclonal antibodies involves the manipulation of the immune
system to increase the number of B cells expressing antigen-specific antibodies, immortalization of these antibody producing cells by cell fusion and identification of stable cell lines secreting an antibody with the desired properties by extensive screening. The experience of Covone et al (1984) serves as a warning to all those using them to sort rare cells since cells adsorbing surface antigens will also be sorted by binding to monoclonal antibodies.

1.5.3 Bacteriophage Technology

Recent advances in the field of antibody generation and selection have bypassed hybridoma technology, making antibody generation and selection much more rapid. The combination of the ability to produce and display functional antibody fragments in bacteria (McCafferty et al 1990), the isolation of antibody variable genes using PCR (Winter et al 1991), and powerful screening systems have been combined to produce monoclonal antibodies.

Antibodies can be stimulated by injection of whole cell antigen into mice. The extracted B cells can then be used to construct a phage-antibody library. This is achieved by using PCR to isolate whole repertoires of antibody variable genes (Winter et al 1991), which are amplified with specific primers (Clackson et al 1991, Marks et al 1991), and cloned into phage vectors which may represent between $10^6$ and $10^9$ different antigen binding specificities. Each recombinant phage genome contains the DNA encoding for the specific antibody displayed on its surface. The desired antibodies are selected by "panning" the phages with the original cells or in petri dishes coated with antigen. This antigen-directed selection is capable of picking out rare antigen-specific phage in huge populations for
example, where only 1 in $10^7$ phage in a population is specific for an antigen (Mc Cafferty et al 1990, Clackson et al 1991). The specific phage antibodies selected are then used to infect *E. coli* to give stable clones producing the desired antibody and liberating it into the culture medium from where it can be used directly for techniques such as ELISA. Phage technology can bring the power of a random approach to the process of restoring the original affinity by the analysis of more intermediates much more rapidly. Such a technique would seem ideally suited to the search for specific trophoblast antibodies.

1.5.4 FACS and Magnetic Cell Sorting

Over the years, researchers have developed different methods using monoclonal antibodies to enrich for fetal cells. Many groups have used flow cytometry, which is capable of using a number of parameters to sort cells. Sorting cells on the basis of cell size, nuclear/cytoplasmic ratio, cell granularity and labelling with fluorescently tagged antibodies has yielded small numbers of cells from the maternal circulation. From these cells, Y-specific sequence of presumed fetal origin has been amplified (Herzenberg et al 1979, Covone et al 1984, 1988, Kozma et al 1986, Bianchi et al 1990, 1991, 1992, 1994, Mueller et al 1990, Bruch et al 1991, Price et al 1991, Wachtel et al 1991, Cacheaux et al 1992, Wessman et al 1992). Fluorescence activated cell sorters are not available outside academic centres and are extremely costly to run and maintain and are not suitable for routine screening and prenatal diagnosis. Because of this, the cheaper and faster method of separating cells with immunomagnetic beads has been developed and used successfully to
identify trisomic fetal cells from maternal blood samples (Ganshirt-Ahlert et al 1993). The use of the MINIMACS developed by miltenyi uses very small magnetic spheres (0.05mm in diameter), which can easily be coated with any antibody or antibodies and attach to cells through antigen-antibody interactions. The presence of six or more of the spheres on a cell surface allows separation of the cell from non-labelled cells by passage through a simple magnetised wool column, to which the labelled cells adhere. When the magnet is removed, the attached cells can then be eluted and because the spheres are so small, the retrieved cells are suitable for culture and FISH analysis. The MACS system has been extremely effective in its isolation of fetal erythroblasts. When tested on cord blood, 81% of all nucleated cells in the positive fraction were erythroblasts (Ganshirt-Ahlert et al 1994). When tested on maternal blood, erythroblasts could be enriched at every gestation from six weeks and the numbers were not increased by a preceding invasive procedure. Ganshirt-Ahlert et al (1994) were also able to demonstrate in pre-eclamptic pregnancies and those with trisomic fetuses, the number of enriched erythroblasts was increased compared to normal pregnancies, with the percentage of trisomic cells between 10 and 17% of the enriched cells.

1.6 Analysis Procedures

Once fetal cells have been enriched, or in future, isolated from maternal peripheral blood, the cells must be accurately and rapidly analysed. This is now possible through the development of PCR and FISH.
1.6.1 PCR

PCR is ideally suited to the analysis of small amounts of DNA in an excess of contaminating DNA. It has been used successfully to identify DNA from single diploid (Arnheim et al 1990), and haploid (Williams et al 1993) cells. It allows the isolation of a specific sequence of DNA and selectively amplifies it in vitro to theoretically unlimited amounts. The use of fetal cells for prenatal diagnosis requires just such a technique and technical improvements continue to be made to enhance its specificity and sensitivity.

PCR is simple in concept and practice and is relatively inexpensive. The principle was first described by Khorana et al in 1974, and the practice by Mullis et al in 1986.

PCR involves the binding of a DNA template with specific oligonucleotide primers in the presence of deoxynucleotide triphosphates and thermostable Taq polymerase in an appropriate buffer. Repeated cycles of heating and cooling then allows logarithmic amplification of the target amplicon. This is possible because oligonucleotides pair specifically with complementary sequences in appropriate conditions and then separate at higher temperatures to allow repriming and amplification.

The specific DNA which is enzymatically amplified is selected by the sequence of the oligonucleotide primers. Their sequences are chosen to be complementary to opposite strands of the template DNA. After template denaturation, the primers anneal to their complimentary sequences and synthesis is initiated by Taq polymerase in a 5'-3' direction. Taq polymerase is a heat stable DNA polymerase which can survive incubation at 95°C which is required for DNA denaturation and has obviated the need to replenish the enzyme at the start of each cycle. In order to increase the specificity
of binding of the primers, the annealing and extension temperatures can be raised by destabilizing mismatch pairing. This further increases the eventual yield by minimising competition by non-specific products for enzyme, dNTPs and primers.

PCR is a highly sensitive technique, which has meant that purification of DNA prior to analysis is minimal and amniocytes and CVS fronds can be rapidly prepared for analysis. PCR is widely used in the diagnosis of human genetic disorders, being first described in sickle cell anaemia using RFLP analysis of fragments generated by PCR of the beta globin gene (Saiki et al 1985). Same day prenatal diagnosis is now possible for some genetic disorders due to the sensitivity and speed of PCR (Williams et al 1988). PCR has also been used for preimplantation diagnosis in cases of IVF at risk of an X-linked disorder (Handyside et al 1990, 1992). A major breakthrough in advancing the field of fetal cells in the maternal circulation was achieved in 1989 by Lo et al. They were able to amplify Y-specific sequence from unenriched maternal peripheral blood using nested primer PCR, which uses two pairs of primers, one pair situated just inside the other and used in separate reactions to increase the specificity of the reaction. This technique has been widely used to amplify Y-specific DNA from maternal blood and cells enriched from maternal blood and has been the basis of proving the presence of fetal cells in the maternal blood.

A major problem with PCR, particularly with its use for prenatal diagnosis, is contamination. The source is most commonly previously amplified material, which then makes an ideal template for amplification in future reactions. Extraneous contamination from other laboratory workers is also possible such that it has been suggested that when Y-specific sequences are sought, only female laboratory staff should handle the samples! Most groups have found
mass male redundancy unnecessary, and their continued presence in the laboratory brings benefits which outweigh the disadvantages.

Where a particular PCR is in daily usage and where single cell analysis is undertaken it is recommended that staff wear theatre clothes, including a mask and gown, and that reactions are set up in a sterile cabinet. Reaction preparation and analysis of products should occur in specific, separate areas and some groups have found the use of UV irradiation and incubation of reaction mixtures with restriction enzymes are efficient in eliminating most sources of contamination (Kwok et al 1989, Lo et al 1989, 1990, Sarkar et al 1990).

One genetic disorder whose prognosis has been greatly improved over the past decade is Rhesus disease. Rhesus D is responsible for most cases of haemolytic anaemia and although sensitisation has been minimised with the development of anti-D, a few cases of sensitisation still occur. Where a Rhesus negative mother has a partner who is heterozygous for Rhesus-D, the fetus could be either Rhesus negative or positive. If positive, the potential for sensitisation or haemolytic disease may ensue. Until recently, the fetal Rhesus status could only be ascertained prenatally by invasive procedures, but as the maternal and fetal genotype may be different, it is a genetic situation which is tailor made for non-invasive PCR diagnosis. With the cloning of the Rhesus-D gene and the demonstration that Rhesus-D negative individuals lack the gene (Le Van Kim et al 1992), the information was available to put theory into practice. Lo et al 1993, were able to detect the Rhesus-D gene in the peripheral blood of rhesus negative mothers using nested PCR and correctly identify the fetal phenotype as Rhesus-D positive in eight out of ten cases and as Rhesus-D negative in 8 of 11 cases. Although not precise enough for routine clinical use, the potential is apparent
and with technical advances, is surely the way forward in the diagnosis and management of Rhesus-D disease.

The detection of Mendelian traits in fetal cells isolated from maternal blood will require the isolation of a pure fetal cell population. In a very specific case, Camaschella et al (1990), studied total DNA obtained from maternal blood in three pregnancies at risk for fetal b-thalassemia/haemoglobin Lepore Boston. Haemoglobin Lepore Boston is a haemoglobinopathy caused by a hybrid db gene that is the result of a 7-kilobase deletion in the b-globin gene cluster. They used PCR to amplify haemoglobin Lepore Boston-specific DNA fragments from maternal blood of women whose partners carried the Lepore Boston gene and correctly identified the haemoglobinopathy in two fetuses. This diagnosis was only possible because the fetal genotype was different from the maternal genotype and would not be applicable to common disorders such as cystic fibrosis, where maternal and fetal genes may be abnormal.

The development of PCR has added to the potential of molecular techniques for prenatal diagnosis and transformed what was once impossible, and influenced our entire approach to genetics.

1.6.2 Quantitative PCR

It is clear that one of the strengths of PCR is its ability to amplify small amounts of DNA exponentially. This fact should also make the technique suitable for quantifying amounts of DNA. The art of quantitative PCR is devoted to minimising interference with the doubling of the target amplicon with each cycle. Quantitative power can be defined as the level of reproducibility, precision and accuracy.
achievable when quantitating the specified amount of nucleic acids.
The key factors which influence the quantitative ability of PCR are:
1. Optimisation of amplification.
   This may involve changes in nucleic acid preparation, primer usage,
   buffer usage and in cycling parameters.
2. Hot start
   The development of the hot start has improved sensitivity by up to
   1000 fold when starting from very few target molecules in the
   presence of 1 mg of genomic DNA (Mullis et al 1991). The target DNA
   is heated for longer to ensure complete denaturation and Taq
   polymerase is added at this high temperature to allow immediate
   annealing to the exposed target. This may disrupt the DNA molecules,
   leaving very few except the target molecules with enough sequence
   homology to bind the primers and thus be amplified. The hot start
   will also increase the reproducibility of the amplification and thus the
   quantification.
3. Linear range of amplification
   If a PCR reaction is 100% efficient, there will be a doubling of the
   target DNA with each cycle. In practice, a typical amplification runs
   at 70-80% efficiency from the 15th to the 30th cycle (Wang et al
   1989). Indeed, the amplification is exponential for a limited number
   of cycles, after which the amplification rate reaches a plateau. Factors
   which contribute to this plateau include saturation of enzyme,
   product strand reannealing and incomplete product strand separation.
   The ultimate goal is to identify the linear range of the reaction in
   which the quantitated amount of amplified target is proportional to
   the initial amount of target molecule. This will depend on the
   number of cycles, the number of target molecules in the starting
   material and on the system of detection and quantitation of the
   amplified product.
In most cases, control or standard samples are used to evaluate the linear range and are run routinely with the unknown reactions and used to construct standard curves.

The method of detection and quantitation is also of importance in determining reproducibility in quantitation with radioactive and fluorescence labels most commonly used. The trend has been towards direct labelling of the amplicons instead of probe detection, because it is easier to run and has greater quantitative power as it allows a more direct reading of the amplicons accumulation. The primers may be end labelled or labelled dNTPs may be used for incorporation into the product.

Analysis techniques will often be governed by what is available, since some approaches require expensive equipment and chemicals, such as systems which include radioimaging, phosphorimaging and fluoroimaging.

Autoradiography is developed by chemical processing and a densitometer is used to quantify image information. The film used to detect the radioactive particles lacks sensitivity and has a nonlinear dynamic range. Less than 5% of B particles emitted by a $^{32}\text{P}$ labelled sample interact with the emulsion of a standard X-ray film and for quantitative purposes the dynamic range of film is limited to 300:1. The density versus log exposure response curve of the film is sigmoid and unreliable at extremes of exposure. The phosphorimager analyses phosphorimage plates which are composed of fine crystals of BaFBr:Eu$^{+2}$ in an organic binder (Amemiya et al 1988). High energy radiation will excite an electron of the Eu$^{+2}$ ion which is trapped by the BaFBr complex with a resultant oxidation of Eu$^{+2}$ to Eu$^{+3}$. By exposing the excited complex to light from a helium-neon laser, the electrons are liberated, reducing Eu$^{+3}$ to Eu$^{+2}$. Eu$^{+2}$ then releases a photon as it returns to ground state. The intensity of this
luminescence is measured and stored digitally in relation to the position of a scanning laser beam, the result is a quantitative representation of the latent image formed on the storage phosphor plate by the original incident radiation (Johnston et al 1990). The dynamic range is linear over a range of 100,000:1 compared with a nonlinear range of 300:1 for film. The sensitivity of the imaging plates for $^{32}$P is 250 times greater than X-ray film and the storage phosphor process is 60-100 times more sensitive than direct autoradiography on film (Johnston et al 1990).

Provided the PCR assay that is to be used quantitatively is optimised, its limits assessed in terms of precision and reproducibility, and suitable standards are used, PCR can be a powerful quantitative tool.

1.6.3 FISH (fluorescence in situ hybridization)

The other technique which makes prenatal diagnosis with fetal cells possible is the development and refinement of FISH.

FISH is a highly sensitive method for the detection of specific nucleic acid sequences in specimens fixed to a microscope slide. It is capable of determining and localising the specific sequences in a morphological context and in so doing, provide quantitative data. In simplistic terms, denaturation of the nuclear DNA allows hybridization with a specific probe (usually a cosmid), which has been fluorescently labelled. Annealing under appropriate conditions allows binding of the probe to specific complimentary DNA sequences. Excess or mismatched probe can be washed off and the nucleus counterstained to define the position of the fluorescence within the
nucleus, when viewed under appropriate fluorescence filter microscopy.

Nuclei in metaphase or interphase can be labelled in this way, allowing direct examination of cells without prior culturing. Prehybridization can be used to reduce binding of repetitive sequences in the labelled probe to target sequences. Probe labelling is most commonly through indirect schemes using biotin or digoxigenin-labelled dNTP's. These are incorporated into the DNA by nick translation, random primed reaction, or PCR. These reporter molecules are fluorescently labelled after hybridization with immunocytochemical steps and can be amplified through subsequent incubation with biotinylated anti-avidin antibody and fluorochrome-conjugated avidin. Fluorochromes can be combined to obtain different colour labelling with different probes within the same nucleus.

The observed sensitivity and specificity of FISH analyses are a function of several parameters including the cell type, probe design, method of sample processing and hybridization efficiency. The responsiveness to hybridization reflects chromatin condensation and in pyknotic cells where the chromatin is highly condensed, minimal hybridization may be seen requiring adjustments in protocol for different cell types. This may influence the percentage of cells that hybridize and more importantly, whether the hybridization pattern reflects the correct genotype. Probe type also affects the hybridization efficiency and signal size. The use of multiple overlapping cosmids (contigs) as probes has given much brighter discreet signals and the development of chromosome painting where an entire target chromosome is labelled gives an extended diffuse hybridization signal, often producing overlapping chromosome domains. The accurate detection of trisomies requires a ratio of
approximately 1 fetal cell to 100 maternal cells, with the hybridization protocol optimised for the fetal and not the contaminating maternal cells. A discreet, rather than a diffuse signal is advantageous when determining aneuploidies in interphase nuclei and probes should be selected accordingly.

FISH is now routinely used for prenatal diagnosis of translocations on fetal material obtained by invasive techniques and has been successfully used for preimplantation diagnosis where very few cells were available for diagnosis (Griffin et al 1992, Chong et al 1993, Delhanty et al 1993).

1.7 Proposed Research

The research undertaken was designed to answer basic questions which remained outstanding from the current literature. When do fetal cells first appear in the maternal peripheral blood? Do they appear at the same time in different pregnancies? Are they present throughout pregnancy? Can they be used for accurate antenatal diagnosis? When do they disappear from the maternal peripheral blood?

In order to answer these questions, a system of PCR amplification of Y-chromosome specific DNA will be devised using the methods described by Lo et al (1989). This will be tested for reliability on clinical samples obtained from women undergoing CVS and results of fetal sex compared. A prospective trial will be instigated involving women who become pregnant through assisted conception. Serial unenriched blood samples from these women collected from four weeks' gestation until eight weeks after delivery
will be analysed for Y-chromosome specific DNA sequences using PCR to determine the presence or absence of fetal DNA at different gestations.

The use of quantitative PCR will be investigated to quantify fetal DNA at different gestations and correlated with pregnancy pathology.

In collaboration with Cambridge Antibody Technology, using phage display antibody technology, antibodies to first trimester trophoblast will be generated. These antibodies will be tested for their specificity, attached to immunomagnetic beads and used to separate putative trophoblast cells from maternal blood cells. Their fetal origin will be determined by the application of dual fluorescence FISH with a contig kit which will identify X and Y chromosomes in interphase nuclei.

Finally, isolation of a fetal nucleus identified by dual X and Y fluorescence FISH will be attempted with a view to performing single gene analysis on a single cell genome using PCR.
CHAPTER 2
MATERIALS AND METHODS

2.1 Materials

2.1.1 Reagents and Suppliers

All reagents apart from those listed were Analar grade supplied by BDH Ltd.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethidium bromide</td>
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</tr>
<tr>
<td>TEMED</td>
<td></td>
</tr>
<tr>
<td>APS</td>
<td></td>
</tr>
<tr>
<td>Melting point bath oil</td>
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<td>Orange G</td>
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<td>Bromophenol blue</td>
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<td>BSA</td>
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<td>AEC</td>
<td></td>
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<td>Calcium chloride</td>
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<td>DAPI/antifade</td>
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<tr>
<td>DMSO</td>
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</tr>
<tr>
<td>EDTA</td>
<td></td>
</tr>
<tr>
<td>Goat α-rabbit FITC</td>
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<td>Harris Haematoxylin</td>
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<tr>
<td>H₂O₂</td>
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<tr>
<td>Rabbit α-mouse FITC</td>
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<td>Pharmacia LKB Biotechnology</td>
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<td>Ficoll-Paque</td>
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<td>Accugel 40</td>
<td>National Diagnostics</td>
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<td>HMP Agarose</td>
<td>BRL</td>
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<tr>
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<td>øX174</td>
<td>Promega</td>
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<td>FCS</td>
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<td>Formamide</td>
<td>International Biotechnologies Inc.</td>
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<td>Avidin-Texas red</td>
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<td>Rabbit α-FITC</td>
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<td>Dual-colour painting kit</td>
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<td>Nail varnish</td>
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<td>Whatman Ltd</td>
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<td>Polaroid, U.K.</td>
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<td>Phosphorimage plates</td>
<td>Molecular Dynamics</td>
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<td>Series 400 Phosphorimage analyser</td>
<td></td>
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<td>Phosphorimage analysis</td>
<td>Imagequant Software</td>
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<td>Sandoglobulin</td>
<td>Sandos</td>
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<tr>
<td>EGFR1 antibody</td>
<td>ICRF</td>
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<tr>
<td>EGFR 425 antibody</td>
<td>Merck</td>
</tr>
<tr>
<td>Goat α-mouse FITC</td>
<td>Dako</td>
</tr>
<tr>
<td>Immunohistochemistry detection kit (VECTAStain)</td>
<td>Vector Labs</td>
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### Reagent

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<td>Premier Beverages</td>
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<tr>
<td>Magnetic microbeads</td>
<td>Miltenyi Biotech</td>
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<tr>
<td>α-mouse IgG</td>
<td></td>
</tr>
<tr>
<td>Human fibroblast cell line</td>
<td>ATTC</td>
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#### 2.1.2 Enzymes

- *Hinf I*
- *Hind III*
- *Taq polymerase*
- Proteinase K
- RNase
- Trypsin/EDTA

#### 2.1.3 Oligonucleotides

Oligonucleotides used as primers were obtained from Oswel DNA Service (Edinburgh) and Genosys (Cambridge). For the sequence details of the primers used see tables 1, 2 and 3.
2.1.4 Radioisotopes

[γ–³³P] dATP (>1000 Ci/mmol) was from Amersham International plc.

2.1.5 Buffers

Electrophoresis buffers

<table>
<thead>
<tr>
<th>Buffer Type</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-acetate (E-Buffer)</td>
<td>40 mM Tris-acetate, 1 mM EDTA, pH 7.0</td>
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<tr>
<td>Tris-borate (TBE)</td>
<td>89 mM Tris-Borate, 1 mM EDTA, pH 8.3</td>
</tr>
<tr>
<td>Agarose loading gel buffer (6x)</td>
<td>0.25% Orange G, 55% glycerol, in TE</td>
</tr>
<tr>
<td>Acrylamide gel loading buffer (6x)</td>
<td>0.25% bromophenol blue, 0.25% xylene cyanol FF, 15% Ficoll 400 in water</td>
</tr>
<tr>
<td>Lysis buffer</td>
<td>0.32 M sucrose, 10 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, 1% Triton X-100</td>
</tr>
<tr>
<td>20xSSC</td>
<td>3M NaCl, 0.3 M tri-sodium citrate, pH 7.0</td>
</tr>
</tbody>
</table>
2.1.6 PCR Equipment and Materials

All PCR reactions were carried out in a 48 well DNA thermocycler purchased from Perkin-Elmer/Cetus company. 0.6 ml PCR tubes used in the thermocycler were purchased from Robbins Scientific Corp. Sunnyvale, CA.

New oligonucleotides used as primers were optimised for magnesium chloride and potassium chloride concentrations and pH using an Opti-prime optimisation kit, supplied by Stratagene.

2.2 PCR Methodology

2.2.1 General Method for PCR

PCR reactions were performed with the following solutions: -

10x PCR buffer: 100 mM Tris-HCl (pH 8.3),
500 mM KCl,
15 mM MgCl$_2$,
0.1% gelatine,
2 mM dNTP's

All primers were used at concentrations of 20 pmol per primer. To reduce the risk of contamination, 10x dNTP's, 10x PCR buffer and diluted primer stocks were divided into small volumes and stored at -20°C. For the incorporation of radioactivity, $^{33}$P labelled dATP at 0.25 µl per reaction was used with 2 mM dCTP, dGTP and dTTP.
2.2.2 Single Cell PCR

For single cell analysis or analysis of patient samples, optimised first round PCR reactions contained;

- 10mM Tris/HCl (pH 8.3)
- 1.5 mM MgCl₂
- 0.01% gelatine
- 5 nmoles dNTPs
- 4 pmoles Y1.5
- 4 pmoles Y1.6
- 4 pmoles 10.1
- 4 pmoles 10.2
- 4 pmoles AMXY-2F
- 4 pmoles AMXY-CR
- 1 U Taq polymerase

in a final volume of 50 µl.

Second round cocktails were prepared in the same way as first round cocktails and included a new blank and a new positive DNA control. AF508 and AMG/AMGL loci were re-amplified independently.

Water for PCR reactions was autoclaved and filtered through a Sartorius mini-sort syringe filter prior to use. All aliquots and PCR cocktails were prepared in a laminar flow hood, with the handler wearing disposable gloves and a gown.

PCR cocktails contained PCR buffer, dNTP's, primers, water and DNA digesting enzymes where appropriate (everything excluding the DNA and Taq polymerase) in the required volume for the total number of reactions in a given experiment. The cocktail was then transferred in small aliquots in a laminar flow hood into PCR reaction tubes and overlayed with one drop of mineral oil. The tubes were then heated in the thermocycler to 95°C for 10 min prior to the addition of the genomic DNA and 1 U of Taq polymerase to each tube.
This served the dual purpose of denaturing the restriction enzyme and preventing pre-PCR mispriming by providing a 'hot start' (Chou et al. 1992). Optimized thermocycling conditions for specific primers are given along with the primer sequences in tables 1, 2, and 3.
Table 1.  
Primer sequences, restriction enzymes and thermocycling conditions for Y-sequence DNA amplification.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Fragment size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Y-specific sexing</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Outer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Y1.5</td>
<td>CTAGACGGCAGAGGCGGCAT</td>
<td>239</td>
</tr>
<tr>
<td>Y1.6</td>
<td>TAGTACCACGCCTGCTCCGG</td>
<td></td>
</tr>
<tr>
<td>Round 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>95°C:1 min; 57°C:1 min; 72°C:1 min x30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preincubate PCR cocktail with 30 U/100 μl of Hinf I for 1 hr.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Denature enzyme by heating to 95°C for 10 min prior to adding genomic DNA and Taq polymerase.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inner</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Y1.7</td>
<td>CATCAGAGCGTCCCTGGCTT</td>
<td>198</td>
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<td>Y1.8</td>
<td>CTTTCCACAGCCACATTTIGTC</td>
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</tr>
<tr>
<td>Round 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>95°C:1 min; 60°C:1 min; 72°C:1 min x30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preincubate PCR cocktail with 30 U/100 μl of Hinf I for 1 hr.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Denature enzyme by heating to 95°C for 10 min prior to adding genomic DNA and Taq polymerase.</td>
<td></td>
<td></td>
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</table>
Table 2.
Primer sequences, restriction enzymes and thermocycling conditions used to amplify AMG and ΔF508 DNA for the first round of nested PCR.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5'-3')</th>
<th>Fragment size (bp)</th>
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<tbody>
<tr>
<td>Outer</td>
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<td></td>
</tr>
<tr>
<td>Amelogenin</td>
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<td></td>
</tr>
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<td>AMXY-2F</td>
<td>CTGGGCTCTGTAAAGAATAG</td>
<td>X 891 (Nakahori et al. 1991)</td>
</tr>
<tr>
<td>AMXY-CR</td>
<td>GTCATGGGGCTCCCTCAAGGCT</td>
<td>Y 705</td>
</tr>
<tr>
<td>ΔF508</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10.1</td>
<td>GGAGCAAGGTGAATCCTGAGCG</td>
<td>normal 352</td>
</tr>
<tr>
<td>10.2</td>
<td>CACAGTAGCTTACCATAGAGG</td>
<td>mutant 349</td>
</tr>
</tbody>
</table>

Round 1
96°C: 10 sec; 56°C: 1.5 min; 73°C: 1.5 min x30
Preincubate PCR cocktail with 30 U/100 μl of Hind III for 1 hr.
Denature enzyme by heating to 95°C for 10 min prior to adding genomic DNA and Taq polymerase.
Table 3.
Primer sequences, restriction enzymes and thermocycling conditions used to amplify AMG and ΔF508 DNA for the second round of nested PCR.

All optimized re-amplification reactions for the ΔF508 and AMG/AMGL loci were performed independently, due to differences in optimal thermocycling conditions for each primer pair.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Fragment size</th>
</tr>
</thead>
<tbody>
<tr>
<td>inner</td>
<td>(5'-3')</td>
<td>(bp)</td>
</tr>
<tr>
<td>AMXY-F</td>
<td>GGTGCAAAACACTGTCCrCA</td>
<td>X 433</td>
</tr>
<tr>
<td>AMXY-NR</td>
<td>ATGAGGAAAACCAGGGTTCCA</td>
<td>Y 253</td>
</tr>
</tbody>
</table>

Round 2
96°C: 25 sec; 60°C: 45 sec; 72°C:1 min  x30
Preincubate PCR cocktail with 30 U/100 μl of Hind III for 1hr.
Denature enzyme by heating to 95°C for 10 min prior to adding genomic DNA and Taq polymerase.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Fragment size</th>
</tr>
</thead>
<tbody>
<tr>
<td>inner</td>
<td>(5'-3')</td>
<td>(bp)</td>
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<tr>
<td>ΔF508</td>
<td>CCTGGATTATGCTGGCACC</td>
<td>normal 98</td>
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<tr>
<td>10.3</td>
<td>GTTGGCATGCTTTGATGACGC</td>
<td>mutant 95</td>
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</table>

Round 2
96°C: 25 sec; 57°C: 45 sec; 72°C:1 min  x30
Preincubate PCR cocktail with 30 U/100 μl of Hind III for 1hr.
Denature enzyme by heating to 95°C for 10 min prior to adding genomic DNA and Taq polymerase.
2.2.3 Electrophoretic Resolution of DNA

Genomic DNA fragments were separated in horizontal 2% or 4% agarose gels 0.5-1 cm thick. 5-10 µl of PCR product with 1 µl of orange G was loaded onto an appropriate gel. The gels were prepared with and run in E-buffer. Electrophoresis times and voltages used were determined by the resolution required and the length of the gel. The size of the resolved fragments was estimated by comparison to the known standard 1Kb ladder which was electrophoresed alongside the test samples. The gels were soaked for 10 min in a solution of E-buffer and 5 µg/100 ml of ethidium bromide solution (10 µg/ml). DNA was visualised under UV light and a polaroid photograph taken to record the results.

Small PCR products, less than 100 bp, were resolved by vertical polyacrylamide gel electrophoresis. 12% acrylamide gels in TBE were used throughout. The gels were polymerised by the addition of ammonium persulphate and TEMED, each at a final concentration of 0.1%. 10 cm plates were used throughout, and all gels were run in 1x TBE buffer at 25 mAmp per plate, at constant voltage, for 2.5 hr at room temperature. DNA was stained with 10 µg/ml ethidium bromide, visualised under UV light and a polaroid photograph taken to record the results.

2.2.4 Phosphorimage Analysis of DNA

DNA amplified with radioactively labelled \(^{33}P\) dATP was run out on a horizontal 2% agarose gel as above. The gel was then transferred onto Whatman 3 MM chromatography paper, overlayed with clingfilm, and dried under vacuum at 80°C in an ATTO rapidry
gel dryer for 2 hr. The time taken for the gel to dry depended upon the thickness of the gel, and it was imperative that the gel was completely dry before it was placed on the phosphorimager plate to avoid damaging the plates. The gels were analysed after 12 hr using the imagequant software package to determine pixel volumes.

The PCR products were analysed and quantified on the series 400 phosphorimager, using imagequant software. The baseline was calculated for each sample on the agarose gel where no PCR products were visualised and the product bands were outlined using the standard rectangle image. The pixel volume was calculated by integration of the rectangular area, and the ratios of the bands calculated.

2.3 Quantitation of Nucleic Acids

The concentration of nucleic acid solutions was determined using a Shimadzu scanning spectrophotometer. Calculations were based on the assumption that an A260 of 1 OD corresponds to a concentration of approximately 50 \( \mu \text{g/ml} \) for double stranded DNA and 40 \( \mu \text{g/ml} \) for single stranded DNA. Serial dilutions were prepared in the standard way from the quantified DNA.

2.4 Isolation of DNA
2.4.1 Isolation of DNA from Whole Blood

Blood was collected in vacutainer sodium citrate tubes and stored at -20°C. After thawing blood at room temperature, 10 ml was added to pre-cooled lysis buffer, left for 30 min on ice and
centrifuged at 10,000 rpm for 10 min at 4°C. The cell pellet was resuspended in 4.5 ml of a solution containing 75 mM NaCl/24 mM EDTA (pH 8.0) by gentle aspiration. 0.5 ml of a solution containing 2 mg/ml proteinase K was then added and the resuspended cell pellet incubated at 37°C for 3 hrs (or at 55°C overnight). The digested sample was purified by phenol extraction followed by phenol/chloroform extraction and a final chloroform extraction. Following extraction of protein the DNA was precipitated with ethanol by adding 1/10th volume 3M sodium acetate and 2 vols of ice cold absolute ethanol, with gentle inverting of the tube. The precipitate was hooked out on a glass rod, air dried and dissolved in 1 ml of sterile water.

2.4.1.1 Isolation of DNA from Patient Blood Samples with Decontamination Precautions

For the preparation of DNA from patient blood samples, 10 ml of blood was collected in vacutainer sodium citrate tubes and stored at -20°C. A DNA extraction kit (Scotlab) was used as described in the kit protocol. All manipulations were carried out in a laminar flow hood, geographically separate from the lab areas used to set up PCR reactions or to run the products of PCR reactions.

2.4.2 Isolation of DNA from First Trimester Placentas

First trimester placentas were collected following vacuum aspiration of the uterine contents at a termination operating list. The placental tissue was sorted from the fetal tissue and chorionic villi identified while floating in sterile normal saline. The tissue was then
washed by agitating in the normal saline and the tissue transferred to a petri dish in a laminar flow hood in a biohazard lab. The tissue was washed in PBS. Chorionic villi were identified macroscopically and any amnion or blood clot was removed and discarded (see figure 1). The villi were roughly dissected using two scalpel blades and the resulting suspension was passed in a syringe through progressively smaller needles, guages 19 to 23, and the volume made up to 25 ml with PBS. 100 µl of the resultant cell suspension was transferred into a 1.5 ml Eppendorf tube and mixed with 800 µl of a solution of 170 mM NH₄Cl by rotating for 20 min. The solution was then spun for 30 sec at 14,000 rpm and the cell pellet resuspended and washed in 500 µl of 10mM NaCl/10mM EDTA. The sample was then centrifuged at 14,000 rpm for 30 sec and the cell pellet resuspended by vortexing in 500 µl of 20 mM NaOH and heated to 95°C for 15 min. Samples were pulse centrifuged to remove any cellular debris and 2.5 µl used for PCR amplification.

2.4.3 Isolation of a single FISHed nucleus

A slide of nuclei which had been subjected to FISH analysis was used to remove a single nucleus for PCR analysis. The coverslip was removed with a pair of forceps and the slide viewed under an inverted microscope at 1000x magnification under epifluorescent filters and a nucleus selected as being of male or female origin according to the fluorescent signal seen (dual FISH method described in section 2.6, see figure 26). The fluorescent filter was then changed to show the nuclei with DAPI staining, since they were easier to visualise. A 10μm diameter closed glass needle (kindly made by Shinya of St. Mary's hospital medical school) mounted on a micro-
manipulator was maneuvered next to the chosen nucleus and lifted from the glass slide onto the needle tip by advancing the needle between the slide and the nucleus. The nucleus could then be viewed under a DAPI filter, adherent to the needle tip and examination of the slide revealed that the nucleus was no longer present on the slide. This technique is extremely time consuming (2-3 hrs to remove 10 nuclei) and requires practice to avoid partial removal of the nucleus or inadvertently making contact with other nuclei on the slide. For this reason, areas sparsely seeded with nuclei were chosen to remove the selected nucleus where possible. When a whole single nucleus was transferred to a needle tip, the needle was transferred directly into the PCR cocktail, and the tip broken off. The PCR mixture, including the needle tip and nucleus was heated to 95°C for 20 min to denature the DNA. *Taq* polymerase was then added to the cocktail and the PCR cycling reaction started.

### 2.5 Preparation of Cells for FISH Analysis

#### 2.5.1 Preparation of Leukocytes from Peripheral Blood

10 ml of fresh blood was taken and diluted 1:1 with PBS. The resultant mix was layered onto ficoll at 4°C in a 50 ml Nunc tube and spun at 2000 rpm for 20 min. The buffy coat, containing the mononuclear blood cells situated at the interface between the PBS and ficoll, was removed with a pipette (approx. 10 ml) and diluted up to 50 ml with PBS (see figure 21). The cells were pelleted by centrifuging at 1000 rpm for 3 min and resuspended in 0.075M KCl for 10 min at 37°C. 10 drops of a 3:1 methanol:acetic acid mix were added to the suspension and the cells pelleted by centrifuging at 1000 rpm for 3 min. The cells were resuspended in 20-30 drops of
the methanol/acetic acid, and 1 drop of the suspension was pipetted onto a methanol cleaned, pre-heated glass slide. The slides were then allowed to air dry and the cells dehydrated by immersion in an ice cold ethanol series, 70%, 70%, 90%, 90%, 100% for 3 min each step and the slides left to dry overnight in an oven at 42°C.

2.5.2 Preparation of Trophoblast Cells

First trimester placentas were collected following vacuum aspiration of the uterine contents at a termination operating list. The placental tissue was sorted from the fetal tissue and chorionic villi identified while floating in sterile normal saline. The tissue was then washed by agitating in normal saline and the tissue transferred to a Petri dish in a laminar flow hood in a biohazard lab. The tissue was washed in PBS. Chorionic villi were identified macroscopically and any amnion or blood clot was removed and discarded. The villi were roughly dissected using two scalpel blades and the resulting suspension was passed in a syringe through progressively smaller needles, gauges 19 to 23 and the volume made up to 25 ml with PBS. The resultant suspension was layered onto 25 ml of ficoll at 4°C, and spun at 2000 rpm for 20 min. The interface layer between the ficoll and PBS (approx. 20 ml) was removed and the cells washed three times in PBS. The cells were treated with 0.075M KCl for 10 min at 37°C and slides were prepared as described in the protocol for preparation of cells from peripheral blood.

2.5.3 Enrichment of trophoblast from blood on a ficoll gradient
Whole blood and trophoblast as prepared in section 2.5.2 were mixed in known proportions with PBS and layered onto 25ml of ficoll at 4°C, and spun at 2000 rpm for 20 min. The interface layer between the ficoll and PBS (approx. 20 ml) was removed and the cells washed three times in PBS (see figure 21). The cells were boiled at 95°C for 10 min to release the DNA and used for PCR analysis.

2.6 FISH Hybridization Protocol

Cells were hybridized to the desired chromosome paints or probes as directed in the Cambio protocol. The key steps in the protocol are as follows:

15 µl of biotin-labelled paint and 15 µl of FITC-labelled paint were mixed per large slide or 5 µl of each per Cytospin slide and denatured at 65°C for 10 min, then transferred to a water bath at 37°C and incubated for 15 min (to 1hr). The nuclei were denatured by incubation for exactly 90 sec (fresh slides), or 2 min for older slides at exactly 75°C, overlayed with the hybridization mix, sealed and incubated overnight at 37°C.

Dual detection of the hybridised probes was achieved using a Cambio dual colour painting kit as described in their protocol. The slides were viewed at 1000X magnification with a fluorescence microscope carrying an epifluorescence filter for FITC, texas red and DAPI (Leitz) (see figure 26).

2.7 Preparation of Cells for Cytospin Analysis
Cells prepared from peripheral blood, trophoblast, or mixtures of the two were attached to glass slides for antibody or histochemical staining by Cytospinning (Cytospin Centrifuge, Shandon) at 750 rpm for 20 min onto glass slides cleaned with ethanol. Each Cytospin slide accommodated up to 500 μl of sample.

2.8 Cell Counting Techniques

Many of the experiments devised required known numbers of cells to be used as an initial step. The following standard technique was used to calculate cell numbers for FISH, PCR, cytopinning, FAC sorting, magnetic cell sorting, artificial mixes of different cell types, and artificial mixes of male and female cells.

The cell suspension to be counted was applied to a standard haemocytometer grid, viewed under a phase-contrast microscope and the number of cells in one large square (9 small squares) counted. This represented the number of cells x10^4/ml, and dilutions of cells were prepared on this basis. Where complex cell mixtures were counted, such as the trophoblast preparation, syncytial cells were counted as a single cell. Cell debris was not phase-bright and was not included in the cell count.

2.9 Magnetic Cell Sorting

Blood or trophoblast cells were prepared by layering on a ficoll gradient as described previously in sections 2.5.2 and 2.5.2), and the
cells resuspended in 1:1 PBS/BSA and incubated on ice for 20 min with 5 mg/ml Sandoglobulin. After washing in PBS the pellet was resuspended in PBS/BSA and incubated with EGFR antibodies (diluted 1:100) on ice for 20 min. The cells were again washed and resuspended in PBS/BSA and mixed at 4°C for 20 mins with magnetic microbeads conjugated to goat anti-mouse immunoglobulin to a dilution of 1:40. The cells were then washed and resuspended in PBS/BSA and passed through a MINIMACS column, attached to a magnet as described in the manufacturers protocol. The eluted mixture was collected as the negative fraction. The column was then removed from the magnet, topped up with PBS/BSA and the positive, labelled fraction eluted by pushing the solution through a column using a plunger.

2.10 Fluorescence Activated Cell Sorting

Blood or trophoblast cells were prepared by layering on a ficoll gradient as described above and washed in five volumes of PBS-CMF. The resulting pellet was resuspended in PBS/BSA and incubated on ice for 20 min with 0.5 mg/ml Sandoglobulin. The cells were resuspended in PBS/BSA and incubated with appropriate EGFR antibodies (each diluted to 1:100) on ice for 20 min. After washing, the cells were treated with rabbit-derived anti-mouse immunoglobulin conjugated to FITC (diluted to 1:20) and incubated for 20 min on ice in the dark. The tubes were then topped up with formalin and left at room temperature in the dark for 20 minutes.

After washing and pelleting, the samples were resuspended in
PBS/BSA. FACS enrichment and analysis of the cells were carried out on a Coulter Epics sorter as described in the instruction manual.

2.11 Histological Staining

Placental tissue was collected by the author and wax embedded and frozen sections kindly prepared by Chris Smith at Charing Cross and Westminster Medical School.

2.11.1 Giemsa

Cytospin slides were prepared as described above and air dried for 10 min. The slides were then fixed in 100% methanol for 12 min and allowed to air dry. The slides were then immersed in Giemsa dye for 30 min and destained in Gurr's buffer for 3 min. The air dried slides were sealed with Apathy's mounting medium, covered with a glass coverslip and viewed under a standard light microscope (Leitz).
2.11.2 Haematoxylin

Air dried Cytospin slides were fixed as described above, and then immersed in Harris haematoxylin for 2 min, rinsed in tap water and mounted in Apathy's mountant medium. The slides were covered with a glass coverslip and viewed under a standard light microscope (Leitz).

2.12 Immuno-histochemistry

Sections of fixed, frozen or cytospun cells were treated with the antibody to be tested and detection of antibody binding was achieved using the ABC method of the VECTA protocol as described in the manufacturer's instructions. This system uses an avidin/biotin horseradish peroxidase complex detection system. The antibodies were used at a dilution of 100:1.

2.13 Dot Blot Antibody Detection

The antigen/antibody to be tested was bound to a cellulose membrane at a concentration of 1 mg/ml for 1 hr at room temperature and washed in PBS. The membrane was then incubated in PBS/3%BSA at room temperature for 2 hr and then washed and
incubated with the primary antibody on parafilm in a moist chamber for 30 min. The membrane was then washed in PBS 3 times for 10 sec and twice for 5 min and incubated with the secondary antibody (ABC VECTA kit) in PBS/3%BSA. Successful binding was indicated by brown horseradish peroxidase staining, while the membrane remained white in the absence of binding.
CHAPTER 3

RESULTS

3.1 Results of PCR Amplification of Y-sequence DNA

One of the methods of detecting fetal DNA in maternal blood is to identify Y-sequence DNA which should be unique to the fetus if it is male. Y-sequence DNA was identified using the method and primers described by Lo et al (1990). The primers amplify a single copy Y-sequence, which is part of a gene expressed in testicular tissue (Arnemann et al 1987). The paired primers Y1.5 and Y1.6 were used as external primers and amplified a 239 base pair Y-specific fragment as described by Lawler et al (1989). An aliquot of amplified DNA from the first round of PCR was used as a template for the internal primers, Y1.7 and Y1.8, to amplify a 198 base pair Y-specific sequence (see figure 4).

Figure 5 shows the resultant DNA fragments when 10 ng of male DNA and 10 ng of female DNA (prepared as described in section 2.4.1) were amplified with the two sets of primers in a nested fashion for 25 and 10 cycles using the conditions described by Lo et al (1990).

In order to amplify the very small amounts of fetal DNA present in maternal blood, it is necessary to increase the number of cycles of PCR (to increase the sensitivity) using the two pairs of primers in a nested fashion (to increase the specificity). Increasing the number of cycles is ultimately limited by contamination, which will give false positive results as shown in figure 6.
Figure 4. Nested Primer PCR Approach to the Detection of Y-Chromosome Specific Sequence.

Figure 5.
Amplification with nested PCR of Y-chromosome specific sequence of male and female DNA.

M - 1kb marker
B - blank (no DNA added)
Lanes 1,3,5,7 - 10ng male DNA
Lanes 2,4,6 - 10ng female DNA
Figure 6.
Effect of increasing the number of cycles of amplification of nested Y-chromosome specific PCR.

M - 1kb marker
E - empty lane (no products loaded)
B - blank (no DNA added)
Lanes 1, 3, 5, -10ng male DNA
Lanes 2, 4, 6, - 10ng female DNA

This PCR was carried out on the same DNA samples as figure 5 and with the same primers, but for 25 and 40 cycles. It demonstrates the presence of false positive bands in the female samples and samples with no DNA, with both sets of primers, when no precautions are taken to avoid contamination. It also demonstrates the crucial importance of running controls in any PCR in order to be able to interpret the results meaningfully.

3.2 Detecting and Minimising Contamination of PCR

Contamination of PCR can occur at any stage of setting up and running the reaction. It can be present in any of the constituent
solutions, the DNA samples and even the oil used to overlay the reaction mixture. Most often, contamination comes from previously amplified material and becomes apparent only after several weeks of routine amplification (Kwok et al 1989). This carry-over contamination can be a significant problem, due both to the abundance of PCR products and to the ideal structure of the contaminant material for re-amplification.

Over a period of several months, the cycling conditions and PCR cocktail were modified to optimise the PCR (tables 1, 2 and 3) and the following procedures were adopted as a routine to minimise and detect contamination problems:

DNA was prepared in a laminar flow hood using a Nucleon II kit as described in section 2.4.1.1.

All solutions used in the PCR (DNA, dNTP’s, primers, buffer, water and oil) were prepared and divided into small volumes in a laminar flow hood in a tissue culture facility where no other PCR reactions were set up. The handler wore disposable gloves and a gown in accordance with the routine procedures of those using the tissue culture facility.

When a solution was prepared it was divided into small volumes and kept in a -20° C freezer. This allowed solutions to be discarded without undue wastage if contamination was identified, to identify the contaminated component quickly and allowed fresh uncontaminated solutions to be readily available.

The water used for any PCR reaction was double distilled and autoclaved and then passed through a Sartorius mini-sort syringe filter into a sterile container in the laminar flow hood. The container was subsequently opened only in a laminar flow hood.

PCR cocktails were incubated with an appropriate enzyme (see tables 1, 2 and 3) which cut DNA within the fragment flanked by the
DNA primers. The enzyme was denatured by heating to 95°C prior to the addition of the DNA to be amplified.

PCR products were loaded onto gels in a geographically separate area specifically set aside for the purpose.

Once these precautions were routinely observed, the sensitivity of the nested Y-specific PCR could be assessed on serial dilutions of male DNA. Following thirty cycles of PCR in round 1 and thirty cycles of PCR in round 2, using the conditions described in table 1, amplified Y sequence DNA was detectable down to 10 pg (see figure 7). This is approximately equivalent to the amount of DNA in one diploid cell (6 pg).

Figure 7.
Sensitivity of nested amplification of Y-chromosome specific sequence DNA.

M  -1kb marker
B  - blank (no DNA added)
1  - 1pg male DNA
2  - 10pg male DNA
3  - 20pg male DNA
4  - 50pg male DNA
5  - 100pg male DNA
6  - 500pg male DNA
7  - 1ng male DNA
8  - 10ng male DNA
9  - 10ng female DNA
3.3 Results of Y-Chromosome Specific Nested PCR on Maternal Blood

Once the PCR was optimised and contamination problems were minimised, the nested PCR technique was tested to see if the fetal sex could be determined correctly from the peripheral blood of pregnant women.

DNA was prepared from the peripheral blood of women (as described in section 2.4.1.1) prior to undergoing CVS for diagnostic purposes in a fetal medicine unit. Informed consent was obtained from all the participating women. 13 women participated in the study and the results are shown in figure 8.

In order to determine the timing of the appearance of fetal and maternal circulation, a prospective study was initiated and ethics committee approval for the study was obtained from the London Gynaecology Research Ethics Committee.

The women were split into two groups: ten ml of blood were taken at fortnightly intervals until 12 weeks gestation (n=26), with sexual intercourse (n=1) or artificial insemination (n=3). Ten ml of blood were stored at -20°C in sodium citrate bottles and stored at -20°C. The samples were analysed for the presence of Y-sequence DNA using nested PCR primers.

Figure 8.

**Nested Y-chromosome specific PCR of peripheral vein blood of women prior to undergoing CVS.**

M - 1 kb marker
1 - 10ng male DNA
2 - 10ng female DNA
3 - 15 DNA prepared from peripheral blood of pregnant women.
Those with a 198 bp product were confirmed to have male fetuses following CVS analysis.
Those without a 198 bp product were confirmed to have female fetuses following CVS analysis.

Gestations 11-13 weeks
The fetal sex was determined correctly using Y-chromosome specific PCR in all the cases when compared to the CVS culture results. Following these encouraging results, the question of the timing of the appearance and disappearance of fetal DNA in the maternal peripheral blood could be addressed.

3.4 The Timing of the Appearance and Disappearance of Fetal DNA in Maternal Peripheral Blood

3.4.1 Experimental Design

In order to determine the timing of the appearance of fetal cells in the maternal circulation, a prospective study was initiated involving women whose date of conception was known accurately. All patients participating in the study gave informed written consent and ethics committee approval for the study was obtained from the London Gynaecology and Fertility Centre ethics committee.

30 women undergoing infertility treatment were recruited. The women were approached when their first hCG pregnancy test was positive: 10 days after embryo transfer (n=26), timed sexual intercourse (n=1), or artificial insemination (n=3). Ten ml of blood was taken into vacutainer sodium citrate bottles and stored at -20°C. Serial blood samples were taken at fortnightly intervals until 12 weeks' gestation, and monthly thereafter until two months after delivery. The gestation was defined as commencing 2 weeks prior to embryo transfer, timed sexual intercourse or artificial insemination.

DNA was prepared from the samples as described in section 2.4.1.1 and the DNA was stored at -20°C. The samples were analysed for the presence of Y-sequence DNA using nested PCR primers.
Y1.5/1.6 and Y 1.7/1.8. The cycling conditions used are described in table 1.

The women completed the questionnaire shown in appendix 1 after delivery and analysis of the blood samples for fetal DNA. The data were then correlated with the timing of the presence of Y-sequence DNA in the maternal blood.

All experiments to identify sequences in maternal blood were carried out blind to the patient, the previous obstetric history and the other data which emerged during the pregnancy.

3.4.2 Results of Y-Sequence DNA Detection in Women Following Assisted Conception

Of the 30 women recruited, thirteen had no previous clinical pregnancies, 5 had male babies previously, and 2 had female babies previously. The remaining 10 women had pregnancies which ended in the first trimester, in ectopic pregnancy, or spontaneous or therapeutic abortions for which fetal sexing was not available.

Nine of the 30 pregnancies were diagnosed on early scan as multiple. One case began as a triplet pregnancy, with intrauterine death of two of the fetuses confirmed on scan at 8 weeks pregnancy. The pregnancy continued uneventfully, with delivery of a singleton male baby at term. Two cases of twin gestation showed intrauterine death of one of the twins at 12 weeks' gestation. The pregnancies continued and resulted in live singleton births. One other patient with a twin pregnancy miscarried following amniocentesis at 17 weeks' gestation. Within the study, 4 women delivered twins and one triplets.
In one singleton pregnancy an intrauterine death was diagnosed at 19 weeks' gestation.

There were no cases of ante-partum haemorrhage or other complications in any pregnancies resulting in live births except in one twin pregnancy with diabetes and hypertension. Of the live births, 17 women (only one of whom had invasive prenatal diagnosis, at 16 weeks' gestation) had male babies, and 11 had only female babies. All babies were delivered with no apparent birth defects between 33 and 42 weeks gestation when the sex was confirmed by inspection. Those delivered at 36 weeks or less were the multiple pregnancies. Six deliveries were by caesarean section and 22 delivered vaginally. None of the placentas required manual removal following the birth of the baby.

In all cases where female babies only were delivered, no Y-sequence DNA was amplified at any gestational age (see figure 9a). In all cases where a male baby, or babies, were born, Y-chromosome DNA sequence was detected in the maternal blood during the pregnancy (Figure 9b, Table 5).
Figure 9a.
Amplification with nested Y-chromosome specific PCR of DNA prepared from serial peripheral blood samples of a woman who subsequently had a female baby.

M - 1kb marker
A - K serial blood samples taken throughout gestation
A - 5 weeks from LMP
B - 7 weeks from LMP
C - 9 weeks from LMP
D - 11 weeks from LMP
E - 15 weeks from LMP
F - 19 weeks from LMP
G - 23 weeks from LMP
H - 30 weeks from LMP
I - 35 weeks from LMP
J - 1 month after delivery
K - 2 months after delivery
b - blank (no DNA added)
cm - control male DNA
cf - control female DNA
Figure 9b.
Amplification with nested Y-chromosome specific PCR of DNA prepared from serial peripheral blood samples of a woman who subsequently had a male baby.

Table 5 shows the time during the pregnancy when Y-chromosome specific DNA was first detected in the 18 women with male fetuses, and when samples were taken throughout the pregnancies. The earliest gestational age at which Y-chromosome specific DNA first appeared was 4 weeks and 5 days (2 patients), and the latest 7 weeks and 1 day. It continued to be amplified from the maternal blood until 4 weeks after delivery (figure 9b), except in one sample (patient 4, table 4) when at 12 weeks'
Table 4—Earliest detection of Y-chromosome-specific DNA in pregnancies with male fetuses (18 patients)

| Patient | 1* | 2† | 3‡ | 4§ | 5 | 6 | 7* | 8* | 9 | 10 | 11 | 12 | 13† | 14 | 15 | 16‡ | 17* | 18 |
|---------|----|----|----|----|---|---|----|----|---|----|----|----|     |----|----|     |----|----|
| r + + + | -  | -  | -  | -  | - | - | +  | +  | - | +  | +  | +  | ( - ) | ( - ) | +  | +  | +  | +  | + |
| 4- 4- 4- | -  | -  | -  | -  | - | - | +  | +  | - | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  |
| 2 t | -  | -  | -  | -  | - | - | +  | +  | - | +  | +  | +  | ( - ) | ( - ) | +  | +  | +  | +  | +  |
| 3 Î | -  | -  | -  | -  | - | - | +  | +  | - | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  |
| 4- 4- 4- 4- 4- 4- 4- | -  | -  | -  | -  | - | - | +  | +  | - | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  |
| 5 | -  | -  | -  | -  | - | - | +  | +  | - | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  |
| 6 | -  | -  | -  | -  | - | - | +  | +  | - | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  |
| 7** | -  | -  | -  | -  | - | - | +  | +  | - | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  |
| 9 | -  | -  | -  | -  | - | - | +  | +  | - | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  |
| 10 | -  | -  | -  | -  | - | - | +  | +  | - | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  |
| 11 | -  | -  | -  | -  | - | - | +  | +  | - | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  |
| 12 | -  | -  | -  | -  | - | - | +  | +  | - | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  |
| 13†† | -  | -  | -  | -  | - | - | +  | +  | - | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  |
| 14 | -  | -  | -  | -  | - | - | +  | +  | - | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  |
| 15 | -  | -  | -  | -  | - | - | +  | +  | - | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  |
| 16‡‡ | -  | -  | -  | -  | - | - | +  | +  | - | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  |
| 17* | -  | -  | -  | -  | - | - | +  | +  | - | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  |
| 18 | -  | -  | -  | -  | - | - | +  | +  | - | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  |

Weeks: 3 3 3 3 4 4 4 4 4 4 4 5 5 5 5 5 5 5 5 6 7 8 12 16 20 24 28 32 36 40 42
Days: 3 4 5 6 0 1 2 3 4 5 6 0 1 2 3 4 5 6 0

All tests for the 18 patients with male fetuses are listed. A plus sign represents detection of the Y-chromosome-specific band, and a minus sign its absence. Gestational ages are shown in weeks plus day after the last menstrual period as calculated 14 days prior to embryo transfer or intrauterine insemination. Pregnancies marked with an asterisk were conceived following intrauterine insemination, all others following IVF and embryo transfer.

*Conception through intrauterine insemination (all other pregnancies achieved through IVF). Case previously quoted (Thomas et al., 1994).
†Intrauterine death diagnosed at 12 weeks' gestation. Last blood sample taken 1 and 2 months after ERPC.
‡Twin pregnancy with one male and one female baby, gestational diabetes, raised maternal blood pressure, amniocentesis at 16 weeks.
§At 12 weeks' gestation only 1 ml of blood was obtained.
¶Initial twin pregnancy, IUD of one embryo at 12 weeks' gestation.
††Twin pregnancy, one male and one female baby.
**Initial triplet pregnancy, IUD of two fetuses at 8 weeks' gestation.
‡‡Twin pregnancy, two male babies. Case previously quoted (Thomas et al., 1994).
†††Triplet pregnancy with one male and two female fetuses.

1 mo.a.d = One month after delivery; 2 mo.a.d. = 2 months after delivery.
gestation, only one ml of blood was available for DNA preparation and analysis. The average first age of detection was 6 weeks +/- 4 days. No Y-chromosome sequence could be detected 8 weeks after delivery, regardless of the method of delivery.

3.5 PCR Amplification of the AMG Loci on the X and Y Chromosomes

A system of nested PCR was developed which amplified the amelogenin (AMG) locus on the X chromosome and the amelogenin-like (AMGL) sequence on the Y chromosome. This gene codes for a tooth enamel protein and the primers are able to amplify the sequences present on both the X and Y chromosomes [(Nakahori et al 1991) see Figure 10]. Although the two sequences share 90% homology, the X-chromosome sequence has an additional 177bp insert (Nakahori et al 1991), making the two products easily distinguishable when run on an agarose gel (Figure 11). This system had the advantage over amplification with Y-sequence specific primers that an X product was also generated. This provided an internal control which distinguished cases where the PCR had not worked versus amplification of female DNA and would allow comparison of the two X and Y products for quantitation.
Figure 10. Nested primer PCR approach to the detection of AMG/AMGL on the X and Y chromosomes.
Figure 11.

Male and female DNA amplified with AMG primers.

4% agarose gel
M - 1kb marker
B - blank (no DNA added)
m - 25 ng male DNA
f - 25 ng female DNA
3.5.1 Results of Amelogenin Amplification of Maternal Blood Samples

Initially, sixty cycles of nested PCR were applied to some of the DNA samples prepared previously from the blood of pregnant women with a male fetus and which had shown amplification of a Y-chromosome specific sequence in previous experiments. The nested primers used, restriction enzymes used to minimise contamination and the cycling conditions for both rounds of PCR are shown in tables 2 and 3.

In agreement with the previous findings of Nakahori et al (1991), Nakagome et al (1991) and Suzumori et al (1992), no Y-specific band was visible after amplification with the AMG primers.

The reaction with the AMG primers was less sensitive than using Y-chromosome specific primers alone. Nakagome et al (1991) have reported that the gene can be detected when a male DNA sample is diluted by female DNA at a ratio of 1:25,000. In order to increase the sensitivity of the detection system $^{33}$P labelled dATP was incorporated into the PCR product and analysed with a phosphorimager as described in section 2.2.4. Despite an estimated increase in the detection sensitivity of 100 fold, Y-specific bands could not be visualised after amplifying DNA from pregnant women with male fetuses (see figure 12).
Figure 12.

Phosphorimage analysis of nested AMG products after 60 cycles of PCR applied to DNA from the peripheral blood of five patients with known male fetuses.

1- 1µg male DNA  
E- empty  
2- 1µg DNA from woman with male fetus, 12 weeks gestation  
3- 1µg DNA from woman with male fetus, 18 weeks gestation  
4- 1µg DNA from woman with male fetus, 23 weeks gestation  
5- 1µg DNA from woman with male fetus, 17 weeks gestation  
6- 1µg DNA from woman with male fetus, 10 weeks gestation

3.6 Quantitative PCR

As discussed in section 1.4.1.5, the number of fetal cells and/or amount of DNA in the maternal circulation at different times during pregnancy may give important information about the health of the pregnancy, in particular, growth retardation and raised blood pressure. Therefore quantitative PCR was investigated to determine these potential relationships.

In order to use PCR for quantitation, the reaction must be as efficient as possible in order to approach the presumed doubling of
the target molecule which theoretically occurs with each cycle of PCR (see section 1.6.2). The conditions for the nested PCR were therefore optimised for magnesium and potassium concentrations using an Optiprime kit as described in the materials and methods and the time and temperature of each step in the cycle were also optimised. A hot start was used to eliminate undesired hybridization events which can occur in the first cycle and thus seriously impair doubling of the target amplicon after a number of cycles. The sequence of the primers used and the cycling conditions used are listed in tables 2 and 3.

Through the incorporation of $^{33}$P labelled dATP into the DNA amplified by the external amelogenin primers (AMXY-2F and AMXY-CR), we were able to compare the relative intensities of the X and Y bands by analysis of the dried gels with the phosphorimager (section 2.2.4). As the X product is 177 bp larger than the Y product, analysis of the sequence (Nakahori et al 1991) shows that it contains more dATP's than the Y product in the ratio of 1.2:1, therefore the relative intensities of the X:Y bands when male DNA is amplified (which is presumed to have equimolar amounts of X and Y templates) should be 1.2:1.

In order to use PCR quantitatively, the reproducibility of the PCR reaction, gel loading and detection systems had to be defined.
3.6.1 Reproducibility of Image Analysis and PCR in the Same Run

In order to assess the reproducibility of the PCR reaction in the same run, gel loading and phosphorimage analysis, 25 ng of male DNA was amplified for 25 cycles with the external amelogenin primers and $^{33}$P labelled dATP in 10 different reaction tubes. Five aliquots from the same tube were loaded onto agarose gels, as well as aliquots from five separate PCR tubes and the results analysed with the phosphorimager (see figures 13 a and 13 b). Repetitive analysis to measure the pixel volume of the same image 100 times gave an error of +/-1.4%.

Five 10 µl aliquots from the first tube were analysed to test the reproducibility of the gel loading and running techniques and gave an X:Y ratio of 1.1 (error +/-2%). The standard deviation of the absolute pixel volume of the X and Y products was 2%. This was maintained over a range of exposure times of the phosphorimage plates to the gels from 6 to 48 hours.

Aliquots from ten separate reaction tubes in the same run were analysed and the ratio of X:Y was 1.1 (error +/-11.5%) with a similar error for the pixel volume of the X and Y products.

It was concluded that the image analysis system, and PCR reaction (within the same run) gave highly reproducible results over a range of exposure times for both the absolute pixel volumes of X and Y products and ratios of X to Y products.
Figure 13.
Repetitive phosphorimage analysis of male and female DNA samples following amplification for 25 cycles with external AMG primers.

a)

Lanes 1-5 25ng male DNA,
10μl aliquots from the same PCR tube
Lane 6 500ng male DNA
Lane 7 500ng female DNA
Lanes 8-11 25ng female DNA,
10μl aliquots from the same PCR tube
Product sizes, 891 and 705 bp

b)

Lanes 1-5 25ng male DNA,
10μl aliquots from different PCR tubes in the same run
Lane 6 500ng male DNA
Lane 7 500ng female DNA
Lanes 8-11 25ng female DNA,
10μl aliquots from different PCR tubes in the same run
Product sizes 891 and 705bp
3.6.2 Reproducibility of PCR in Different Runs

When analysing a large number of samples, it is necessary to perform the analyses in different batches or runs of PCR. It was therefore important to ascertain reproducibility between runs of PCR so that quantitative results could be compared.

By amplifying 25 ng of male DNA for 25 cycles using the external AMG primers, $^{33}$P labelled dATP and conditions described in table 2, the reproducibility of the PCR reaction was assessed in runs at weekly intervals. Different batches of $^{33}$P dATP were used in the runs.

The pixel volumes obtained for the X and Y products were widely variable between the five runs of PCR (up to 50% variation) when analysed with the same software parameters. However the ratio of X to Y products from the amplified male DNA showed good reproducibility with the average ratio 1.1 and an error of +/- 18%.

It was hoped that by comparing ratios of pixel volumes of X and Y products (rather than the absolute pixel volumes) in a given PCR reaction that variations between individual runs of PCR could be compensated for and used to generate comparable quantitative data.

3.6.3 Defining the Quantitative Phase of a PCR Reaction

Using PCR to quantify DNA relies upon a doubling of the target amplicon with each cycle of PCR. In practice, up to 80% efficiency can be achieved over a limited number of cycles, after which the amplification rate reaches a plateau. The factors that contribute to
this plateau phenomenon include substrate saturation of enzyme, product strand reannealing and incomplete strand separation. To use PCR for quantitative analysis a balance must be struck between a constant efficiency in each cycle and an exponential phase in the amplification process. For each PCR reaction, the linear range in which the quantitated amount of amplified target is proportional to the initial amount of target molecules must be identified.

By amplifying a known amount of male DNA (25 ng) for an increasing number of cycles with external AMG primers, $^{33}$P dATP using the conditions described in table 2, the logarithmic and plateau phases of the PCR were identified and the range of cycles in the logarithmic phase where quantitative evaluation was possible was defined.

Figure 14 shows the increasing intensity of the X and Y-chromosome AMG products with increasing cycle number, with an approximate doubling of the intensity of the products between 24 and 25 cycles. In the plateau phase, the rate of amplification of the X and Y products did not increase consistently. Figure 15 shows that beyond 25 cycles, there is no longer a doubling in the intensity of the bands with each cycle and there is an increasing discrepancy between the amount of X and Y product. The ratio of the pixel volumes of X to Y is decreasing, representing a relative increase in the amount of Y chromosome product. The logarithmic range for the above reaction was up to 25 cycles.
Figure 14.
Defining the logarithmic phase of AMG PCR (external primers) with 25ng male DNA amplified for different numbers of cycles.

Lane 1 - 25ng female DNA amplified for 24 cycles
Lane 2 - 25ng male DNA amplified for 20 cycles
Lane 3 - 25ng male DNA amplified for 24 cycles
Lane 4 - 25ng male DNA amplified for 25 cycles

Figure 15.
Plateau effect seen when the number of cycles of AMG PCR is increased beyond 25.

Lane 1 and 2 - 25ng male DNA amplified for 25 cycles
Lane 3 and 4 - 25ng male DNA amplified for 26 cycles
Lane 5 and 6 - 25ng male DNA amplified for 27 cycles
If PCR results in the exponential accumulation of target DNA, the following relates the initial concentration of target DNA to the concentration of the amplified target DNA in a mathematical form.

\[ Y = X(1+E)^n \]

where \( Y \) is the concentration of PCR-amplified DNA (pixel volume), \( X \) is the concentration of target DNA prior to PCR (pixel volume), \( E \) is the average reaction efficiency for each cycle and \( n \) is the number of amplification cycles.

However, in order to use this equation, the concentration of target DNA prior to PCR in pixel volumes needed to be known and with such a small amount of DNA no product was seen until after 12 cycles of PCR.

By using the equation

\[ \log(Y) = \log(X) + n\log(1+E) \]

and plotting the logarithmic values of the PCR product yield, \( \log(Y) \) against the number of amplification cycles, \( n \), a linear curve can be generated with the intercept equal to the logarithmic values of target DNA, \( \log(X) \) and the slope equal to \( \log(1+E) \) (see figure 16). This equation can also be used to assess cycle to cycle efficiency of amplification, where \( n=1 \). It follows that if the PCR product quantity is experimentally measured after sequential numbers of cycles, a graph similar to figure 16 can be plotted and that both the efficiency of a given PCR amplification, \( E \), and the concentration of target DNA, \( X \), can be determined.
Figure 16.
Graph of $\log(Y) = \log(X) + n\log(1+E)$

$Y$ is the concentration of PCR-amplified DNA (pixel volume)
$X$ is the concentration of target DNA prior to PCR (pixel volume)
$E$ is the average reaction efficiency for each cycle
$n$ is the number of amplification cycles
Figure 17. Graph of Log(Y) versus n [where Y is the PCR product yield (pixel volume) and n is the number of cycles of PCR], for AMG PCR amplification of male DNA.

Only the X-chromosome product is plotted on the above graph since the Y-chromosome product gave similar data, with the same efficiency of 77% as the X-chromosome product (see graph a).
By plotting such a graph from the results of sequential AMG PCR amplifications (figure 17b), the line was extrapolated back to its intercept on the Y axis and (X) the amount of target DNA in pixel volume was calculated and used in the original equation.

Using this equation, the average efficiency for AMG PCR as described above was 77%. This efficiency varied by up to 10% over five separate PCR runs. The cycle to cycle efficiency ranged from 80% to 65% in the logarithmic phase, becoming less efficient with an increasing number of cycles, as the plateau phase was approached. If efficiency is less in the early rounds of PCR, it will have a much more profound effect on the final amount of product than if efficiency is less in the later rounds.

Variations in efficiency between runs and between cycles, as well as variations in the reaction reagents (in particular $^{33}$P) account for the poor reproducibility of the absolute pixel volume seen in the experiment in section 3.6.2. However these variations should affect the amount of both the X-chromosome and Y-chromosome products equally, explaining why the ratio of X to Y product gave good reproducibility.

3.6.4 Quantitative Analysis of Artificial Mixes of Male and Female DNA

To test the accuracy and reproducibility of quantifying ratios of X:Y DNA, artificial mixes of male and female DNA were prepared ranging from 1:1 to 1001:1 and subjected to 25 cycles of PCR with the external AMG primers and conditions described in table 2 and the ratios obtained from five separate runs were analysed using the phosphorimager. The results obtained are shown in table 5. From
the data displayed, it is apparent that the greater the ratio of X:Y DNA analysed, the greater the error in the result.

Table 5.

Ratios of X:Y AMG DNA products obtained from artificial mixes of male and female DNA.

<table>
<thead>
<tr>
<th>DNA mix ratio X:Y</th>
<th>phosphorimage ratio</th>
<th>Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:1</td>
<td>1.1:1</td>
<td>11.5%</td>
</tr>
<tr>
<td>11:1</td>
<td>12:1</td>
<td>20%</td>
</tr>
<tr>
<td>101:1</td>
<td>116:1</td>
<td>32%</td>
</tr>
<tr>
<td>1001:1</td>
<td>1400:1</td>
<td>53%</td>
</tr>
</tbody>
</table>

These data can be explained by understanding the PCR reaction. The plateau phase of the reaction no longer produces a constant efficiency of amplification with the rate of amplification plateauing. The mechanisms producing this effect include substrate saturation of enzyme, product strand reannealing and incomplete strand separation. We can therefore predict that the amount of starting target DNA will affect how quickly this plateau is reached, with a larger amount reaching the plateau more quickly.

Where the quantities of DNA amplified vary over orders of magnitude, (as in the last experiment), the amount of Y-chromosome target may be in the logarithmic phase of amplification, while the X-chromosome product is in the plateau phase. This would lead to inaccuracies in quantitation in particular of the X-chromosome product. If applied to maternal blood, in the case of a male fetus, this is exactly the situation which would be expected. The X-chromosome contribution would come from amplification of the vast excess of maternal X chromosomes with a minute contribution.
from the fetal X-chromosome. The Y-chromosome product would come from the amplification of the minute quantity of fetal Y-chromosome in the maternal blood. We could not expect that both the X and Y chromosome amplifications would be in the logarithmic phase if unenriched genomic maternal DNA was the substrate. This method is therefore not suitable for the quantitation of fetal DNA from whole maternal blood.

3.6.5 Construction of a Standard Curve for Increasing Amounts of Male DNA

In order to quantify absolute amounts of DNA, a standard curve showing the pixel volume of the amplified product must be constructed for different amounts of DNA in each PCR run. To test this system, increasing amounts of male and female DNA ranging from 1 ng to 10 μg were amplified in the same run. The genomic DNA was amplified with the external amelogenin primers (AMXY-2F and AMXY-CR) using $^{33}$P labelled dATP for 25 cycles under the conditions described in table 2. The log pixel volume of the X-chromosome AMG product and the Y-chromosome AMGL product were plotted against the log DNA target concentration (see figure 18). This showed accurate quantitation was possible only in the range of target DNA concentrations from 10 ng to 1000 ng after 25 cycles of amplification. In this range, the gradient of the line is constant and was approximately 1, reflecting the logarithmic phase of the PCR.
Figure 18.
Standard curve of quantity of AMG PCR products (pixel volume versus quantity of starting (male) target DNA (ng) and analysis) for different quantities of male DNA.

Log starting target [DNA] ng

Under 10 ng and over 1000 ng the gradient was not constant and did not approach 1, indicating that the reaction was not in the logarithmic phase and making quantitation inaccurate at the extreme quantities of target DNA amplified.

Five male DNA samples were prepared by another laboratory worker. The quantity of DNA in these samples was known only to the colleague who prepared them. The samples were amplified in the same AMG PCR run as the standard samples and analysed blind on a phosphorimager. The target DNA in the unknown samples was quantified by plotting on the standard curve (see figure 18). The samples could be quantified accurately (error 10%) provided they
were in the range 10-1000 ng. Outside this range the errors were too great to make quantitation meaningful.

Repetition of construction of the standard curve showed good reproducibility, with an average error of 10% from 10 ng to 1000 ng of DNA. This error increased up to 30% at 1000 ng of DNA, reflecting less efficient amplification of larger amounts of DNA. Above 1 μg, the amount of starting target DNA could not be quantified, since when 10 μg of DNA was amplified the amount of product did not increase. Indeed a decrease in the amount of product was seen, reflecting decreased efficiency in the early cycles of the PCR. This was probably due to the large amount of target DNA causing substrate saturation of Taq polymerase, product strand reannealing and incomplete strand separation. It was not possible therefore to quantify accurately the amount of DNA in the unknown samples if it was over 1 μg. This problem could probably have been overcome by reducing the number of cycles of PCR prior to analysis, but would make it incomparable with the results obtained for smaller amounts of DNA.

3.7 Trophoblast Preparation

For the reasons discussed in the introduction (section 1.4.1), the fetal cell type which we chose to try to isolate was trophoblast. In order to test any retrieval system, it was necessary to develop a method of preparing populations of trophoblast cells from first trimester placentas. Initially, the method described by Kliman et al (1986) for the preparation of cytotrophoblast from term placentas was adapted. This was a very time consuming method which aimed to produce cytotrophoblast which could be cultured and studied as it
differentiated into syncytiotrophoblast. When applied to first trimester placentas the method produced very few cells in very poor condition.

The proposed physiological dissemination of trophoblast into maternal blood (via the shedding of syncytial knots and the invasion of cytotrophoblast) meant that if a pure population of cells was used for a test system, one of the cell types potentially present in the maternal blood may not be detected. Similarly, if the cells were cultured prior to using them in a test system, non viable cells would have been selected out and changes in cell surface markers may occur, making it a poor analogy for the physiological situation and leading to abandoning systems which may work in vivo. A simple mechanical method was developed to produce a mixed cell population from first trimester placentas which was used as a test system without culturing or selecting specific cell types as soon as the cells were prepared (see section 2.5.2). The disadvantage of this system is that cell types other than those potentially present in maternal blood will be included, in particular, the mesenchymal cells. The histology of the placental tissue used to prepare the trophoblast cells is shown in figure 2.

The mixed cell population generated by the mechanical preparation was analysed histologically (see figure 19) and by FACS (see figure 20). It was found to consist of numerous cell types of which syncytiotrophoblast and cytotrophoblast could be histologically identified after Giemsa staining. Mesenchymal cells must also have been present, but were difficult to distinguish from other cells using routine stains. FACS analysis similarly suggested a heterogenous cell population with a large variation in size and granularity.
Figure 19.
Light micrograph of cells prepared from first trimester trophoblast, cytospun and stained with Giemsa. X100 magnification.

SC - Syncytial cells 20-50μm
CC - Cytotrophoblast cells 11-20μm
BS - Blood and Stromal cells 5-10μm
Figure 20.
FACS sorting of cells prepared from first trimester trophoblast.

EPICS profile analysis of a) 40,000 cells and b) 25,000 cells sorted according to increasing granularity along the X axis and size along the Y axis.
Note the three distinct cell populations corresponding to the histological populations in figure 19.

There appeared to be three defined cell populations which became more apparent when sparser cell suspensions were analysed (see figure 20). These corresponded to the blood cells/mesenchymal cells (small cell population near the X and Y axes), intermediate size cells (cytotrophoblast and small syncytial elements) and large granular cells (syncytiotrophoblast).
3.8 Enrichment of Trophoblast from Blood by Separation on a Ficoll Gradient

Ficoll is routinely used to isolate lymphocytes from other populations of blood cells. By diluting whole blood with PBS and layering it onto ficoll and spinning the sample in a centrifuge, the populations separate according to density. Lymphocytes migrate to the interface or "buffy coat" layer and red blood cells, membrane fragments and granulocytes sediment at the bottom of the ficoll (see figure 21). Since this is a simple widely used technique, which might be used to separate trophoblast from blood elements, we decided to investigate the migration of trophoblast on a ficoll gradient.

Figure 21.
Separation of blood elements on a ficoll gradient

Before centrifuging

<table>
<thead>
<tr>
<th>Supernatant (blood in PBS)</th>
<th>Ficoll</th>
<th>Sediment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interface (buffy coat)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

After centrifuging

<table>
<thead>
<tr>
<th>? trophoblast</th>
<th>No cells</th>
<th>Lymphocytes</th>
<th>Granulocytes and red blood cells</th>
</tr>
</thead>
</table>
Trophoblast from five terminations was collected separately, processed as described in section 2.5.2, DNA prepared from the samples as described in section 2.4.2 and the sex analysed using the amelogenin primers AMXY-2F and AMXY-CR as described in table 2. (see figure 22).

**Figure 22.**
AMG PCR analysis of first trimester trophoblast cells from five separate placentas.

<table>
<thead>
<tr>
<th>Lane</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>1 Kb marker</td>
</tr>
<tr>
<td>1</td>
<td>DNA from 5 separate placentas</td>
</tr>
<tr>
<td>2</td>
<td>Male</td>
</tr>
<tr>
<td>3</td>
<td>Female</td>
</tr>
<tr>
<td>4</td>
<td>Male</td>
</tr>
<tr>
<td>5</td>
<td>Female</td>
</tr>
<tr>
<td>Cf</td>
<td>Control female DNA</td>
</tr>
<tr>
<td>B</td>
<td>Blank (no DNA added)</td>
</tr>
<tr>
<td>Cm</td>
<td>Control male DNA</td>
</tr>
</tbody>
</table>

891 bp
705 bp
20 μl of one of the trophoblast samples from which X and Y DNA was amplified was mixed with 10 ml of whole blood from a female volunteer and enriched on a ficoll gradient as described in section 2.5.3. 500 μl samples from the supernatant, the interface and the sediment were removed from the gradient taking care not to disturb the other layers. The samples were centrifuged, washed and DNA prepared by boiling the cells for 20 min. The DNA prepared from each fraction was amplified with the external amelogenin primers for 25 rounds and the results are shown in figure 23.

No DNA was amplified from the supernatant fraction, a clear X band and weak Y band were visible from the sediment fraction and clear X and Y bands were visible in the interface (buffy coat) fraction. These data suggest that most of the trophoblast mixed with whole blood separated into the interface of the ficoll gradient with the PBS and that isolation of this layer could provide an initial step in the enrichment of trophoblast from maternal blood samples. The presence of a weak Y band in the sediment fraction after PCR implies the presence of some trophoblast cells in the sediment and these would be lost to analysis if that layer was discarded. It was therefore important to know which trophoblast cells were migrating to the sediment layer.
Artificial female blood/male trophoblast mix separated on a ficoll gradient.

The 4% agarose gel above shows the results of AMG PCR amplification of DNA from different fractions of the ficoll gradient.

- M - 1Kb marker
- Su - supernatant
- Se - sediment
- I - Interface
- Cm - control male DNA
- Cf - control female DNA

When a suspension of trophoblast alone was layered and spun on ficoll and the sediment washed and examined histologically, small (15 μm) cells similar to the blood/mesenchymal cells seen in figure
Figure 23.
Segregation of trophoblast and blood on a ficoll gradient.

M Su Se I Cm Cf

891bp
705bp

Artificial female blood/male trophoblast mix separated on a ficoll gradient.
The 4% agarose gel above shows the results of AMG PCR amplification of DNA from different fractions of the ficoll gradient.
M - 1Kb marker
Su - supernatant
Se - sediment
I - Interface
Cm - control male DNA
Cf - control female DNA

When a suspension of trophoblast alone was layered and spun on ficoll and the sediment washed and examined histologically, small (15 μm) cells similar to the blood/mesenchymal cells seen in figure
19 were found, with no cytотrophoblast or syncytiotrophoblast elements. Since these latter are the cell types predicted to be present in maternal blood, selecting the interface layer following ficoll separation should enrich for trophoblast cells in maternal blood without significant loss of these very rare cells.

3.9 Bacteriophage-Selected Trophoblast Antibodies

As discussed in the introduction, the technique of phage display selection of trophoblast antibodies was used to generate antibodies which might be used to enrich for trophoblast in maternal blood. The initial results when the selected antibodies were tested by the group at CAT with good binding of two antibodies to cultured choriocarcinoma cell lines and no detectable binding to blood elements.

These antibodies were made available to us for further testing on wax embedded and frozen sections of first trimester trophoblast. No binding with either antibody was detected after analysis with the VECTA ABC kit. We therefore used a dot blot test to check the binding of the antibodies myc tag to the anti-myc antibody, using the VECTA ABC kit (see section 2.13) the results of which are shown in table 6.
Table 6.

**Dot blot analysis of Bacteriophage-generated antibodies.**

Binding to cellulose membrane detected with an anti-mouse IgG biotin/avidin peroxidase detection system.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>$^0$ antibody</th>
<th>result</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 none</td>
<td>none</td>
<td>-</td>
</tr>
<tr>
<td>2 αEGFR</td>
<td>none</td>
<td>+</td>
</tr>
<tr>
<td>3 α Von Willebrand (rat)</td>
<td>none</td>
<td>-</td>
</tr>
<tr>
<td>4 αmyc</td>
<td>none</td>
<td>+</td>
</tr>
<tr>
<td>5 CAT α1</td>
<td>αmyc</td>
<td>-</td>
</tr>
<tr>
<td>6 CAT α2</td>
<td>αmyc</td>
<td>-</td>
</tr>
<tr>
<td>7 CAT α3</td>
<td>αmyc</td>
<td>-</td>
</tr>
<tr>
<td>8 none</td>
<td>αmyc</td>
<td>-</td>
</tr>
</tbody>
</table>

The results show that the antibodies generated did not have the expected myc tag which was intended to be an integral part of the antibody and explains the failure to detect any binding to the frozen or wax embedded trophoblast. This proved to be a considerable problem, and despite the continued work of the Cambridge group, no suitable antibody with a recognisable tag was generated using the phage-display method of antibody selection.

### 3.10 EGFR Antibodies

Trophoblast is an actively dividing tissue and the cell surface is rich with growth factor receptors. The EGFR is a 170 kD
transmembrane receptor with an intrinsic tyrosine kinase activity found on all actively dividing cells, but absent from blood cells. The EGFR antibody was chosen as a candidate which might be suitable for enriching trophoblast cells from blood. Three types of EGFR antibody were used, a 29-1 EGFR antibody from Sigma, EGFR-425 antibody from Merck and EGFR-1 from ICRF. These are directed against different epitopes of the EGFR.

Firstly, it was important to define the distribution of the receptors throughout first trimester trophoblast and whether they were present on all first trimester trophoblast tissue. It was also important to identify any binding to blood elements, since in the in vivo maternal blood samples, even the rare blood cells would greatly outnumber the trophoblast cells.

To use the antibody to separate trophoblast from blood, it was attached to immunomagnetic beads as described in section 2.9 and cells expressing EGFR adhered to the beads and were separated from non-attached cells on a magnetic column (see figure 24).

3.10.1 EGFR Antibody Binding to Trophoblast

Staining with a VECTA ABC kit as described in section 2.12 of frozen sections of first trimester trophoblast and wax embedded sections of first trimester trophoblast showed that both syncytiotrophoblast and cytotrophoblast were labelled by all three EGFR antibodies used. A very small proportion of the mesenchymal cells also appeared to bind EGFR antibodies (see figure 25).
**Figure 24**

**Magnetic Cell Sorting**

- Magnetic beads (antibody-labelled)
- Target cell suspension
- + anti-trophoblast antibody
- Wash
- + secondary antibody
- Wash
- + magnetic beads
- Wash
- Magnet retains labelled cells
- Elution of magnet, labelled cells

**Example Text:**

**a)** Frozen section x 50 magnification

**b)** Wax embedded section x 50 magnification

Note the brown staining of both the syncytiotrophoblast and cytrophoblast layers. There is no apparent staining of mesenchymal cells in the frozen section, however, some do exhibit staining in the wax section.
Figure 25. Frozen and wax embedded sections of first trimester trophoblast stained with horse radish peroxidase for the presence of EGFR.

a) Frozen section X 50 magnification
b) Wax embedded section x 50 magnification

Note the brown staining of both the syncytiotrophoblast and cytotrophoblast layers. There is no apparent staining of mesenchymal cells in the frozen section, however some do exhibit staining in the wax section.
However, the 29-1 EGFR antibody did not stain all the placentas tested. Three placentas failed to bind the antibody and further investigation revealed that it recognised a blood group A antigen (Gullick and Lemoine 1990) implying that the placenta which stained strongly with the antibody was of blood group A or AB, while those which did not stain were of other blood groups. Because of this, the 29-1 EGFR antibody was not used for magnetic sorting samples.

FACS sorting of the trophoblast preparation with the EGFR antibodies confirmed the histological findings (see table 7). When EGFR was fluorescently labelled, 50% of cells in the trophoblast suspension (as prepared in section 2.5.2) were identified. Most of these (80%), were in the larger cell populations (see figure 20) corresponding to cytotrophoblast and syncytiotrophoblast cells.

Table 7.
Trophoblast Immunohistochemistry of EGFR antibodies

<table>
<thead>
<tr>
<th>Fractions Enriched For</th>
<th>EGFR1</th>
<th>EGFR425</th>
<th>EGFR 29-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Syncytiotrophoblast</td>
<td>+</td>
<td>+</td>
<td>+/-</td>
</tr>
<tr>
<td>Cytotrophoblast</td>
<td>+</td>
<td>+</td>
<td>+/-</td>
</tr>
<tr>
<td>Mesenchyme</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
3.10.2 EGFR Antibody Binding to Leukocytes

In order to identify any binding of the EGFR antibodies to blood elements, leukocytes were incubated with the antibodies at a dilution of 1:100 and then incubated with goat derived anti-mouse immunoglobulin conjugated to FITC and analysed by FACS as described in section 2.10. The binding of the antibodies to the various blood elements is shown in table 8.

FACS labelling sorts the cells based on the fluorescent label, size and the granularity of the cells. 95% of the monocytes were labelled with the EGFR antibodies, while there was no labelling of lymphocytes or granulocytes. Monocytes have Fc receptors which bind antibodies non-specifically and are not known to express EGFR's.

Table 8.

<table>
<thead>
<tr>
<th>FRACTIONS ENRICHED FOR</th>
<th>EGFR1</th>
<th>EGFR425</th>
<th>EGFR 29-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphocytes</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Monocytes</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Granulocytes</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
3.10.3 Effect of Pretreatment of Blood and Trophoblast with Sandoglobulin Prior to Sorting

Leukocytes were treated by incubation with Sandoglobulin at concentrations from 0-10 mg/ml, (see section 2.10) as it is known to block non-specific sites such as the monocyte Fc receptor. After incubation with fluorescently labelled EGFR antibodies, analysis with FACS showed that at a concentration of 5 mg/ml, Sandoglobulin blocked the non-specific binding of monocytes to a level comparable to the background binding seen in the lymphocyte population (2.2%). When trophoblast was similarly pretreated with Sandoglobulin, the percentage labelled with EGFR antibodies did not alter, at 50% (see table 9). This shows that the Sandoglobulin did not interfere with specific binding to the EGFR receptor.

Table 9.
FACS analysis of trophoblast pretreated with Sandoglobulin and incubated with EGFR antibodies.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% trophoblast labelled by FITC anti-mouseIgG</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 mg/ml SG/-α-EGFR</td>
<td>2.0</td>
</tr>
<tr>
<td>0 mg/ml SG/+α-EGFR</td>
<td>50.5</td>
</tr>
<tr>
<td>5 mg/ml SG/-α-EGFR</td>
<td>1.9</td>
</tr>
<tr>
<td>5 mg/ml SG/+α-EGFR</td>
<td>50.3</td>
</tr>
</tbody>
</table>

* % of cells in a trophoblast preparation (as prepared in section 2.5.2)
3.10.4 EGFR Antibody Sorting of Artificial Mixes of Blood and Trophoblast

Artificial mixes of leukocytes and trophoblast were prepared in a ratio of 100:1, incubated with EGFR antibodies and separated with goat anti-mouse immunoglobulin coated magnetic beads. The number of cells following sorting was counted under phase-contrast microscopy. The sorted cells were cytopspun and counted following Giemsa staining and the syncytial and cytotrophoblast elements counted and calculated as a percentage of the total number of cells (see table 10).

Table 10.
a) Total cell counts and percentage of trophoblast cells before and after EGFR MACS.

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>No. cells before MACS</th>
<th>No. cells after MACS (+ve and -ve fractions)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>$2 \times 10^6$</td>
<td>$1.8 \times 10^5$</td>
</tr>
<tr>
<td>2</td>
<td>$2 \times 10^6$</td>
<td>$1.76 \times 10^5$</td>
</tr>
<tr>
<td>3</td>
<td>$2 \times 10^6$</td>
<td>$1.68 \times 10^5$</td>
</tr>
<tr>
<td>4</td>
<td>$2 \times 10^6$</td>
<td>$1.87 \times 10^5$</td>
</tr>
<tr>
<td>5</td>
<td>$2 \times 10^6$</td>
<td>$1.74 \times 10^5$</td>
</tr>
</tbody>
</table>

% cells lost during MACS 10%
Table 10.

b) Percentage trophoblast on cytospin (syncytio and cytotrophoblast) following Giemsa staining

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>before MACS</th>
<th>after MACS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.4%</td>
<td>72%</td>
</tr>
<tr>
<td>2</td>
<td>0.3%</td>
<td>65%</td>
</tr>
<tr>
<td>3</td>
<td>0.5%</td>
<td>77%</td>
</tr>
<tr>
<td>4</td>
<td>0.31%</td>
<td>64%</td>
</tr>
<tr>
<td>5</td>
<td>0.24%</td>
<td>58%</td>
</tr>
</tbody>
</table>

Average enrichment factor 150 fold.

3.10.5 EGFR Antibody Sorting of Maternal Blood Samples

In view of the promising results achieved with EGFR antibody MACS sorting, we applied the technology to maternal blood samples to enrich for any trophoblast present. Pregnant women attending a clinic for prenatal diagnosis were approached and informed consent obtained for participation in the study. Ethics committee approval had been obtained and 15 patients were enrolled.

10-25 ml of blood was collected into sodium citrate treated vacutainers prior to the invasive diagnostic procedure. The blood elements were separated on a ficoll gradient and the leukocytes incubated with 5 mg/ml Sandoglobulin and then incubated with EGFR-1 antibodies and goat anti-mouse immunoglobulin labelled magnetic beads. The cells were then sorted on a magnetic column and the positive fraction subjected to dual FISH with a FITC labelled
Y chromosome paint and a texas red labelled X chromosome paint (see section 1.6.3). The cells were then scanned for dual X and Y signals in the interphase nuclei which may represent fetal cells. The results are shown in table 11.

Initially, a Y-chromosome paint was used to identify male cells, but results were very variable from run to run, including a three month period when no signals were found in control male cells. This was found to be due to a failure of the detection kit. The other disadvantage was that cells without a Y signal could have resulted from either analysis of a female cell or hybridization/detection failure. For these reasons, the dual fluorescence kit was used to detect X and Y chromosomes simultaneously in the same nucleus. This system avoided inaccurate sexing, gave better hybridization and was more reliable from run to run (see figure 26).
Table 11.
Patient blood samples after EGFR MACS analysed by dual X and Y FISH for fetal cells.

<table>
<thead>
<tr>
<th>Gestational age (weeks and days)</th>
<th>Volume of blood (ml)</th>
<th>Sex of fetus</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>19+2</td>
<td>10</td>
<td>male</td>
<td>-</td>
</tr>
<tr>
<td>11+5</td>
<td>10</td>
<td>female</td>
<td>-</td>
</tr>
<tr>
<td>11+6</td>
<td>20</td>
<td>male</td>
<td>-</td>
</tr>
<tr>
<td>9+1</td>
<td>15</td>
<td>Female</td>
<td>-</td>
</tr>
<tr>
<td>18+2</td>
<td>15</td>
<td>male</td>
<td>-</td>
</tr>
<tr>
<td>16+3</td>
<td>18</td>
<td>male</td>
<td>-</td>
</tr>
<tr>
<td>12+6</td>
<td>18</td>
<td>male</td>
<td>-</td>
</tr>
<tr>
<td>15</td>
<td>22</td>
<td>male</td>
<td>+ (1 cell)</td>
</tr>
<tr>
<td>10+3</td>
<td>18</td>
<td>male</td>
<td>+ (3 cells)</td>
</tr>
<tr>
<td>10+4</td>
<td>18</td>
<td>female</td>
<td>+ (1 cell)</td>
</tr>
<tr>
<td>16+3</td>
<td>15</td>
<td>male</td>
<td>-</td>
</tr>
<tr>
<td>10+5</td>
<td>18</td>
<td>female</td>
<td>-</td>
</tr>
<tr>
<td>16+4</td>
<td>18</td>
<td>male</td>
<td>+ (1 cell)</td>
</tr>
<tr>
<td>11+4</td>
<td>18</td>
<td>male</td>
<td>-</td>
</tr>
<tr>
<td>11+2</td>
<td>18</td>
<td>female</td>
<td>-</td>
</tr>
</tbody>
</table>
Figure 26. FISH analysis of interphase and metaphase lymphocytes from a male volunteer with a) Y chromosome paint and b) dual X and Y chromosome probes.

a) Magnification X 1000 FITC painting of the Y chromosome
Hybridization efficiency 70%

b) Magnification x1000 Dual FITC and texas red labelling of male interphase lymphocytes. A red and green signal are seen in both nuclei.
Hybridization efficiency 75%
3.10.6 Quantitative Analysis of Artificial Mixes of First Trimester Trophoblast and Leukocytes Separated with EGFR Antibodies

Trophoblast from a male fetus and leukocytes from a female volunteer were prepared as described in sections 2.5.1 and 2.5.2. They were mixed in a ratio of 100 leukocytes : 1 trophoblast cell in PCR tubes and DNA was prepared by boiling the cell mixture for 5 mins. The resultant DNA was amplified with AMG primers AMXY-2F and AMXY-CR using $^{33}$P labelled dATP for 25 cycles under the conditions described in table 2. In the same PCR run, known amounts of male and female DNA were also amplified to construct a standard curve as described in section 3.6.5. The amplified DNA was analysed using the phosphorimager and the resultant absolute amounts of DNA and the X:Y ratio were calculated and the results are shown in figure 27.
The initial mix of 100,000 female leukocytes and 1000 male trophoblast cells gave an absolute X value of 1.0 μg and an absolute Y value of 20 ng, giving a ratio of 50:1. From the previous quantitative analysis in section 3.6.4, it is apparent that the X value may be considerably more than 1 μg, since amplification of larger amounts of DNA did not yield any more product. The number of cycles (25), was chosen because the greater accuracy was required with smaller amounts of DNA since it was anticipated that they would show the greater change. The experiment was then repeated with an EGFR-1 antibody sorting step added prior to PCR amplification of the positive and negative fractions. The method used for sorting is described in section 2.9. Following enrichment, the amplified positive fraction gave an absolute X value of 5 ng and an absolute Y value of 15 ng, giving a ratio of 1:3. The negative fraction gave an absolute X value of 1 μg and an absolute Y value of 2 ng giving a ratio of X:Y of 500:1.
The enrichment factor following EGFR antibody sorting of leukocytes and trophoblast was 50X.

3.11 Co-Amplification of a Single FISHed Nucleus with \(\Delta F508\) and Amelogenin Primers.

Techniques for cell enrichment, such as EGFR-antibody attached to magnetic beads may be suitable for the diagnosis of aneuploides if enrichment can achieve a ratio of 1:100 of fetal to maternal cells. However, no enrichment strategy can achieve 100% pure fetal cells which would be necessary for the diagnosis of single gene disorders, such as cystic fibrosis. Any maternal or extraneous DNA amplification in such a case could lead to the wrong diagnosis. For such diagnoses from cells retrieved from the maternal blood, the isolation of a single cell or nucleus, which is known to be of fetal origin, offers the best hope for the future. For this reason, the technique of co-amplification with PCR of a single nucleus which had previously been subjected to FISH was investigated.

Cystic fibrosis is inherited as an autosomal recessive disease in normal Mendelian fashion. It is responsible for extensive and varied pathology, with the significant clinical manifestations resulting from abnormal ion transport in the exocrine glands, leading to secretion of thick, sticky mucus in the organs affected. Recurrent infections of the lungs and reduced protein absorption from the diet lead to a markedly reduced life expectancy and impaired quality of life.

\(\Delta F508\) is the commonest mutation in the caucasian population (Keren et al. 1989). A 3 bp deletion in exon 10 of the CFTR gene on chromosome 7 leads to deletion of a phenylalanine residue at
codon 508. This results in changes in chloride ion transport and is the underlying defect responsible for the clinical manifestations.

PCR amplification of genomic DNA from individuals with appropriate primers can be used to identify normal (homozygous), carrier (heterozygous) and affected (homozygous) individuals (see figures 28 and 29).

Figure 28. Nested Primer PCR Approach to the Detection of ΔF508.
Figure 29.
Analysis of PCR amplification of AF508 using polyacrylamide gel electrophoresis.

C - heterozygote
N - normal heterozygote
A - affected homozygote

A test system was devised to be analogous with a clinical situation which may require prenatal diagnosis (see figure 30). The model system chosen was a female carrier of the AF508 gene mutation, representing the maternal genotype and a homozygous normal male, representing an unaffected fetus. Leukocytes from 2 volunteers with the above genotypes were prepared from 10 ml of peripheral blood as described in section 2.5.1. A 100:1 female to male mix was prepared and subjected to dual FISH analysis with a Texas red labelled X probe and a FITC labelled Y probe as described in section 2.6.
Figure 30.
Test system for analysis of a single nucleus for ΔF508 and AMG status.

Nuclei selected from slides of lymphocytes prepared in 100:1 mix of female heterozygous ΔF508 cells and male homozygous normal cells. Nuclei selected as male or female from the dual FISH signals detected in the nucleus.

The nuclei were viewed at x1000 magnification under the appropriate epifluorescence filters on a Leitz microscope and a nucleus selected which demonstrated dual red and green signals representing the X and Y chromosomes of a male cell (representing the fetus, see figure 26). The nucleus was then removed from the slide as described in section 2.4.3 and subjected to a nested co-
amplification with primers detecting the ΔF508 mutation and the AMG locus (see tables 2 and 3). Stringent precautions to minimise contamination were observed during the amplifications, as described in section 2.2.2.

Co-amplification with AMG and ΔF508 primers provided an internal control for the FISH, by checking that a male nucleus had been selected (represented by X and Y bands after amplification with AMG primers) and determining its ΔF508 status. An example of the resultant gels is shown in figure 31. Nuclei with two red X chromosome signals were also selected for isolation and analysis and results are also shown in figure 31. The technique identified the ΔF508 and amelogenin genotype correctly in 60% of the 20 male and 20 female nuclei analysed. In 12% an incorrect ΔF508 genotype was amplified, in 10% the genotype was unclear with difficulty identifying heteroduplexes and normal and mutant bands. In 8%, the amelogenin amplification gave no result, while the ΔF508 amplification did. In the remaining 10%, no amplified DNA was visualised, either because the PCR failed, or there was no, or insufficient DNA transferred to the PCR reaction tube. Table 12 shows the results obtained for both male and female isolated nuclei.
**Figure 31.**
PCR co-amplification analysis of FISHed single nuclei for AMG and ΔF508.

**AMG**

- B - Blank
- F - Female DNA or nucleus as identified by FISH
- M - Male DNA or nucleus as identified by FISH
- 433 bp
- 253 bp

**ΔF508**

- B - Blank
- A - Affected homozygote
- C - Heterozygote ΔF508
- N - Normal homozygote
- 98 bp
- 95 bp

F - Female DNA or nucleus as identified by FISH
M - Male DNA or nucleus as identified by FISH
C - Heterozygote ΔF508
N - Normal homozygote
A - Affected homozygote
B - Blank
* - Single nucleus
Table 12.
Results of co-amplified, nested PCR with ΔF508 and AMG primers applied to single nuclei.

Nuclei were selected from slides of lymphocytes prepared in 100:1 mix of female heterozygous ΔF508 cells and male homozygous normal cells.
Nuclei were selected as male or female from the dual FISH signals detected in the nucleus.

<table>
<thead>
<tr>
<th></th>
<th>Male nuclei (N=20)</th>
<th>Female nuclei (N=20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMG &amp; ΔF508 bands in agreement with FISH nuclear signals</td>
<td>14</td>
<td>10</td>
</tr>
<tr>
<td>ΔF508 bands in disagreement with FISH nuclear signals</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>ΔF508 bands unclear</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>No AMG bands detected</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>No AMG or ΔF508 bands detected</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>
CHAPTER 4

DISCUSSION

The enormous emotional, social and financial impact of babies born with congenital abnormalities has driven forward developments in molecular techniques for prenatal diagnosis. With the number of disorders which can be diagnosed prenatally increasing almost daily, the quest to obtain representative fetal tissue without putting the pregnancy at risk has been similarly driven forward. The potential information obtained about pathophysiological processes in pregnancy through such research are also invaluable to our understanding and prevention of other fetal problems including PIH and IUGR.

When I began working on this thesis, many questions regarding the suitability of fetal cells retrieved from the maternal circulation remained to be answered. In the ensuing years, some of these questions have been answered while others remain, and of course new questions have arisen.

4.1 Consideration of the Optimal Fetal Cell Type for Prenatal Diagnosis and Screening

The ideal fetal cell for prenatal screening and diagnosis would become available (and continue to be available) in sufficient numbers during the first trimester of the pregnancy.

It would be representative of the fetal genotype and have a number of surface antigens unique to the fetus and/or cell type which are distinct from maternal blood cells. This would enable
selection with a number of monoclonal antibodies to enrich for fetal cells to a minimum level of 1:100 for the screening of the common aneuploidies, and to diagnose specific chromosome rearrangements.

It would be unrealistic to expect that any antibody, or set of antibodies could select a population of pure fetal cells since some maternal cells would inevitably adhere to fetal cells or separation columns, yet this is what would be required for the screening or diagnosis of single gene disorders. A method of identifying a fetal cell with certainty, followed by isolation and PCR analysis seems to offer the best chance of non-invasive diagnosis of these disorders.

The fetal cell type should be cleared from the maternal blood soon after delivery to avoid diagnostic confusion in subsequent pregnancies.

So how do the cell types available measure up to the ideal characteristics presented above and how does this affect the future of prenatal diagnosis and screening?

4.2 The Timing of the Appearance and Disappearance of Fetal Cells in Maternal Blood for Prenatal Diagnosis and Screening

It is perhaps not surprising that none of the available fetal cell types fit all the above criteria. From the results of the prospective study of timed pregnancies presented in section 3.4.2, some conclusions regarding fetal cells can be drawn. Although the number of patients was small, their follow up was complete and represented a considerable amount of laboratory investigation for each pregnancy.
It was most encouraging to have identified correctly the fetal sex in all cases despite the potential for contamination, and this justifies the time spent developing stringent precautions prior to processing the samples collected. The single most important factor in controlling contamination was pre-incubation of the PCR reaction mixture with restriction enzymes which cut DNA within the region to be amplified. Without introducing this, contamination was a persistent problem which threatened to render any results meaningless. In previous studies, only 80% accuracy was achieved in 600 cases analysed by nested PCR (Simpson and Elias 1993). The improvement reflects increasingly stringent precautions taken to minimise contamination and will continue to improve in the future with the development of the incorporation of dUTP in PCR products, and uracil DNA-glycosylase (UDG) sterilisation (Longo et al 1990). UDG cleaves the uracil base from the phosphodiester backbone of uracil-containing DNA, but has no effect on natural (thymine-containing) DNA. The resulting apyrimidinic sites block replication by DNA polymerases. As UDG is inactivated by heat denaturation prior to PCR amplification, carry-over contamination can be controlled if the contaminant contains uracils in place of thymines. This method can sterilise up to 20,000 molecules of carry over PCR product and is claimed to be much more efficient than restriction enzyme digestion which was used in my study (Lo et al 1989).

Another promising development in minimising contamination is the prevention of pre-PCR mispriming with the 'hot start' process (Chou et al 1992). While I found that this was necessary to achieve sufficient sensitivity (Mullis 1991) to detect fetal DNA in unenriched maternal blood, I was unable to dispense with the use of nested primers, although Lo et al (1994) have been able to, thus still achieving detection when the feto-maternal ratio is as low as 1 in
10^6, while avoiding the risk of introducing contamination between the two rounds of amplification.

As such developments continue and contamination is minimised, PCR results from amplified fetal DNA will become more reliable and match the standards currently achieved through invasive techniques.

Although the analysis of fetal DNA does not provide information about the cell type(s) contributing to the DNA detected, it does infer that some fetal cells must be present in the maternal blood and should be available for prenatal diagnosis provided enrichment strategies for the appropriate cell type are used. From the study of assisted conception pregnancies, the timing of the detection of fetal (Y-chromosome specific sequence) DNA was assessed accurately at a gestation when many women would be unaware they were pregnant.

I was able to establish that fetal DNA became detectable in the pregnancies over a two week period, and was detected by 7 weeks' gestation in all cases with a male fetus. There is no reason to expect that fetal cells from a female fetus would have given different results from a male fetus, and so it is reasonable to extrapolate these results to all pregnancies, regardless of the fetal sex. This implies that fetal cells are available in the maternal peripheral blood for first trimester screening and diagnosis.

In the two cases of multiple pregnancy with one male baby (one set of twins and one set of triplets), fetal DNA was not detected earlier in the pregnancy than in singleton pregnancies, suggesting from this small sample that the timing of appearance of fetal cells in maternal blood is not affected by multiple gestation.

Previous studies on DNA extracted from unsorted peripheral blood by Lo et al (1990) describe amplification of Y-chromosome
DNA sequences in the blood of one pregnant woman recorded as 6 weeks' gestation. Liou et al (1993) studied 19 pregnancies with male fetuses, and amplified Y-chromosome specific DNA at 6 weeks' gestation in one pregnancy. They were able to diagnose male fetal sex in only half the cases by 8 weeks' gestation, and in one case not until 12 weeks. These scatters of gestational ages of first appearance of fetal sequences may reflect inaccuracies in dating the pregnancies. Gänshirt-Ahlert et al (1993) detected 10-100 nucleated erythrocytes after density gradient and magnetic cell sorting of 10 ml samples of maternal blood at 8-12 weeks' gestation. Other groups have described detection of trophoblast cells at 9 or more weeks' gestation using specific antibodies, but blood samples from earlier gestations were not analysed (Mueller et al 1990, Hawes et al 1994).

The continued detection of fetal DNA for up to one month after delivery suggests that the fetal cells continue to be available throughout pregnancy and could be used for prenatal diagnosis at later gestations as may arise with late booking for antenatal care, or when ultrasound findings suggest an abnormality.

Partuition is a time when fetal cells are released into the maternal circulation in large quantities. It is known to be the time when Rhesus iso-immunisation is most likely to occur and the Kleihauer test can be used to demonstrate fetal cells in the maternal blood as they do not lyse in alkali. The clearance of fetal DNA from the maternal blood between 4 and 8 weeks after delivery was not influenced by the method of delivery. The process of caesarean section may reasonably be expected to release more fetal cells (both trophoblast and fetal blood) into the maternal blood than vaginal delivery, since it involves handling the baby in utero, manipulation of the uterus to gain access the baby, and digital removal of the
placenta rather than waiting for signs of physiological separation. The uterus is swabbed out routinely after delivery of the fetus and placenta and this may be expected to introduce more trophoblast cells into the maternal circulation.

This expected increase in the fetal cell load was not reflected by delayed clearance of the fetal DNA from the maternal blood following caesarean section compared with vaginal deliveries. If the mechanism of clearance involves immunological mechanisms, then a larger 'dose' of antigen would provoke a larger immune response, and this may explain why the time taken to clear the cells is not affected by the mode of delivery.

The quantity of DNA detected by the PCR chosen (which was shown to be down to single cell levels in section 3.2), suggests that diagnostic confusion by contamination of cells from a previous pregnancy should not be a problem. This is reinforced by the observation that in the study, of the 5 women who had previously had male infants, 4 delivered female babies and no Y chromosome-specific DNA fragments were amplified.

In one case, an intrauterine death was detected at a routine scan at 19 weeks. The previous scan at 12 weeks' gestation had shown that the fetus was alive and well. Y chromosome-specific DNA was amplified from the maternal blood from 6 weeks' gestation until 12 weeks. Samples taken at 19 weeks and after suction curettage were negative. Unfortunately no fetal material was available to determine the fetal sex. The fact that a suction curettage was undertaken implies that the fetus died well in advance of the 19 week scan. If fetal cells are cleared from the maternal blood at the same rate in early pregnancy as following term delivery, then the disappearance of the Y-chromosome specific sequence from the maternal blood would imply that the fetus died between 12 and 14 weeks gestation.
The data about clearance of fetal cells after delivery from previous studies has been conflicting. Schroder (1974) described the persistence of male lymphocytes in maternal blood for up to one year after birth of a male infant using Y chromatin analysis. Ciarafini (1977) reported continued circulation of male fetal cells up to 5 years after delivery again using Y chromatin analysis. Using PCR, Lo et al (1993) failed to detect them in maternal blood postpartum. It has been suggested that their method was not sufficiently sensitive to detect the DNA. However, as in this study, single cell sensitivity should theoretically have been achieved. Hsieh et al (1993) used PCR analysis and were unable to detect Y-sequence DNA in over half of the women studied 4 months after delivery of a male infant, but found that 2 of 23 women still had amplified Y-specific DNA sequence 8 months after delivery. More recently, Bianchi et al (1994) have identified Y-sequence DNA in circulating haematopoietic stem cell precursors (using stem cell antigen markers and antibody flow sorting) up to 27 years post partum.

It would appear that the question remains to be resolved, since those using PCR techniques on unenriched blood have not been able to detect fetal DNA sequences in most cases, while those applying an enrichment procedure for lymphocytes are. Theoretically, if fetal nucleated red cells, or trophoblast are the enriched cell population, this potential problem could be overcome. Clearly the methodology may be influencing the results. For PCR analysis of 10-20 ml of whole blood, DNA is prepared and only 1-2 μg of that DNA is used for amplification. It may be that the chances of having DNA from one of the lymphocytes or haematopoietic cells in the DNA to be amplified is very low, while specifically selecting such cells from a 10-20 ml sample increases the chances of finding the cells and this may explain the apparent discrepancy. It seems most likely that the
ongoing clinical trials involving enrichment for fetal nucleated red cells will provide the practical answer to this question, as significant persistence of cells from a previous pregnancy would give rise to diagnostic error in many cases when compared to amniocentesis.

It is interesting to speculate about the source of the fetal DNA amplified in the study, since the enrichment strategy employed to retrieve fetal cells obviously relies upon the cell type intended to be retrieved. The possible origins of fetal DNA in the maternal circulation are from fetal blood cells and trophoblast. The presumed source of the fetal blood cells is feto-maternal haemorrhage. Certainly, feto-maternal haemorrhage leading to transfusion of fetal cells is well documented throughout pregnancy at amniocentesis, CVS therapeutic and spontaneous miscarriage, threatened miscarriage and during labour. However all these conditions arise in abnormal pregnancies or are iatrogenic. In normal, undisturbed pregnancies such haemorrhage has not been proven to occur and it is possible that fetal red cells are actively transferred to the mother as some maternal lymphocytes gain access to the fetus. Fetal blood cells are formed initially in the yolk sac from 4 weeks' gestation, but do not circulate until the heart begins to beat at 5 weeks. Fetal vessels are not present within the chorionic villus stroma until 8 weeks. It is therefore unlikely that fetal blood elements would be able to escape into the maternal circulation as early as 5 weeks' gestation. Ganshirt et al (1994) have detected fetal erythroblasts in maternal samples as early as the fifth week of gestation (though this was not an assisted conception pregnancy and so the gestation was not known accurately) and they point out that the highest percentage of erythroblasts in fetal blood occur at earlier gestations.

The most likely source of the Y chromosome DNA sequence detected during weeks five to eight is trophoblast. Von Kuskull and
Gahmberg (1995) used 2,3-bisphosphoglycerate (a marker for HbF) and ISH with Y-specific probes to identify fetal cells. They identified Y-signal in BPG negative cells only at 6 weeks' gestation though they were present in erythroid cells after this gestation. Histologically the cells were epithelioid and were thought to be cytotrophoblasts, although immunocytochemistry was not performed and so their origin remains questionable. Trophoblast development in the early embryo is very rapid, and by 4 weeks, the lacunae of the trophoblast are filled with maternal blood into which knots of syncytiotrophoblast and underlying trophoblast may be shed directly making physiological dissemination even at this early gestation a possibility.

After 8 weeks' gestation the relative contribution of fetal blood and trophoblast cells to the fetal DNA detected in maternal blood is unknown; it may change as the pregnancy advances. Bianchi et al. (1991) presented data that there were no detectable fetal nucleated red blood cells at 16-20 weeks' gestation compared with 11-12 weeks when analysed for Y-DNA sequences, while Chua et al (1991) have shown that trophoblast (both syncytiotrophoblast and cytotrophoblast) are disseminated into uterine vein up to term.

4.3 The Suitability of Fetal Cells in Maternal Blood for Prenatal Diagnosis and Screening

4.3.1 Fetal Erythroblasts

Although most cell types in fetal blood have been demonstrated to be present in maternal blood, fetal erythroblasts have been most studied of the possible cell types as they are thought to be easier to purify using cell sorting or immunological techniques.
They have the advantage over other fetal blood elements of being present in small but sufficient numbers (5-20 cells per 20 ml blood sample) during the first trimester, rare in maternal blood, and relatively well differentiated with a limited life span. Nucleated erythroblasts, once identified as fetal, should be representative of the fetal genotype, while trophoblast with potential problems of mosaicism may not. As they are nucleated and not terminally differentiated, they have the (at present) theoretical possibility of being suitable for culturing which would provide more cells for analysis and offers the prospect of a pure fetal cell population to study.

From the many studies published, there have been no reports of an absence of these cells in the first trimester and it therefore seems likely that they are present in all pregnancies, although precise timing of their detection has yet to be studied. The number of cells available has been found to differ widely between apparently normal pregnancies at the same gestational ages (CD71 transferrin positive cells ranged from 0.2% to 8.5% of maternal mononuclear cells at 11 weeks of gestation, Bianchi et al 1994a). This is not a surprising finding since the degree of feto-maternal transfusion will depend on many fetal and maternal factors. Indeed, invasive procedures such as amniocentesis are known to increase the number of fetal cells present in the maternal blood.

Problems with contamination by maternal cells remain, since the erythroblast receptors chosen for antibody enrichment also select a population of maternal cells. Zheng et al (1993, 1995) and Von Koskull and Gahmberg (1995) used BPG to identify cells containing HbF and combined this with Y-specific ISH in an attempt to identify fetal erythroblasts with certainty. Fetal haemoglobin ($\alpha_2\gamma_2$) is the predominant cytoplasmic protein found in fetal erythrocytes after 7
weeks gestation, but pregnancy stimulates synthesis of small amounts of fetal haemoglobin (Pembury et al 1973) albeit in much smaller amounts than in fetal erythrocytes. This system has the advantage of using an intracytoplasmic marker, thus avoiding potential problems with adsorption of antigen onto maternal cells and greatly reduces the number of cells sorted (48-240) compared with using CD7 to sort erythroblasts (230-685,000), increasing the sorted cell purity of fetal cells when analysed with FISH (4.5%-44.5%) (Zheng et al 1995). However, these data demonstrate that the detection of fetal Hb in sorted erythroblasts is not sufficient proof that the cells are of fetal origin.

There is currently no certain way to identify fetal cells and until a reliable method is found, contamination will remain a confounding problem. With continued improvements in antibodies, the use of groups of antibodies, and the power of negative selection being increasingly recognised (Zheng et al 1993), maternal cell contamination will decrease, but pure fetal erythroblasts are unlikely to be the end result.

Fetal nucleated erythroblasts have been successfully used for prenatal diagnosis of aneuploidies (Price et al 1991, Elias et al 1992 Bianchi et al 1992, Ganshirt-Ahlert et al 1993), in the first instance, this was detected prior to any invasive procedure being performed.

It is interesting and fortuitous that there appear to be increased numbers of nucleated erythroblasts (resulting in a greater percentage of fetal cells) in maternal samples enriched for erythroblasts in aneuploid pregnancies (up to 20% of enriched erythroblasts in the case studied by Simpson and Elias in 1993 and an average of 10% by Ganshirt-Ahlert et al 1993 compared with 3-5% in normal pregnancies). This may reflect altered placentation or immune responses at a fundamental level in chromosomally
abnormal pregnancies and may aid non-invasive diagnosis in affected pregnancies.

### 4.3.2 Trophoblast

Trophoblast, whether cytotrophoblast or syncytiotrophoblast, is another potential tissue present in maternal blood which may be suitable for prenatal screening and diagnosis of chromosome disorders.

There is the obvious potential problem of mosaicism which may mean that the trophoblast genotype does not reflect the fetal genotype, but the drawbacks may be more fundamental as investigators find trophoblast universally difficult to find. Despite the apparent physiological dissemination of trophoblast into maternal blood throughout pregnancy described in the introduction (sections 1.4.1-1.4.1.5) when attempts are made to retrieve them, there appear to be very few trophoblast cells in the peripheral maternal blood. This may reflect the efficiency of the maternal immune system in clearing the cells as soon as they enter the general circulation via the uterine veins, internal iliac veins, inferior vena cava and thence to the heart and lungs. The lungs are known to be a major site of clearance of deported trophoblast and hence it is isolated and destroyed before reaching the peripheral veins of the antecubital fossa, where clinical blood samples are usually taken. Indeed, this swift isolation, both anatomically and in time, may be of vital importance in preventing immune recognition of the trophoblast which might jeopardise future pregnancies with the same partner. The other possibility is that the techniques used so far to identify trophoblast are not sufficiently sensitive or specific so
that not all trophoblast cells in the maternal blood are being identified.

Trophoblast contains many cell types, distinct both morphologically and immunologically, with sub-populations responsible for different physiological functions (e.g. invasion of spiral arterioles by extra-villus cytotrophoblast) and this heterogeneity makes it particularly challenging to retrieve from maternal blood.

Despite adopting a novel approach to antibody generation (section 1.5.3) which should have been able to select for rare and specific antibodies to trophoblast, bacteriophage-selected antibodies failed to provide an antibody or antibodies suitable for isolating trophoblast from blood. Three promising antibodies were identified by CAT which bound to cultured choriocarcinoma cells, but not to blood cells. Binding of these antibodies could not be demonstrated on frozen or wax embedded sections of first trimester trophoblast. This was found to be due to a failure of incorporation of a myc tag into the antibody which was to be an integral part of the antibody and was used for detection of antibody binding (section 3.9).

Despite continued work, this problem could not be overcome and further preparation and investigation of the antibodies was suspended while funding for the project was reviewed. CAT are a commercial company whose scientists are keen to collaborate on projects. However results are expected in a relatively short time since work on other more lucrative projects could be undertaken by the scientists involved. For this reason the project was halted and alternative antibodies which might be suitable for trophoblast retrieval identified.

Antibodies to EGFR were identified as an alternative candidate to sort trophoblast from maternal blood since EGFRs were reported to be
present in placental tissue and absent from blood cells. A test system was devised which offered all possible trophoblast cell types in first trimester placenta for labelling with the antibody (section 3.7). The preparation was mechanical, avoiding the use of enzymes to disaggregate cells (which may remove EGFR from the cell membrane) and approximating the physiological dissemination of syncytiotrophoblast into blood. Histological and FACS analysis of the resultant cell suspension showed that it contained syncytiotrophoblast, cytotrophoblast and smaller blood and mesenchymal cells, 50% of which bound EGFR antibodies by FACS analysis, most of which were syncytio and cytotrophoblast populations (section 3.10.1).

EGFR antibodies (with the exception of EGFR 29-1, which was found to bind to a blood group A antigen and not EGFR) bound to the syncytio and cytotrophoblast layers of first trimester placenta (section 3.10.1), with minimal staining of mesenchymal cells.

EGFR antibodies were found by FACS analysis to bind to monocytes in blood (10.3.2) which would represent a considerable source of contamination if sorted with trophoblast cells. This problem of non-specific binding of EGFR antibody to the Fc receptors of monocytes was overcome by pre-incubation with sandoglobulin, without affecting trophoblast binding (section 3.10.3).

From these results, EGFR antibody appeared to fulfil the criteria for successfully enriching trophoblast from blood, binding syncytio and cytotrophoblast and not binding to blood cells. Separation of trophoblast from red blood cells and granulocytes was achieved through a ficoll gradient (section 3.8) and provided an initial enrichment step prior to MACS. The process of magnetic cell sorting with MINIMACS accounted for the loss of approximately 10% of cells (3.10.2).

Separation of artificial mixes of 100:1 blood and trophoblast with EGFR antibodies and MACS achieved good enrichment of 150
fold for relatively rare cells. To sort trophoblast from maternal blood would of course require identification of very rare cells (estimated to be less than 1 in \(10^6\)) however, it was not possible to prepare accurately such dilutions of trophoblast and to assess the resultant enrichment following MACS in a test system.

With this accumulation of knowledge, success in sorting trophoblast from maternal blood with EGFR antibody seemed a possibility provided there was trophoblast to sort. The results of maternal blood EGFR MACS sorting were very disappointing (section 3.10.3), but in keeping with the findings of other groups. Putative fetal cells were identified in only four of the ten pregnancies with male fetuses analysed, with correct prediction of the fetal sex in three cases. Such poor identification of fetal cells is clearly not reliable enough to form the basis of a prenatal diagnostic or screening test.

Recently, Durrant et al (1995) have had success retrieving trophoblast and fetal nucleated red blood cells from peripheral maternal blood using a specific monoclonal antibody: 340. This, (like EGFR) recognises a cell surface antigen on both syncytio and cytotrophoblast cells and was applied to ficoll gradient sorted cells with MACS (the same methodology used in this thesis). They were able to isolate trophoblast cells from 20 ml of maternal blood and predict the fetal sex correctly using PCR in 10 of 14 pregnancies from 10 to 14 weeks gestation. There were no false positives, but only 3 of 7 male pregnancies were detected. A major criticism of the study is that blood samples were taken after CVS procedures, which could have led to the detection of iatrogenic cells.

The poor results achieved with EGFR sorting may have been due to limits or sub-optimal use of the technology, or because trophoblast cells are simply not present in sufficient numbers in the
peripheral blood to sort. Because the optimal gestation for
trophoblast deportation was not known, a range of gestational ages
were sampled. Fetal cells were thought to be identified in samples
from 10 to 16 weeks' gestation.

The initial ficoll enrichment should not have resulted in the
loss of significant numbers of syncytio or cytotrophoblast (section
3.8), and loss of 10% of the cells through MACS sorting is within the
loss expected for most procedures. However even loss of this small
proportion could have accounted for the loss of some trophoblast
cells. If only one or two such cells were present in the original blood
samples they could have been lost during the procedure. Bianchi
(1994) compared FACS and MACS sorting and concluded that FACS
gave better recovery for positive sorting (70-100%), with MACS
unable to retrieve fetal cells when only 10 - 100 cells were present.
Johansen et al (1995) studied retroplacental blood samples and
found that while anti-CD 16 coated immunomagnetic beads gave the
best immunocytochemical identification (cytokeratin antibodies) of
trophoblast cells isolated, negative selection with anti-CD 45 gave a
higher recovery rate (78% versus 68%). FACS sorting achieved a
3250 fold enrichment of trophoblast cells, but a very poor recovery
rate of only 8%. An additional problem with the FACS sorted cells
was that they could not then be analysed with cytokeratin antibodies
since they had already been antibody-labelled. Neither of these
studies have addressed the question of loss of cells during analysis
procedures, particularly harsh multi-stage procedures such as FISH
and immunocytochemical staining which may account for the poor
final results.

In my studies, the EGFR antibody binding to 80% of syncytio
and cytotrophoblast could again account for the loss of some
trophoblast into the negative sorted fraction. Additionally, FISH
analysis itself is likely to result in the loss of some cells during processing (the percentage was not ascertained) and a hybridization efficiency of 75% implies that 25% of trophoblast cells were not labelled fluorescently. This highlights the problems in searching for rare-event cells with multiple step analyses during any one of which the sought-for cells could be lost.

As with erythroblasts, the apparent success of some groups in identifying chromosomal abnormalities through analysis of trophoblast from maternal blood (Cacheaux et al 1992) may reflect the fortuitous increased deportation seen in such abnormal pregnancies, rather than a technique that is applicable to normal pregnancies. This in itself may be utilised as the basis of future screening programmes.

4.4 Quantitation of Fetal DNA in Maternal Blood

Accurate quantitation of DNA and of numbers of cells of different types in maternal blood is fundamental to deciding the optimum time in pregnancy for isolating given fetal cell types for screening and diagnosis and may provide important information about physiological and pathophysiological processes in pregnancy.

There are many methods that might be used to provide this information including isolating the cells and counting them. As has been described in the previous section, this may be technically difficult and if many steps are involved in the isolation and identification of the cells, the final data will probably be inaccurate through loss of the cells during processing.

For this reason, the use of quantitative PCR was investigated as a simple two step procedure (DNA preparation and PCR) which might
provide accurate data about the quantity of Y-specific sequences from the small numbers of fetal cells in the maternal blood.

Anyone who has experience of PCR will know the familiar sinking feeling when a PCR has failed to work for no apparent reason and so making such a reaction quantitative is approached with trepidation and not a little scepticism! Using a PCR reaction for quantitation requires patience and a thorough understanding of the methodology as well as painstaking optimization of the reaction so that its strengths and limitations can be realised.

The system chosen involved simultaneous amplification with a single pair of primers of AMG/AMGL sequences present on both the X and Y chromosomes. This approach could provide absolute quantitation of amounts of X and Y DNA as well as comparative ratios of X and Y DNA. It also provided a reliable standard for analysis in each run - male DNA containing equimolar amounts of X and Y DNA.

The incorporation of $^{33}\text{P}$ labelled dATP into the product was used to quantify it, alternatively this could have been achieved through end-labelling the primers rather than the product, with both methods being used successfully by other groups (Oka et al 1990, Noonan et al 1990). Direct labelling of the product is faster and easier to run and potentially has greater quantitative power because it allows a more direct reading of product accumulation. The band intensities were analysed through phosphorimage analysis which has a linear dynamic range over a range of 100,000:1 compared with a non-linear range of 300:1 for film.

The reproducibility of image analysis and the PCR reaction in the same run was found to be acceptable. However variations in results between runs were too great for comparisons to be made. As the efficiency of the PCR for amplification of the X-chromosome AMG product was affected by different PCR runs, so was the Y-
chromosome AMGL product, so that ratios of X:Y product were comparable between runs of PCR.

At extremes of ratios of X:Y product, the results became much more variable because the large amount of starting X-chromosome DNA meant that the reaction was in the plateau range and therefore not suitable for quantitation. Of course, this is the exact situation in maternal blood, with a very large amount of X template and a very small amount of Y template. In addition to this, 60 cycles of nested PCR was required to detect fetal Y-sequence in unenriched maternal blood. This would clearly push the PCR reaction into the plateau phase making it unsuitable for quantitative analysis. For these reasons, the methodology was not suitable for quantifying the amount of fetal DNA in the maternal blood. Quantitative analysis was therefore carried out on much larger amounts of DNA than the amounts of fetal DNA which might be present in maternal blood, amounts which may be achievable after enrichment for fetal cells and which make it suitable for assessing the success of enrichment strategies.

Within the confines of a given range of cycles of PCR and a given range of target DNA, the PCR reaction could be used for quantitation of absolute amounts of DNA to within 10%. The system was then used to assess the enrichment of trophoblast from blood in artificial mixes following EGFR MACS (section 3.10.4). The initial mix of 100,000 female leukocytes and 1000 male trophoblast cells gave absolute values of 1 μg of X and 20 ng of Y, a ratio of 50:1, though the value of the X product was at the upper end of the range of quantitation and may therefore have been underestimated. Following EGFR MACS the positive fraction gave a ratio of 1:3, an enrichment factor of over 50 fold. This is in keeping with the 150
fold enrichment quantified by counting syncytio and cytotrophoblast elements (3.10.2).

Discrepancies would be expected between the two analyses since syncytial cells are multinucleate and whereas they were counted as one cell in the cell counting method, all the nuclei in one cell would contribute separately to the final X and Y products when analysed with quantitative PCR (see figure 32).

Also, it was not possible to distinguish between blood and mesenchymal cells in the cell counting method so that they would all be counted as blood cells (this is reasonable since mesenchymal trophoblast elements would not be expected to enter the maternal circulation in the physiological situation), while in the quantitative PCR they would contribute to the Y product in both the negative and positive fractions. These factors should make quantitative PCR more accurate than morphological analysis in determining the amount of fetal DNA in a sample and highlight increases in syncytial knot shedding (giving an increase in fetal nuclei in the blood) a process known to be stimulated by hypoxia.

Because of the differences in the two techniques, quantitative PCR would have been expected to identify a greater proportion of the DNA as Y-specific than the morphological method and therefore give a greater enrichment factor when in fact the reverse was the case. The enrichment factor calculated with morphological analysis may have over-estimated the proportion of syncytial and cytotrophoblast cells to blood cells, since blood cells are much smaller and therefore difficult to quantify accurately.
Figure 32.
Cartoon showing EGRF MACS of blood and trophoblast and analysis by morphology and quantitative PCR.

Composition of Female blood and male trophoblast

Blood cells
Mesenchymal cells
Cytotrophoblast
Syncytiotrophoblast

EGFR MACS +ve fraction
50% of trophoblast cells selected

Morphology
cannot distinguish blood from mesenchyme.
Counts syncytial cell as one unit despite multiple nuclei.

Quantitative PCR
Counts all the nuclei in a syncytial cell.
Can distinguish mesenchymal cells from blood cells
Blanchi (1994) used quantitative PCR to quantify fetal cells in samples FACS enriched for erythroblasts, using a Y-specific sequence and an autosomal sequence on chromosome 7, and found this both reliable and sensitive with fetal cell purity ranging from .015% to 4.8%. This method of quantitative PCR may give inaccurate results if the primers used to amplify the autosomal sequence have a significantly different efficiency from the Y-primers or if the logarithmic phase of PCR does not coincide for the two sets of primers and the amounts of DNA analysed. Careful assessment of these parameters is required before reliable data can be generated.

Electrochemiluminescent detection and quantification could provide more accurate quantitation of very small amounts of DNA (in the attomole range 10^{-18} mole, Wages et al 1993) and it avoids running the product on a gel thus abolishing potential errors in lane loading and inconsistencies in the gel. However this method was not available for use and the equipment and electroluminescent probes are very expensive.

4.5 Assessing New Enrichment Strategies

At the outset of this project, the weight of research in the field had been undertaken using flow cytometry with only a few groups using magnetic sorting. The decision was made to investigate its use further, since it was cheaper, simpler and most importantly would be readily available to all laboratories if fetal cell screening from maternal blood were to become the standard in antenatal care.

Since then, many groups have switched to magnetic sorting, convinced by its ability to sort rare cells, particularly in aneuploid
pregnancies (Ganshirt Ahlert et al 1994, Zheng et al 1994). This conviction has not been born out in the EGFR sorting experiments undertaken in this thesis and I would share the reservations expressed by Bianchi et al (1994) regarding loss of rare cells with MACS sorting in chromosomally normal pregnancies.

It is however the technique of choice for quick evaluation of new enrichment strategies involving positive and negative selection with new antibodies or panels of antibodies. The results could be assessed morphologically or with quantitative PCR and will surely be exploited in the future to advance our understanding and knowledge at a faster rate than was previously possible.

4.6 The Role of FISH for Identification of Fetal Cells and Prenatal Diagnosis

The development of the technique of FISH over the past ten years has provided another powerful molecular tool to complement other molecular techniques.

The ability of FISH to identify chromosome-specific sequences in interphase as well as metaphase chromosomes has been exploited for prenatal diagnosis so that rapid results from uncultured cells can achieve a diagnosis within 48 hours.

Dual X and Y chromosome FISH was used as one of the multistep procedures to assess enrichment of fetal trophoblast on EGFR MAC sorted cells. Inefficiencies in the hybridization technique, as well as loss of cells through FISH processing may have contributed to the poor results obtained with maternal blood samples.

However, the technique of dual fluorescence analysis in an interphase nucleus has allowed the identification of male and female
nuclei and provides a means of identifying a nucleus as fetal, provided the fetus is male. Exploitation of this technique has allowed us to undertake diagnosis of a single gene disorder (cystic fibrosis ΔF508) on a single nucleus, something that cannot be achieved through enrichment procedures alone as they cannot isolate pure fetal cells. This technique has provided an important internal control through using FISH and PCR analysis of the same single nucleus to identify a male (or female nucleus) with certainty, and thus potentially identify it as fetal (section 3.11).

Fish analysis will continue to provide a basic diagnostic technique for analysis of chromosomes, as well as a potentially important step in the identification of fetal cells. At present it is not possible to analyze the small three bp deletion (identified with PCR) which is involved in the ΔF508 lesion with FISH, since the resolution is not sufficient to identify single bases.

With the development of confocal microscopy and computer enhanced techniques FISH analysis of interphase chromosomes may in future be able to identify the genotype of single gene disorders thus obviating the need for PCR and allowing one step in situ analysis of both X and Y chromosomes and ΔF508 genotype. This would reduce the risk of contamination and of misrepresentation of the fetal genotype that could currently occur in two step FISH/PCR analysis.

4.7 The Role of PCR for Identification of Fetal Cells and Prenatal Diagnosis

Currently, PCR is the only technique which can identify single gene disorders which may be identified as a risk factor from the
family history of the pregnant women. Disorders which may be identified in this way increase in number daily and such diseases form an increasing proportion of pregnancies subjected to invasive diagnostic procedures. The application of PCR analysis of single gene disorders to fetal cells isolated from maternal blood would therefore represent a significant advance to the obstetric well-being of both mother and fetus.

By using dual FISH male (fetal) cells could be identified in artificial mixes and a single nucleus analysed simultaneously for both sex specific DNA and ΔF508 (section 3.11).

This technique, although time consuming, generated promising data which when refined could form the basis of a most important area of prenatal diagnosis. The main problems identified were of loss of one of the AMG or ΔF508 bands in the case of known heterozygotes or failure of the PCR either through non-transference of the nucleus to the PCR tube or PCR failure. This latter result is of less significance clinically, since it would not jeopardise a potentially normal fetus. However unreliable amplification of the ΔF508 genotype might.

The reason for these anomalous results may be disruption of one of the alleles through FISH processing, removing the nucleus from the slide, DNA preparation, or a combination of all three. The problem might be overcome by a reduction in the boiling time prior to PCR or abolishing this step altogether since the DNA should be accessible to the primers through previous processing. If damage is occurring during FISH analysis then the processing may have to be modified, however any changes must not deleteriously affect the hybridization efficiencies which would make identification of fetal cells less likely.
Improvements in nucleus isolation would come with familiarity of technique so that less damage and incomplete nuclear transfer may be anticipated with more experienced operators. Further technical refinements are obviously therefore required to make the technique reliable enough for prenatal diagnosis, but offer hope for the future.

4.8 Future Developments

Continued refinements of molecular technology are needed to provide reliable screening and diagnosis for antenatal chromosomal disorders and to provide basic information about physiological and pathophysiological processes during pregnancy.

Enrichment procedures using single or multiple monoclonal antibodies for both negative and positive selection need to be evaluated, with particular care to minimise the loss of cells during processing and looking to achieve enrichment to about 1 in 100 fetal to maternal cells for the diagnosis of aneuploides. Simple, single stage procedures offer the most reliable tests, with gradient and magnetic sorting emerging as the potential front runners. The success of these procedures, both in evaluating new enrichment strategies and their application needs to be assessed accurately. Currently, the procedures being used (FISH and immunocytochemistry) are labour intensive, multistage procedures which themselves result in loss of some of the cells. Cell loss needs to be evaluated so that successful techniques are not labelled as unsuccessful due to limits in procedures to identify the cells as fetal. To this end, the search must continue for a unique fetal marker which allows us to identify fetal cells in a population of maternal cells with certainty. This is without doubt the single most
important advance that could be contributed to the field of non-invasive prenatal diagnosis.

While this is not yet available, refinements in PCR technology has allowed analysis of a single nucleus in a test system so that when a reliable fetal marker is available prenatal diagnosis of single gene disorders will be possible.

Advances in quantitative PCR, in particular very sensitive chemiluminescence techniques will soon allow quantitation of the very small amounts of fetal DNA that I have shown are present in maternal blood throughout pregnancy. This may provide the basis of future understanding of normal pregnancy physiology and pathophysiology in the clinically vital areas of PIH and IUGR. Quantitation of fetal DNA in serial samples from timed pregnancies will need to be analysed once reliable PCR quantitation of very small amounts of DNA is possible.

Finally, in collaboration with electrical engineers at the University of Glasgow, I am looking at a novel approach to cell isolation which should theoretically be capable of isolating homogeneous groups of cells and single cells. The approach has the great advantages that it does not use any type of antibody and is a simple, fast, one step procedure which could provide a population of pure cells. It is based on the behaviour of cells in non-uniform electric fields and we have already demonstrated its ability to sort rapidly (within 15 mins) red blood cells from white blood cells.

Whole cells placed in an alternating current migrate towards electrodes depending upon the behaviour of the charged proteins within the lipid bilayer that forms their membranes, with the force generated dependant upon the radius, so that cell size is clearly an important parameter. Because cells types have unique membrane properties they migrate at different speeds and in different directions.
in magnetic fields. Various parameters including the strength of the field, the AC frequency and the ionic composition of the suspension medium can be manipulated to isolate single cells or cell populations.

Experiments are currently underway to separate populations of blood cells and to observe the behaviour of different types of trophoblast with a view to assessing the sorting efficiencies of rare event cells and the application of the technique to maternal blood for trophoblast isolation.

Non-invasive prenatal diagnosis remains the vision for the future. Within the next decade this is likely to become a reality, when new problems of who should be screened and for what diseases will require the urgent attention of doctors, prospective parents and the whole of society, as once again scientific advance precedes the wider ethical issues.
Appendix 1.

Questionnaire completed by women participating in the assisted conception study.

<table>
<thead>
<tr>
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<th>Date of Birth</th>
</tr>
</thead>
<tbody>
<tr>
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<td>Telephone number</td>
<td>Weight</td>
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<tr>
<td></td>
<td>Height</td>
</tr>
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</table>

Booking Hospital/ Consultant in charge of obstetric care

**Past Obstetric history**
- Number of previous pregnancies
- Type of conception
- Gestation if miscarriage
- Date of deliveries/gestation
- Mode of delivery
- Complications/diseases during pregnancy

| Sex | Birthweight |

**Maternal medical history**
- Hypertension
- Diabetes
- Kidney disease
- SLE
- Smoker
- Reason for assisted conception

**This Pregnancy**
- Type of assisted conception
- Date of conception (embryo transfer/IUI)
- Multiple pregnancy
- Dates of scans
- Dates of blood samples
- Complications of pregnancy
- Missed abortion (gestation)
- Complete/incomplete abortion (gestation)
- Ectopic pregnancy (gestation)
- Raised blood pressure
- Proteinuria
- Diabetes
- Growth retardation
- Mode of delivery and reason
- Gestation at delivery
- Sex
- Birthweight
Appendix 2.
Recruitment of patients for timed appearance of fetal cells. Women were recruited by Dr. M. Thomas and exclusively followed up by her at fortnightly or monthly intervals depending on the gestation.

At the initial visit, an information sheet informing them of the purpose of the study and a written consent form were provided and signed (see below).
The questionnaire in appendix 1 was completed at the final blood sampling with the help of Dr. Thomas.

Consent form for study.
From Professor I. Craft (London Fertility Centre), Professor R. Williamson (St. Mary's Hospital Medical School) and Dr. Margot Thomas (Study Co-ordinator).

We are conducting a study to isolate cells form the fetus which may be released during pregnancy into the mother's blood stream. If we are able to find a way to retrieve these cells, it may be possible to carry out genetic analysis of the fetus so that abnormalities such as Down syndrome may be detected.

At present, the only way to do this is to take some of the amniotic fluid (amniocentesis) or to obtain placental tissue (chorion villus sampling). Both these procedures involve a small risk to the pregnancy. If we could obtain fetal cells from the mother's blood, then it would not be necessary to expose the pregnancy even to the small risks of amniocentesis or chorion villus sampling.

We are asking you to take part in this study. It would mean donating a small sample of blood from your arm every two weeks from four to sixteen weeks of pregnancy, then monthly thereafter, including some samples after the delivery. This is a research project
and we do not expect to obtain definitive results for your pregnancy, but just to test whether the technique works in principle; your reward will be the knowledge that you may be able to help others in future. You will not receive results yourself since it is only when all the data are put together that we will know whether this technique works or not.

I agree to participate in the above trial to isolate fetal cells from maternal blood

signed:------------------  date:------------------

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References


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Von Koskull, H., and Gahmberg, N. (1995) " Fetal erythroblasts from maternal blood identified with 2,3-bisphosphoglycerate (BPG) and in situ hybridization (ISH) using Y-specific probes." *Prenat Diagn* 15:149-154


Addendum


Y chromosome sequence DNA amplified from peripheral blood of women in early pregnancy

Sir—We are studying a cohort of women who became pregnant through in-vitro fertilisation. Serial blood samples taken at fortnightly intervals from 4 weeks' gestation (14 days after embryo transfer) have been analysed for the appearance of DNA sequences from the Y chromosome by use of a sensitive method of gene amplification, the nested polymerase chain reaction, as described by Lo et al.

To date, the sex of babies of five of the women in the study is known after amniocentesis or ultrasound analysis. In two cases, a male fetus was identified. Y chromosome sequences were first detected at gestations of 4 weeks 5 days, and 5 weeks 5 days, respectively. The analysis of maternal blood before these gestations did not demonstrate any Y chromosome DNA sequences, nor were such sequences amplified from the blood of the three women with female fetuses. Y sequence DNA continued to be amplified from the blood of the two women with male fetuses up to 12 weeks' gestation. The analyses were done blind before the fetal sex was determined.

The earliest reported detection of fetal DNA in maternal blood previously was at about 6 weeks by Lo et al., and the exact timing of conception of that pregnancy is not known. The detection of fetal DNA at 5–6 weeks' gestation raises the question of the cellular origin of the amplified fetal DNA, which might come from fetal blood cells (erythroblasts or leucocytes) and/or trophoblast, all of which have been shown to be present in maternal blood. Fetal blood cells are formed initially in the yolk sac at 4 weeks' gestation but do not circulate until 5 weeks when the heart begins to beat. Furthermore, fetal vessels are not present within the villous stroma until 8 weeks. It is unlikely, therefore, that fetal blood elements would be able to escape into the maternal circulation as early as 5 weeks' gestation.

Trophoblast development in the early embryo is very rapid, and by week 4 the lacunae of the trophoblast are filled with maternal blood in direct contact with syncytiotrophoblast. The physiological process of invasion of the maternal arterioles by the cytotrophoblast is said to occur from 6 weeks' gestation, but the shedding of knots of syncytiotial cells into the maternal circulation is known to occur throughout pregnancy.

From this small patient sample, it is not possible to estimate whether fetal DNA will be detectable in all pregnancies at such early gestations, or whether there is marked variation between individuals. It is, however, encouraging that detection of fetal DNA in the maternal circulation may be possible within 1–2 weeks from the expected date of the missed period, because this provides additional hope that this procedure may form the basis of future early antenatal screening.

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The Time of Appearance, and Quantitation, of Fetal DNA in the Maternal Circulation

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INTRODUCTION

In the future, fetal cells in the maternal blood may be a source of material for routine screening for prenatal cytogenetic or DNA diagnosis in the first trimester for any woman who wishes to be tested. In order for this to become a reality, the timing of the appearance of fetal cells in the maternal circulation must be defined accurately. We must be able to define at what stage of gestation we can obtain fetal cells reliably, and whether they are then present and available in sufficient numbers for analysis throughout the pregnancy. Fetal cells from maternal blood have already been successfully identified as having a 47, XY, + 21 karyotype in a woman whose amniocentesis analysis independently showed the same karyotype, demonstrating that the possibility of routine testing of maternal blood may become a reality once the basic questions relating to timing, reproducibility, and accuracy are answered.

One of the methods employed for identifying the presence of fetal cells in the maternal blood has been the amplification of Y-sequence DNA, which would be predicted to be present in the case of a male fetus. This has been applied to both enriched cell samples and to unsorted maternal nucleated blood cells. However, there have been no studies in which the duration of the pregnancy has been accurately known from conception, so that serial samples could be analyzed for Y sequence. The presence of Y sequences can be reliably detected using the nested primers

This research was supported by Life-Force Research Ltd.
described by Lo et al. By using appropriate decontamination procedures, contamination can be minimized.

We describe the use of PCR to amplify a Y-specific repeat sequence from the peripheral blood of women who have achieved a pregnancy through an IVF program. The duration of the pregnancy was known exactly and confirmed with serial ultrasound scans.

Another important and as yet unanswered question is what quantity of fetal material is available at different gestational stages throughout a pregnancy and the variability in amount between pregnancies. Such information is of importance in defining the best gestational age(s) for maternal blood sampling for fetal cells and for correlation with pathological processes such as pregnancy-induced hypertension and intrauterine growth retardation. A failure of early trophoblast invasion of the spiral arterioles of the placental bed is thought to be the underlying pathology in both these conditions and may be reflected by altered amounts of trophoblast in the maternal circulation. As new antibodies are developed to target the types of fetal cells available in the maternal blood, quantitation of fetal DNA could be used to evaluate their enrichment potential against existing enrichment techniques.

Previous estimates of the quantity of fetal DNA have been made using cytological analysis (range 1 in 27 to 1 in 25,000), and using DNA studies by PCR amplification and slot blot analysis or Southern blot hybridization (range 1 in 100,000 to 1 in 1,000,000 at various gestations). We describe below a method of quantitative PCR that may be refined for measuring the amount of fetal DNA present in maternal blood at serial gestations following enrichment procedures.

PATIENTS AND METHODS

Women attending a clinic for fertility treatment were approached when they had a positive βhCG pregnancy test and asked to participate in the study. The pregnancy test was performed 12 days after egg collection and corresponds to 4 weeks' gestation. Ethics committee approval had been obtained for the study, and informed written consent was obtained from each woman taking part. Ten milliliters of blood was collected in citrate from the antecubital veins of 20 women at fortnightly intervals from 4 to 12 weeks of gestation. None of the women recruited had previous clinical pregnancies. The blood was frozen at -70°C until DNA was prepared from the sample. The gestation was defined as 2 weeks at the time of in vitro fertilization (IVF), gamete intrafallopian transfer (GIFT), zygote intrafallopian transfer (ZIFT), or embryo transfer (ET).

DNA was extracted from maternal blood by standard techniques under class II containment conditions. Each DNA extract was vortexed for 30 seconds to fragment long DNA strands and to mix the DNA before samples were taken for PCR. Two micrograms of DNA were subjected to 30 cycles of PCR with primers Y1.5/1.6 amplifying a Y-specific 239 base pair fragment. After the initial amplification, 2 μl of the PCR product was reamplified for 35 cycles with primers Y1.7/1.8 amplifying a Y-specific 198-bp fragment. Denaturation was at 94°C for 30 sec, with annealing at 57°C for 1 min and extension at 72°C for 2 min. Each 100 μl of reaction mixture contained 10 μl of sample DNA, 2 U Taq DNA polymerase, 200 μmol of each
deoxynucleotide triphosphate, 0.8 μmol of each primer, 50 nmol/l KCl, 10 nmol/l Tris-HCl (pH 8.3), 1.5 nmol/l MgCl₂, and 0.1% (wt/vol) gelatin. Initial decontamination of the reagents was done by UV irradiation for 30 min and aliquotting in a laminar flow hood in a room separate from the area where products were analyzed. The reaction mixture (excluding the DNA sample) was then incubated with HindIII (30 units per 100 μl of PCR reagent at 37°C for 1 hour), which cleaves within the region flanked by the Y 1.5/1.6 primers. The enzyme was then destroyed by heating at 95°C for 10 min. before addition of the DNA samples. The products of the PCR amplification were subjected to 4% agarose gel electrophoresis and visualized by exposure to ultraviolet light.

Quantitative PCR was performed using primers from the amelogenin gene AMXY-2F and AMXY-CR, which amplify a homologous region of the gene that is found on both the X and Y chromosomes. The sizes of the amplified fragments were 809 base pairs from the X chromosome and 621 base pairs from the Y chromosome. Denaturation was at 95°C for 20 sec, with annealing at 60°C for 1 min, 30 sec and extension at 72°C for 2 min. Each 100 μl of reaction mixture contained 2 U Taq DNA polymerase, 200 μmol of each deoxynucleotide triphosphate, 1 μmol of each primer, 50 nmol/l KCl, 10 nmol/l Tris-HCl (pH 8.3), 1.0 nmol/l MgCl₂, and 0.1% (wt/vol) gelatin. [³²P]dATP (0.25 μl) was added to 25 μl of reaction mixture. The PCR products were visualized after 4% agarose gel electrophoresis. The dried gels were applied to a phosphorimage plate for 24 hours.

**Phosphorimage Analysis**

The PCR products were analyzed on a series 400 phosphorimager using Imagequant software. The baseline was calculated for each sample from an area of the lane on the agarose gel where no PCR product was visualized, and the product bands were outlined using the standard rectangle image. The pixel volume was then calculated by integration of the rectangular area, and ratios of the X : Y products calculated. Repetitive analysis of the same image using this technique gave an error of 1.4%.

**RESULTS**

**PCR Products**

The nested PCR used to amplify single-copy Y-sequence DNA (Y1.5/1.6, Y1.7/1.8) generated amplified products of the expected molecular weights. Stringent decontamination precautions were maintained with regular checks for any contamination problems (Fig 1). Preliminary data from the analysis of a triplet pregnancy shows that the Y sequence was not detected at 3 weeks, 5 days of gestation and was first detected at 5 weeks, 5 days of gestation. Y sequence could be amplified throughout the 12 weeks that were monitored. The current gestation of the pregnancy is only
FIGURE 1. Amplification of male and female DNA using nested Y primers. Full decontamination precautions. Lanes 1 and 11, 1-kb marker; lane 2, female DNA (1 μg); lane 3, female DNA (1 μg) + male DNA (10 pg); lane 4, female DNA (1 μg); lane 5, female DNA (1 μg) + male DNA (50 pg); lane 6, female DNA (1 μg); lane 7, female DNA (1 μg) + male DNA (100 pg); lane 8, female DNA (1 μg); lane 9, female DNA (1 μg) + male DNA (200 pg); lane 10, blank.

14 weeks, and therefore it is not possible to check the fetal sexes by ultrasound scanning.

The PCR used to amplify single-copy X and Y sequence DNA (AMXY-2F and AMXY-CR) also generated amplified products of the expected molecular weight (FIG. 2).

Standardization of PCR Conditions

Depletion of PCR components, saturation of Taq polymerase activity, or incomplete strand denaturation, evident as a loss of amplification efficiency (plateau), appears to bias the ratio of X : Y product towards Y product amplification, as seen in Figure 3.

The logarithmic phase of amplification was identified by amplifying 25 ng of template DNA for a variable number of cycles, and the quantity of product was measured by the incorporation of radioactivity labeled [32P]dATP into the product. Amplification was found to be logarithmic up to 25 cycles, after which the amount of product plateaus, and there is considerable distortion of the X : Y product ratio.

DNA prepared from male blood was presumed to have equimolar amounts of X and Y templates. From analysis of the sequence of the amelogenin gene, the number of A-T pairs in the X product is 257 and in the Y product 215. The relative intensity of the 32P-labeled X and Y products from male DNA should therefore be 1.2. Repetitive

[Note added in proof: The patient described has recently given birth to two girls and one boy. Mother and babies are doing fine.]
analysis of male DNA amplified using the amelogenin primers for less than 25 cycles consistently gave an X/Y ratio of 1.1 (error 11.5%).

Artificial mixes of female and male DNA up to 1000:1 were analyzed after amplification (Fig. 4). For 11:1 mixes of X and Y DNA, the results were again consistent, with average ratios after amplification of 12:1 (error 15%). At increased ratios, the error became much greater, up to 50% at 100:1 ratios.
FIGURE 3. Effect of increasing rounds of PCR on amplification of male DNA using amelogenin primers. Data analysis from phosphorimager. Lanes 1-3, 20 rounds of PCR; lane 4, blank; lanes 5-10, 25, 26, 27, 28, 29, and 30 rounds of PCR, respectively.
FIGURE 4. Quantitation of mixes of male and female DNA after 20 rounds of PCR using amelogenin primers. Data analysis from phosphorimager. Lane 1, female DNA (2.5 mg); lane 2, female DNA (250 ng); lane 3, female DNA (25 ng); lane 4, male DNA (2.5 mg); lane 5, male DNA (2.5 ng); lane 6, 1 : 1 ratio of X : Y DNA; lane 7, 101 : 1 ratio of X : Y DNA; lane 8, 1001 : 1 Ratio of X : Y DNA.
Addressing the question of the timing of the appearance of Y sequence (fetal) DNA in the maternal circulation requires a precise knowledge of the timing of conception, and it is for this reason that women on an IVF program were chosen as the study group. Previous studies by Lo et al. describe amplification of Y sequence DNA in the blood of one pregnant woman recorded at 6 weeks' gestation, and several groups described detection at 8+ weeks gestation on maternal blood enriched for trophoblast cells, but the timing of conception was not accurately known in these cases. The potential sources of fetal DNA in the maternal circulation are fetal blood cells and trophoblast cells. The presumed source of fetal blood cells is fetomaternal hemorrhage. However, at 6 weeks' gestation the volume of fetal blood would be very small and unlikely to be contributing to the Y sequence DNA detected. The most likely source of the detected Y sequence is trophoblast cells.

Because the Y sequence was detectable in the blood of a woman pregnant with triplets at 5 weeks, 5 days gestational age, we must ask whether this is representative of all pregnancies. The placental area exposed to the maternal circulation would be expected to be greater (though not three times greater) than for a singleton pregnancy, and therefore the dissemination of trophoblasts into the maternal circulation may also be greater. Likewise, fetomaternal hemorrhage may be greater in a triplet pregnancy; and if all three, or two of the three, fetuses were male, then a greater contribution would be made than in a singleton pregnancy. Invasion by cytotrophoblast of the maternal spiral arterioles is said to occur at 6 to 8 weeks of gestation, and there is no physiological reason why the timing of invasion should be altered in a triplet pregnancy. Further analysis of the blood samples collected is required to answer these questions.

Quantitative PCR is becoming widely used to measure the appearance of small amounts of target DNA in a background of excess genomic DNA. It has been particularly successful when applied to detection of HIV DNA. Mutter et al. used quantitative PCR of ZFX and ZFY DNA to measure aneuploidies in cultured cell populations and were able to quantify Y target sequences accurately down to 10% of the population; these results are comparable with our current data. The loss of accuracy below this level can be explained by the requirement of quantitative PCR to be optimized for a given amount of target DNA. As the ratios of X : Y DNA become greater, the amount of either X or Y target DNA will be outside the linear range for quantitation, resulting in inaccurate analysis through plateauing of the X product. For this reason, the quantitative PCR technique at present is not suitable for application to maternal blood samples in which only 1 in 100,000 cells are of fetal origin. It could be applied to achieve quantitative analysis on samples of maternal blood enriched for fetal cells, or for assessing enrichment strategies.

It is clear that fetal cells appear in the maternal circulation as early as six weeks of pregnancy, at least in some cases; further experiments are under way to determine how much variation there is from one person to another. However, the use of fetal cells for predictive testing, whether using cytogenetic or DNA techniques, will require considerable enrichment of fetal cells from maternal nucleated cells even with the most sensitive existing techniques. Continued work on quantification strategies is essential in order to provide the answers to basic questions about the presence of
fetal DNA in the maternal circulation that are required before testing can become routine.

REFERENCES

THE TIME OF APPEARANCE AND DISAPPEARANCE OF FETAL DNA FROM THE MATERNAL CIRCULATION

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SUMMARY

A single copy Y-chromosome DNA sequence was amplified using the polymerase chain reaction (PCR) from the peripheral blood of 30 women who had achieved a pregnancy through an in vitro fertilization (IVF) programme. The time of conception was known precisely and was confirmed by serial ultrasound scans. Conceptions were dated as the number of weeks after fertilization plus 2, to give a time equivalent to the obstetric menstrual dating of the pregnancy (LMP). Y-chromosome-specific DNA was detected in all pregnancies with a male fetus (18/30). The earliest detection was at 4 weeks and 5 days, and the latest at 7 weeks and 1 day. Y-chromosome-specific sequences were no longer detected in any of the male pregnancies 8 weeks after delivery. No Y-chromosome sequences were detected in any of the pregnancies where only female babies were delivered. This demonstrates that fetal DNA appears in the maternal circulation early in the first trimester, that it can be identified in all pregnancies tested by 7 weeks, that it continues to be present throughout pregnancy, and that it has been cleared from the maternal circulation 2 months after parturition. Early non-invasive prenatal diagnosis for aneuploidies and inherited disorders will be possible in all pregnancies if fetal cells can be isolated free from maternal contamination (or identified accurately in the presence of maternal cells) without problems of contamination from previous pregnancies.

KEY WORDS: fetal cells; maternal peripheral blood; timed serial samples.

INTRODUCTION

The retrieval of fetal cells from the maternal circulation offers the possibility of non-invasive prenatal cytogenetic or DNA diagnosis. Two cell types have been demonstrated in maternal blood; fetal blood cells and trophoblast.

Several groups have isolated fetal erythroblasts from the maternal blood and have correctly deter-
et al., 1989; Adinolfi et al., 1989). The presence of Y-chromosome-specific sequences can be reliably detected in unenriched peripheral maternal blood samples without contamination, using nested primers as described by Lo et al. (1990).

For non-invasive prenatal screening to become routine, it is necessary to determine whether fetal cells are present in sufficient numbers for analysis throughout the pregnancy and whether there is significant variation in the time of appearance of such cells. It is also necessary to know whether cells from previous pregnancies persist in the blood stream and, if not, how soon after pregnancy the cells are cleared from the blood. These data are difficult to obtain from normal pregnancies presenting for prenatal care, as the time of conception is rarely known with accuracy and women usually present several weeks into a pregnancy. Reviews of the literature reveal that these questions have not yet been adequately answered (Adinolfi, 1992; Morris and Williamson, 1992). This study analyses serial blood samples obtained from a large series of accurately dated pregnancies for Y-chromosome-specific DNA with nested PCR.

**PATIENTS AND METHODS**

**Patients**

In order to determine the timing of the appearance of fetal cells in the maternal circulation, a prospective study was initiated involving women undergoing treatment for infertility whose date of conception was accurately known. All patients participating in the study gave informed written consent, and ethics committee approval for the study was obtained from the London Gynaecology and Fertility Centre Ethics Committee.

Thirty women undergoing infertility treatment were approached when their first beta human chorionic gonadotrophin (βhCG) pregnancy test was positive: 10 days after embryo transfer (n=26), timed sexual intercourse (n=1), or artificial insemination (n=3). Ten ml of blood was taken into vacuumer sodium citrate bottles and stored at −20°C. Serial blood samples were taken at fortnightly intervals until 12 weeks' gestation, and monthly thereafter until 2 months after delivery. The gestation was defined as commencing 2 weeks prior to embryo transfer, timed sexual intercourse, or artificial insemination.

DNA was prepared from the samples under class II containment conditions and the DNA was stored at −20°C. The samples were analysed for the presence of a single copy Y-chromosome-specific DNA sequence, using a nested PCR strategy first described by Lo et al. (1990). The women also completed a questionnaire regarding their past obstetric history and details of the present pregnancy. All experiments to identify sequences in maternal blood were carried out blind to the patient, the previous obstetric history, and the other data which emerged during the pregnancy.

**DNA preparation and PCR**

For the preparation of DNA from patient blood samples, a DNA extraction kit (Nucleon II, Scotlab) was used following the instructions in the kit protocol. All manipulations were carried out in a laminar flow hood geographically separated from the lab areas used to set up PCRs or to run the products of PCRs. Each DNA extract was vortexed for 30 s to fragment long DNA strands and to mix the DNA before samples were taken for PCR. Two μg of DNA (approximately 1–2 per cent of the extracted DNA) was subjected to 40 cycles of PCR with primers Y 1-5/1-6 amplifying a Y-chromosome-specific 239 base pair (bp) fragment (Lawler et al., 1989). After the initial amplification, 2-5 μl of the PCR product was reamplified for 25 cycles with primers Y 1-7/1-8 amplifying a 198 bp fragment (Lo et al., 1990). Denaturation was at 94°C for 30 s, with annealing at 57°C for 1 min and extension at 72°C for 2 min. Each 100 μl of reaction mixture contained 10 μl of sample DNA, 2 U of Taq DNA polymerase, 200 μmol of each deoxynucleoside triphosphate, 0-8 mmol of each primer, 50 mmol/l KCl, 10 mmol/l Tris–HCl (pH 8.3), 1-5 mmol/l MgCl₂, and 0-1 per cent (weight/volume) gelatin.

To avoid contamination, the reagents were pipetted in a laminar flow hood in a room separate from the area where products were analysed, followed by incubation of the reaction mixture (excluding the DNA sample) with HinI (30 U per 100 μl of PCR reagent at 37°C for 1 h), which cleaves within the region flanked by the Y-chromosome 1-5/1-6 primers. HinI was denatured by heating at 95°C for 10 min before addition of the maternal DNA samples. The products of the PCR amplification were run using 4 per cent agarose gel electrophoresis and visualized by exposure to ultraviolet light after ethidium bromide staining. The samples were analysed blind.
RESULTS

Of the 30 women recruited, 13 had had no previous clinical pregnancies, 5 had had male babies previously, and 2 had had female babies previously. The remaining ten women had had pregnancies which had ended in the first trimester, in ectopic pregnancy, or spontaneous or therapeutic abortions for which fetal sexing was not available.

Nine of the 30 pregnancies were diagnosed on early scan as multiple. One case began as a triplet pregnancy, with intrauterine death of two of the fetuses confirmed on scan at 8 weeks' pregnancy. The pregnancy continued uneventfully, with the delivery of a singleton male baby at term. Two cases of twin gestation showed intrauterine death of one of the twins at 12 weeks' gestation. The pregnancies continued and resulted in live singleton births. One other patient with a twin pregnancy miscarried following amniocentesis at 17 weeks' gestation. Within the study, four women delivered twins and one triplets.

In one singleton pregnancy, an intrauterine death was diagnosed at 19 weeks' gestation. There were no cases of ante-partum haemorrhage or other complications in any pregnancies resulting in live births, except in one twin pregnancy with diabetes and hypertension. Of the live births, 17 women (only one of whom had invasive prenatal diagnosis, at 16 weeks' gestation) had male babies and 11 had only female babies. All babies were delivered with no apparent birth defects between 33 and 42 weeks' gestation when the sex was confirmed by inspection. Those delivered at 36 weeks or less were the multiple pregnancies. Six deliveries were by Caesarean section and 22 delivered vaginally. None of the placentae required manual removal following the birth of the baby.

In all cases where a male baby or babies were born, a Y-chromosome DNA sequence was detected in the maternal blood during the pregnancy (Table I). In all cases where female babies only were delivered, no Y-sequence DNA was amplified at any gestational age. Table I shows the time during the pregnancy at which Y-chromosome-specific DNA was first detected in the 18 women with male fetuses, and when samples were taken throughout the pregnancies. The earliest gestational age at which Y-chromosome-specific DNA first appeared was 4 weeks and 5 days (two patients), and the latest 7 weeks and 1 day. It continued to be amplified from the maternal blood until 4 weeks after delivery (see Fig. 1), except in one sample (patient 4) when, at 12 weeks' gestation, only 1 ml of blood was available for DNA preparation and analysis. The average first age of detection was 6 weeks ± 4 days. No Y-chromosome sequence could be detected 8 weeks after delivery, regardless of the method of delivery.

DISCUSSION

It is necessary to know the precise time of conception if the time of appearance of fetal DNA in the maternal circulation is to be determined. For this reason, a cohort of women participating in an IVF programme was chosen as the group for this study. Previous studies on DNA extracted from unsorted peripheral blood by Lo et al. (1990) describe the amplification of Y-chromosome DNA sequences in the blood of one pregnant woman recorded as 6 weeks' gestation. Liou et al. (1993) studied 19 pregnancies with male fetuses and were able to amplify Y-chromosome-specific DNA at 6 weeks' gestation in one pregnancy. They were able to diagnose male fetal sex in only half the cases by 8 weeks' gestation, and in one case not until 12 weeks. These scatters of gestational ages of first appearance of fetal sequences may reflect inaccuracies in dating the pregnancies.

Ganshirt-Ahlert et al. (1993) detected 10–100 nucleated erythrocytes after density gradient and magnetic cell sorting of 10 ml samples of maternal blood at 8–12 weeks' gestation. Other groups have described the detection of trophoblast cells at 9 or more weeks' gestation using specific antibodies, but blood samples from earlier gestations were not analysed (Mueller et al., 1990; Hawes et al., 1994).

The possible origins of fetal DNA in the maternal circulation are from fetal blood cells and trophoblast. The presumed source of the fetal blood cells is feto-maternal haemorrhage. Fetal blood cells are formed initially in the yolk sac from 4 weeks' gestation, but do not circulate until the heart begins to beat at 5 weeks. Fetal vessels are not present within the chorionic villous stroma until 8 weeks. It is unlikely that fetal blood elements would be able to escape into the maternal circulation as early as 5 weeks' gestation. The most likely source of the Y-chromosome DNA sequence detected during weeks 5–8 is trophoblast. Trophoblast development in the early embryo is very
Table I—Earliest detection of Y-chromosome-specific DNA in pregnancies with male fetuses (18 patients)

<table>
<thead>
<tr>
<th>Patient</th>
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<th>3‡</th>
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<th>16††</th>
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<td>Days</td>
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<td>5</td>
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<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
</tr>
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</table>

All tests for the 18 patients with male fetuses are listed. A plus sign represents detection of the Y-chromosome-specific band, and a minus sign its absence. Gestational ages are shown in weeks plus day after the last menstrual period as calculated 14 days prior to embryo transfer or intrauterine insemination. Pregnancies marked with an asterisk were conceived following intrauterine insemination, all others following IVF and embryo transfer.

1 mo.a.d. = One month after delivery; 2 mo.a.d. = Two months after delivery.

*Conception through intrauterine insemination (all other pregnancies achieved through IVF). Case previously quoted (Thomas et al., 1994).
†Intrauterine death diagnosed at 12 weeks' gestation. Last blood sample taken 1 and 2 months after ERPC.
‡Twin pregnancy with one male and one female baby, gestational diabetes, raised maternal blood pressure, and amniocentesis at 16 weeks.
§At 12 weeks' gestation only 1 ml of blood was obtained.
¶Initial twin pregnancy, IUD of one embryo at 12 weeks' gestation.
††Twins pregnancy, one male and one female baby.
**Initial triplet pregnancy, IUD of two fetuses at 8 weeks' gestation.
†††Triplet pregnancy, two male babies. Case previously quoted (Thomas et al., 1994).
‡‡Triple pregnancy with one male and two female fetuses.
rapid, and by 4 weeks, the lacunae of the trophoblast are filled with maternal blood into which knots of syncitial trophoblast may be shed directly. After 8 weeks' gestation, the relative contribution of fetal blood and trophoblast cells to the fetal DNA detected in maternal blood is unknown; it may change as the pregnancy advances. Bianchi et al. (1991) presented data indicating that there were no detectable fetal nucleated red blood cells at 16-20 weeks' gestation, compared with 11-12 weeks when analysed for Y-chromosome DNA sequences.

Our data indicate that fetal DNA is detectable in all pregnancies, beginning in a narrow time band between 5 and 7 weeks' gestation. These fetal cells can be used for prenatal diagnosis, provided that enrichment strategies for the appropriate cell type are used.

Contamination of maternal blood with Y-chromosome-specific DNA from the previous pregnancies was not seen at the level of detection which can be achieved with nested PCR. In our study, of the five women who had previously had male infants, four delivered female babies and no Y-chromosome-specific DNA fragments were amplified.

In one case, an intrauterine death was detected at a routine scan at 19 weeks. The previous scan at 12 weeks' gestation had shown that the fetus was alive and well. Y-chromosome-specific DNA was amplified from the maternal blood from 6 weeks' gestation until 12 weeks. Samples taken at 19 weeks and after suction curettage were negative. Unfortunately, no fetal material was available to determine the fetal sex. The fact that a suction curettage was undertaken implies that the fetus died well in advance of the 19-week scan. If fetal cells are cleared from the maternal blood at the same rate in early pregnancy as that following term delivery, then the disappearance of the Y-chromosome-specific sequence from the maternal blood would imply that the fetus died between 12 and 14 weeks' gestation.

In this small prospective study, we were able to identify the fetal sex correctly in all 29 women for whom the sex was known, either by subsequent testing or after birth. We were also able to amplify Y-chromosome-specific DNA in all cases with male fetuses by 7 weeks +1 day gestation. This early detection in all cases, together with the clearance of Y-chromosome-specific DNA by 8 weeks after delivery, indicates that fetal DNA in maternal blood may provide the basis for non-invasive early prenatal screening for aneuploidies and inherited disorders.
ACKNOWLEDGEMENTS

We thank all the doctors and technical staff at the London Gynaecology and Fertility Centre for their help in collecting and storing samples, and all those women who were good enough to give their time and blood to make this study possible. This research was funded by Life-Force Research Ltd.

REFERENCES

Zusammenfassung


Nichtinvasive Pränataldiagnostik an fetalen Zellen im mütterlichen Blut

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Fetale Zellen im mütterlichen Blut sind seit über 100 Jahren Gegenstand medizinischer Forschung [59, 60], und die Ansätze zu ihrer Gewinnung und Untersuchung spiegeln die Entwicklung morphologischer, zytogenetischer, zellbiologischer und molekularbiologischer Methoden wider [1, 3, 37, 49]. Invasive Techniken zur Erlangung fetalen genetischen Materials bei der pränatalen Diagnostik von chromosomalen und genetischen Störungen tragen ein, wenn auch kleines, durch den Eingriff bedingtes Risiko sowie nicht unerhebliche Kosten; sie können deswegen nur Patientinnen mit erhöhtem Risiko angeboten werden (Tabelle 1).


Historischer Überblick

Die erste Beschreibung fetaler Zellen in der mütterlichen Blutbahn geht bis ins letzte Jahrhundert zurück. Mit dem Blutstrom verschleppte Trophoblastzellen wurden in mütterlichen Lungenkapillaren gefunden [59, 60], und auch auf Röntgenaufnahmen der Lungengefäße waren solche Emboli sichtbar [40]. Die Trophoblastzellen sind auch im Uterus bei einer Schnittentbindung gewonnenen unteren Venenblut nachweisbar [2].

Versuche, fetale Lymphozyten aus nicht spezifisch angereicherten mütterlichen Blutproben anzuzüchten, waren letztlich nicht erfolgreich [3]. Beim Vorliegen bestimmter Konstellationen im Leukozytenantigensystem („human leukocyte antigens“, HLA-System) können väterlich ererbte Antigene, die sich von den mütterlichen unterscheiden, zur Isolierung fetaler Lymphozyten mit spezifischen Antikörpern verwendet werden [72]. Am erfolgversprechendsten scheinen die Ergebnisse der Untersuchungen an durch spezifische Sortierung angereicherten fetalen kernhaltigen roten Blutzellen zu sein („nucleated red blood cells“, nRBC). Verschiedene Gruppen haben auf diesem Weg bereits fetale Aneuploidien nachweisen können [9, 24, 29].

Eine besondere Bedeutung kommt der Anwendung der Polymerasekettenreaktion („polymerase...
Invasive und nichtinvasive Techniken zur pränatalen genetischen Diagnostik

<table>
<thead>
<tr>
<th>Methode</th>
<th>Zeitpunkt</th>
<th>Bedeutung</th>
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<tbody>
<tr>
<td>Amniozentese</td>
<td>&gt; = 15 SSW</td>
<td>Abortrisiko, späte selektive Abruption mit möglichen medizinischen und</td>
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<td>psychologischen Komplikationen</td>
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<tr>
<td>Kordozente</td>
<td>&gt; = 18 SSW</td>
<td>Abortrisiko, späte selektive Abruption mit möglichen medizinischen und</td>
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<td>psychologischen Komplikationen</td>
</tr>
<tr>
<td>Chorionzottenbiopsie</td>
<td>&gt; = 10 SSW</td>
<td>Abortrisiko, frühe selektive Abruption mit weniger Komplikationen</td>
</tr>
<tr>
<td>Fetales Zellen im mütterlichen Blut&lt;sup&gt;a&lt;/sup&gt;</td>
<td>ab 5.-7. SSW</td>
<td>kein fetales Risiko, selektive Abruption, zuverlässige Diagnose?</td>
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<tr>
<td>Präimplantationsdiagnostik&lt;sup&gt;b&lt;/sup&gt;</td>
<td>vor Implantation</td>
<td>keine Abruption nötig, Fruchtschädigung?, zuverlässige Diagnose?</td>
</tr>
<tr>
<td>Serumscreening&lt;sup&gt;c&lt;/sup&gt;</td>
<td>15-16 SSW, evtl. auch im ersten Trimenon</td>
<td>kein fetales Risiko, aber keine definitive genetische Diagnose, sondern Angabe von Risikoziffer</td>
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</table>

<sup>a</sup> Experimentelle Forschung.  
<sup>b</sup> Angewandte Forschung.  
<sup>c</sup> Serumscreening etabliert für Zwittrimesterscreening, weitere Untersuchungen zum Ersttrimesterscreening evtl. in Kombination mit Ultraschallmarkern. (Mod. nach [12])

Das Übertreten fetalen Bluts in die mütterliche Zirkulation wird als Einschwemmung fetaler Blutzellen betrachtet. Ein klassischer Beweis für diesen Umstand, dessen physiologische Bedeutung und zeitliches Auftreten nicht endgültig geklärt sind, ist die Bildung von Antikörpern gegen kindliche Blutgruppenantigene. Verschiedene kernhaltige fetale Zellarten, die bei einer fetomaternalen Blutung in die mütterliche Zirkulation gelangen können, sind hinsichtlich einer nichtinvasiven Pränaldiagnostik untersucht worden: kernhaltige rote Blutzellen (nucleated red blood cells, nRBC) und Leukozyten (Lymphozyten). Daneben haben in den letzten Jahren auch Trophoblastzellen besonderes Interesse gefunden [57].

**Lymphozyten**


Ein Bericht existiert über die Identifikation fetaler Granulozyten mittels der Durchflußzytometrie („fluorescence activated cell sorting‘, FACS) gelang erstmals die Anreicherung fetaler Zellen in Schwangerschaften, in denen eine vom Vater ererbte männliche Zellpopulation vorhanden war [3]. Mittlerweile kann die Fetalzelle-DNA in nahezu jedem weiteren Schwangerschaftstest bestimmt werden.

**Kernhaltige rote Blutzellen**

Im ersten Trimenon der Schwangerschaft stellen kernhaltige rote Blutzellen („nucleated red blood cells“, nRBC) die Mehrzahl der kernhaltigen Zellen im fetalen Blut (30–70%), werden also bei einer fetomaternalen Bluttransfusion nachweisbar. Die fetale Blutmenge erreicht bis zur Geburt einen Wert von etwa 100 ml. Das Chorion frondosum, das in der 12. bis 17. SSW eine Oberfläche von bis zu 25 m² erreicht [4], besteht aus einer Schicht von Blutzellen, die die Lungenkapillarbetten abfangen, verursacht durch die Hemmung der Zellwanderung der Zellen. Dies zusammen mit Informationen über die Zellgröße und Zellgranularität wurde zur Abgrenzung der nRBC aus [54]. Auch mit dieser Methode ließen sich an fetalen nRBC aus mütterlichem Blut kernhaltige Zellen nachweisen [24].

Das Chorion frondosum, das in direktem Kontakt mit der mütterlichen Blutbahn steht und eine Oberfläche von bis zu 25 m² erreicht [4], besteht aus einer Schicht von kernhaltigen Zellen, die die Lungenkapillarbetten abfangen. Dies zusammen mit Informationen über die Zellgröße und Zellgranularität wurde zur Abgrenzung der nRBC aus [54]. Auch mit dieser Methode ließen sich an fetalen nRBC aus mütterlichem Blut kernhaltige Zellen nachweisen [24].

**Anreicherung und Untersuchung: Techniken und molekularbiologische Methoden**

Zellgranularität
(side scatter)

Zellgröße
(forward scatter)

Zu untersuchende Zellen werden einzeln senkrecht durch den Laserstrahl geleitet


Ein weiterer Ansatz zur Ergänzung der physikalisch-biochemischen ist die biologische Anreicherung durch Ausnutzen des selektiven Wachstumsreizes fetaler Blutzellen. Durch spezifische Wachstumsreize wie z. B. Inkubation von fetalen Blutzellen mit Wachstumsfaktoren der roten Zellreihe lassen sich im mütterlichen Blut enthaltene fetale Zellen in Kultur mit Erythropoetin vermehren [45]. Es muß noch weiter untersucht werden, ob es sich dabei nur um eine relative Vermehrung durch Absterben nicht ansprechender mütterlicher Blutzellen oder um eine absolute Zunahme der fetalen Zellzahl handelt.

Dem ersten Anreicherungsschritt schließt sich in den meisten Verfahren eine antikörpervermittelte Sortierung an. Zwei Methoden werden angewandt: die Durchflußzytometrie (FACS) und die magnetische Zellsortierung. Das Prinzip der Sortierung mittels FACS (s. Abb.1) beruht auf der spezifischen Bindung fluoreszenzmarkierter Antikörper, z. B. anti-CD 71 oder anti-GPA für nRBC. Wird die Suspension mit den fetalen
Spezifische Erst-Antikörper / Antikörperbeschichtete Magnetpartikel

Waschen der Zellen

Abb. 2a–d. Schematische Darstellung der magnetischen Zellsortierung (MACS) [48].


Die wichtigste Alternative für die fetale Zellsortierung ist die magnetische Zellsortierung (s. Abb. 2 [14, 23, 26, 72]). Sie beruht auf der Bindung von spezifischen Antikörpern, nur sind hier – je nach Hersteller – mikroskopisch bis submikroskopisch kleine Magnetpartikel (Durchmesser 0,05–5 µm) an die Antikörper gebunden. Wird dann die Zellsuspension z.B. durch eine magnetisierte Matrix geleitet, werden die spezifisch markierten Zellen zurückgehalten und können erst nach Entfernen des magnetischen Felds eluiert werden. Ein Vorteil dieses Systems sind die vergleichsweise geringen Kosten, die einfache Handhabbarkeit und der geringe Zeitaufwand für die Sortierung einer Probe. Der Name des meistverwendeten Produkts, MACS („magnetic cell sorting“ [48]), wird synonym mit dem Verfahren verwendet.

Prinzipiell werden bei der Sortierung mittels Antikörper verschiedenere Strategien verfolgt, um fetale Zellen anzureichern. Siehe für die fetalen Zellen verschiedene spezifische Antikörper zur Verfügung, kann eine positive Anreicherung durchgeführt werden, bei der fetale Zellen direkt gebunden und die mütterlichen Zellen ausgewaschen werden (z.B. Bindung an den Transferinrezeptor oder Glycophorin A der nRBC, HLA-Epitope fetaler Lymphozyten, trophoblastspezifische Epitope [10, 23, 26, 72]). Es gibt eine große Zahl gut charakterisierter Antikörper gegen Leukozytenantigene, von denen viele auf den relativ unreifen fetalen Zellen nicht exprimiert werden, so daß durch ihre Anwendung eine sog. „negative Sortierung“ durchgeführt werden kann [18, 74]. Eine dritte Möglichkeit schließlich ist die Kombination beider Verfahren, z.B. durch Depletion weißer Blutzellen, gefolgt von einer spezifischen Anreicherung fetaler Zellen [14].

Wie bereits angesprochen, sind aus dem mütterlichen Blut gewonnene fetale Zellen selbst nach den besten Anreicherungsverfahren im Vergleich mit mütterlichen kernhaltigen Zellen noch in der Minderzahl. Abgesehen von mehrkernigen Synzytiothrophoblastzellen, die sich bei mikroskopischer Betrachtung deutlich von allen im mütterlichen Blut zirkulierenden kernhaltigen Zellen unterscheiden, kann die allein morphologische Identifikation durch verschiedene Verfahren ergänzt werden. Sind die Zellen z.B. auf einem Objekträger aufgebracht, kann die Auswertung nach spezifischer Markierung der fetalen Zellen auf diese beschränkt werden. Der am häufigsten verwendete Marker für fetale Zellen ist der Nachweis des Y-Chromosoms mittels FISH (Abb. 3, 4), der allerdings nur bei männlichen Fetten die Zellen identifiziert. Eine weitere Möglichkeit, z.B. nRBC unabhängig vom fetalen Geschlecht spezi-
Zu untersuchendes genetisches Material: doppelsträngige DNS

...ATGCCCCGATAGGCT....

+ ATGC

...ATGCGTACGGCTATCCGA....


fisch hervorzuheben und so eine Auswertung auf die fetalen Zellen in der angereicherten Fraktion zu konzentrieren, ist die sog. Immunphänotypisierung, bei der mit einem Antikörper die γ-Kette des fetalen Hämoglobins angefärbt wird (s. Abb. 4 [74]). Auch der Nachweis der Expression gewebespezifischer Gene in einzelnen Zellen über die Bestimmung der Messenger-RNS (mRNS, z.B. Hämoglobin-F-mRNS in nRBC, hCG-mRNS in Trophoblastzellen) könnte zur Identifikation fetaler Zellen genutzt werden. Unter Untersuchungstechniken


Das klassische Untersuchungsverfahren für chromosomale Störungen besteht in der Anzüchtung der Zellen und der Betrachtung des Chromosomenmusters des in der Metaphase künstlich angehaltenen Zellzyklus. Dabei werden numerische und strukturelle Aberrationen erkennbar. Allerdings sind die aus dem Blut isolierten fetalen Zellen in der Regel nicht mehr teilungsfähig, so dass eine Methode zur Untersuchung an Zellkernen in der Interphase benutzt werden muss. Die Fluoreszenz-in-situ-Hybridisierung (FISH, s. Abb. 3) wurde ursprünglich entwickelt, um Gene physikalisch bestimmten Chromosomen zuzuordnen und ihre Lage zueinander zu bestimmen. Ihr Prinzip beruht auf der Doppelsträngigkeit und Komplementarität der DNS. Wird der DNS-Doppelstrang aufgetrennt („denaturiert“), nehmen die beiden Stränge nach Möglichkeit wieder die einander gegenüberliegende ursprüngliche und durch chemische Brücken verstärkte Stellung ein, die durch die sich gegensinnig ergänzende (komplementäre) Sequenz auf den Strängen vorgegeben ist. Wird aber die DNS-Doppelhelix unter Zugabe kurzer, fluoreszenzmarkierter DNS-Stücke definierter Sequenz, den DNS-Sonden, denaturiert, so lagern sich diese Sonden unter Bildung eines Hybriddoppelstranges statt des gegenläufigen Strangs an die komplementäre Sequenz an („Hybridisieren“). Dadurch kann eine bestimmte, bekannte DNS-Sequenz mikroskopisch sichtbar gemacht werden (s. Abb. 3, 4). Hybridisiert also z.B. eine mit grüner Fluoreszenz markierte DNS-Sonde, die spezifisch für eine Sequenz ist, die nur auf dem Y-Chromosom vorkommt, mit einer auf fetale Zellen angereicherten Blutprobe, werden
A b b .4 a - f. K e r n h a l t i g k i n d l i c h e r o t e  B l u t z e l l e n  ( n R B C ). F e t a l e s  H a r a o g l o b i n  ( H b - F ) w i r d d u r c h e i n e n  s p e z i f i s c h e n  A n t i k ö r p e r u n d  r o t e  F a r b u n g  d e s  Z y t o p l a s m a s  n a c h g e w i e s e n  ( I m -
phänotypisierung). I n f e t t e n  Z e l l e n  z u s a z l i c h d u r c h s p e z i f i s c h e  G e n s o n d e n  d i e  X - u n d  X - C h r o m o s o m e n  d e r Z e l l e n  a n g e f a r b t  ( F I S H .
A b b .4 b , d , f). a  n R B C  a u s  d e m  N a b e l s c h n u r b l u t e i n e n m a n n l i c h e n  N ' e u g e b o r n e n  m it
H b F - N a c h w e i s  ( r o t e  Z y t o p l a s m a f a r b u n g ). b  D i e s e l b e  Z e l l e  w i e  a m it  F I S H - N a c h w e i s  d e s
Y - C h r o m o s o m s  d u r c h  e i n e  g r ü n e  m a r k i e r t e  Y - D N S - S o n d e . c  A u s  d e m  B l u t e i n e r  S c h w a n g e-
rere n  m i t  m a n n l i c h e m  F e t e n  i s o l i e r t e  f e t a l e  n R B C  m it  H b F - F a r b u n g . d  D i e s e l b e  Z e l l e  w i e  c
m it  F I S H - N a c h w e i s  v o n  z w e i  X - C h r o m o s o m e n  d u r c h  A n w e n d u n g  e i n e r  g r ü n e n  m a r-
k i e r t e n  X - D N S - S o n d e . ( A u s  [ 7 4 ] , m i t  f r e u n d l i c h e r  G e n e h m i g u n g  d e s  V e r l a g s  u n d  d e r  A u t o-
nur in männlichen Zellen grüne Flu-
oreszenzsignale sichtbar; je ein grüner
Punkt für jeden männlichen Zell-
kern. kein grüner Punkt in Kernen
weiblicher Zellen. Aufgrund der ho­
hen Spezifität der DNS-Sonden kön-
nen durch Verwendung verschiede-
der Fluoreszenzfärbbstoffe (Fluo-
chrom) Sonden gegen zwei oder
mehr verschiedene Chromosomen
eingesetzt und dadurch die Anzahl
der untersuchten Chromosomen in
einer großen Zahl von Zellkernen re-
lativ schnell ermittelt werden, ohne
daß die gesamten Chromosomen in
der verdichteten Form der Meta-
phase sichtbar sein müssen. Eine An-
wendung der FISH-Technik ist in
Abb.4 dargestellt: Eine aus dem müt-
erlichen Blut sortierte fetale nRBC
ist mit Sonden für das X- und
das Y-Chromosom hybridisiert. Ein
grünes Y-Signal zeigt einen normalen
männlichen XY-Status dieser
Zelle an, was gleichzeitig den fetalen
Ursprung beweist; zusätzlich ist in
dieser Aufnahme das fetale Hämoglo-
bolin zur weiteren Bestätigung des
fetalen Zellcharakters mittels eines
spezifischen Antikörpers gegen die
γ-Kette des Hämaglobins rot ange-
färbert ([74] mit freundlicher Geneh-
migung durch Autoren und Verlag).
Mittels der FISH-Technik sind an fe-
talen Zellen aus dem männlichen
Blut bereits erfolgreich verschiedene
Aneuploidiediagnosen gestellt wor-
den (s. Tabelle 2).
Eine Weiterentwicklung der
FISH-Technik ist die gleichzeitige
Anwendung mehrerer, mit unter-
dieschiedlichen Fluorochromen mar-
kerter Sonden, wodurch an einem
Zellkern die Anzahl von bis zu fünf
verschiedenen Chromosomen (z. B.
21, 13, 18, X und Y) bestimmt wer-
den kann [21, 41]. Ein Problem der
FISH-Technik besteht darin, daß die
tzu untersuchenden Zellen als fetal
identifiziert werden müssen, bevor
aus FISH-Signalen eine zuverlässige
Diagnose gestellt werden kann. So
kann z. B. ein Zellkern, der mit einer
Chromosom-21-spezifischen Gen-
sonde untersucht wird und zwei Si-
gale zeigt, einer normalen fetalen,
aber auch einer normalen mütterli-
chen Zelle, entsprechen. Mögliche
Abhilfe kann eine statistische Aus-
wertung großer Anzahlen von Zel-
nen die gleichzeitige Anwen-
dung eines fetalspezifischen Mar-
kers, wie etwa der Antikörperfärb-
zung für fetales Hämaglobin in
nRBC, schaffen (s. Abb.4 [74]). Die
Anwendung fluoreszenzmarkierter
Sonden an Interphasenzellkernen
kann aber nur eine Aussage über nu-
merische Aberrationen der im indivi-
duellen Fall untersuchten Chromoso-
men machen; alle anderen Chromo-
somen sowie strukturelle Anomalien
werden nicht erfaßt.
Eine Methode zur Untersuchung
genetischer Veränderungen, die auch
### Tabelle 2

<table>
<thead>
<tr>
<th>Autor</th>
<th>Fetales Zellart</th>
<th>Anreicherung</th>
<th>Technik</th>
<th>Diagnose</th>
</tr>
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<td>Mueller et al. [50]</td>
<td>Trophoblast</td>
<td>MACS</td>
<td>PCR</td>
<td>Y-Chromosom-Sequenz</td>
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<tr>
<td>Lo et al. [46, 47]</td>
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<td></td>
<td>PCR</td>
<td>Y-Chromosom-Sequenz</td>
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<td>Carman et al. [16]</td>
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<td>PCR</td>
<td>Laporte-Hämoglobins/β-Thalassämie</td>
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<td>Lymphozyten</td>
<td>FACS</td>
<td>PCR</td>
<td>HLA-DR4</td>
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<tr>
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<td>FACS</td>
<td>PCR</td>
<td>Y-Chromosom-Sequenz</td>
</tr>
<tr>
<td>Bianchi et al. [7]</td>
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<td>FACS</td>
<td>PCR</td>
<td>Y-Chromosom-Sequenz</td>
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<tr>
<td>Price et al. [54]</td>
<td><em>nRBC</em></td>
<td>FACS</td>
<td>PCR</td>
<td>Y-Chromosom-Sequenz</td>
</tr>
<tr>
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<td></td>
<td>PCR</td>
<td>Rhesus-D-Gen</td>
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<tr>
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<td>FACS</td>
<td>PCR</td>
<td>HLA-DQα</td>
</tr>
<tr>
<td>Thomas et al. [64, 65]</td>
<td></td>
<td></td>
<td>PCR</td>
<td>Y-Chromosom-Sequenz</td>
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<tr>
<td>Geifman-Holtzman et al. [32]</td>
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<td>FACS</td>
<td>PCR</td>
<td>Rhesus-D-Gen</td>
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<tr>
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<td>MACS</td>
<td>PCR</td>
<td>β-Thalassämie</td>
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<tr>
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<td><em>nRBC</em></td>
<td>FACS</td>
<td>FISH</td>
<td>Trisomie 21</td>
</tr>
<tr>
<td>Weismar et al. [71]</td>
<td>Granulozyten, Lymphozyten</td>
<td>nur Dichtegradient</td>
<td>FISH</td>
<td>Y-Chromosom</td>
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<td>Bianchi et al. [9]</td>
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<td>FACS</td>
<td>FISH</td>
<td>Trisomie 21</td>
</tr>
<tr>
<td>Chueh et al. [17]</td>
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<td>FACS, MACS</td>
<td>FISH</td>
<td>Trisomie 21</td>
</tr>
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<td>Simpson und Elias [61]</td>
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<td>FACS</td>
<td>FISH</td>
<td>Trisomien 18 und 21</td>
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<tr>
<td>Ganshirt-Ahlert et al. [29]</td>
<td><em>nRBC</em></td>
<td>FACS</td>
<td>FISH</td>
<td>Trisomien 21 und 18</td>
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</tbody>
</table>

### Tabelle 3

<table>
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<tr>
<th>Patienten</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>13</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wochen</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>5</td>
<td>6</td>
<td>7</td>
<td>8</td>
<td>12</td>
<td>16</td>
<td>20</td>
<td>24</td>
<td>28</td>
<td>32</td>
</tr>
<tr>
<td>Tage</td>
<td>3</td>
<td>1</td>
<td>3</td>
<td>5</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*a* In der 19. SSW wurde ein missed abortion diagnostiziert. Die letzte Blutprobe wurde 4 und 8 Wochen nach Abortabrasio durchgeführt.  
*b* In der 12. SSW wurde nur 1 ml mütterliches Venenblut entnommen und untersucht  
*c* Mo.pp: Monate post partum

An kleinsten Mengen genetischen Materials innerhalb weniger Stunden durchgeführt werden kann, ist die sog. Polymerasekettenreaktion, PCR (*polymerase chain reaction* [55]). Das Prinzip der PCR, durch die DNS-Sequenzen spezifisch vervielfältigt werden können, ist in Abb. 5 dargestellt. Durch Erhitzen des Untersuchungsmaterials wird die DNS in Einzelstränge aufgetrennt (*denaturiert*), so daß sich im Über-
Zu untersuchendes genetisches Material: doppelsträngige DNS

^ ^ Primer A: komplementär zum unteren Strang
...ATGCCATGCCGATAGGCT....
...TACGTTACGGCTATCCGA....

^ ^ Primer B: komplementär zum oberen Strang
CCGA

1. Zyklus

ATGC
...ATGCCATGCCGATAGGCT....
...TACGTTACGGCTATCCGA....

2. Zyklus

von Primersequenzen flankierte PCR-Produkte
ATGCCATGCCGATAGGCT....
...TACGTTACGGCTATCCGA....

3. Zyklus

4ff. Zyklus

wdh. 20-60 Zyklen

denaturieren, annähern, verlängern


schoß hinzugegebene definierte DNS-Stücke (die sog. Primer) beim Abkühlen an ihre komplementäre Sequenz anlagern („annahern“) können. Durch ein im Reaktionsmedium enthaltenes Enzym (Taq-Polymerase) werden dann die Primer zur vollen Länge der Gensequenz verlängert. Durch vielfaches Wiederholen dieses dreiteiligen Zyklus wird die im Untersuchungsmaterial enthaltene Zielsequenz exponentiell vervielfacht, so daß sie mit konventionellen Methoden, wie z.B. Auftrennung der DNA in einzelne Banden durch Elektrophorese, sichtbar gemacht werden kann. Besteht die vererbbare Genstörung z.B. im Fehlen oder einer Verkürzung des untersuchten Genabschnitts, wird dies durch Fehlen oder veränderter Wanderungsverhalten des PCR-Produkts der Elektrophorese erkennbar.


letzten Periodenblutung, und spätestens ab der 7. SSW war bei allen Patientinnen fetale DNA nachweisbar (s. Tabelle 3, [64]). Weitere Anwendungen der PCR in der Diagnostik an fetalen Zellen aus dem mütterlichen Blut sind in Tabelle 2 aufgelistet.


Schlußfolgerungen

Verschiedene Gruppen haben, technisch und in Hinsicht auf die fetalen Zellarten mit teils ähnlichen, teils unterschiedlichen Methoden, eindeutig gezeigt, daß die Isolierung fetaler Zellen aus dem mütterlichen Blut und eine nichtinvasive pränatale genetische Diagnostik prinzipiell möglich sind. Fetale Aneuploidien und Einzelgenstörungen sind so bereits diagnostiziert worden (s. Tabelle 2).

Jedoch bedeutet die Detektion fetaler Zellen nicht unmittelbar die Möglichkeit zur Diagnosestellung. Wesentliche Fragen, wie z.B. nach dem optimalen Zeitpunkt der Probenentnahme, der optimalen Zielzelle und dem optimalen Anreicherungsverfahren bleiben noch offen. Können fetale Zellen in allen Schwangerschaften in ausreichendem Maß gefunden (Sensitivität) und eindeutig identifiziert und iso-
weiterten diagnostischen Möglichkeiten. Allerdings stehen diesen er­
diagnostizierbaren Störungen ge­
Zunahme der durch DNS-Analyse
medizin bereits zu einer deutlichen
vollständig sein, so daß in der Pränatai­
ternationale Kooperation möglich
in der letzten Dekade und durch in­
durch die Entwicklung der moleku­
Dies wird alle Aspekte der Medizin
wendbarkeit überprüft. In 10 bis 15 Jahren wird die Kartie­
untersucht und auf eine klinische An­
diesem Zweck in einer Studie in den
Amniozentese, Kordozentese), ver­
trotz bereits erzielter, ermutigender
Untersuchungen (s. Tabelle 1), muß
Ergebnisse noch bestimmt werden. Dazu müssen die verschiedenen Techniken in einer größeren Unter­
suchungsreihe miteinander und mit dem „Goldstandard“, der Untersuchung invasiv gewonnenen fetalen Materials (Chorionzottenbiopsie, Amniozentese, Kordozentese), ver­
glichen werden. Zur Zeit werden zu diesem Zweck in einer Studie in den USA und in Deutschland die Verfah­
en an einer größeren Patientenzahl untersucht und auf eine klinische An­
wendbarkeit überprüft.

Ausblick

In 10 bis 15 Jahren wird die Kartie­
rung des menschlichen Genoms, die durch die Entwicklung der moleku­
argenetischen Methoden besonders in der letzten Dekade und durch in­
ternationale Kooperation möglich geworden ist, vollständig sein, so daß alle monogenetischen Erbgänge un­
tersucht werden können [25,70]. Dies wird alle Aspekte der Medizin beeinflussen und hat in der Pränatal­
medizin bereits zu einer deutlichen Zuname der durch DNS-Analyse
diagnostizierbaren Störungen ge­
führt. Allerdings stehen diesen er­
weiterten diagnostischen Möglich­
keiten noch stark limitierte therapeuti­sche Optionen für die pränatal
diagnostizierbaren chromosomalen und genetischen Störungen gegen­
über. Die ethischen und sozialen Fra­
gen werden kontrovers diskutiert, besonders wenn die genetische Un­
tersuchung von der Erkennung des Anlagenträgerstatus bei den Eltern auf die direkte Erkennung der Stö­
rung beim Feten vorverlagert und im nächsten Schritt über den Einsatz gentherapeutischer Maßnahmen
nachgedacht wird.

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